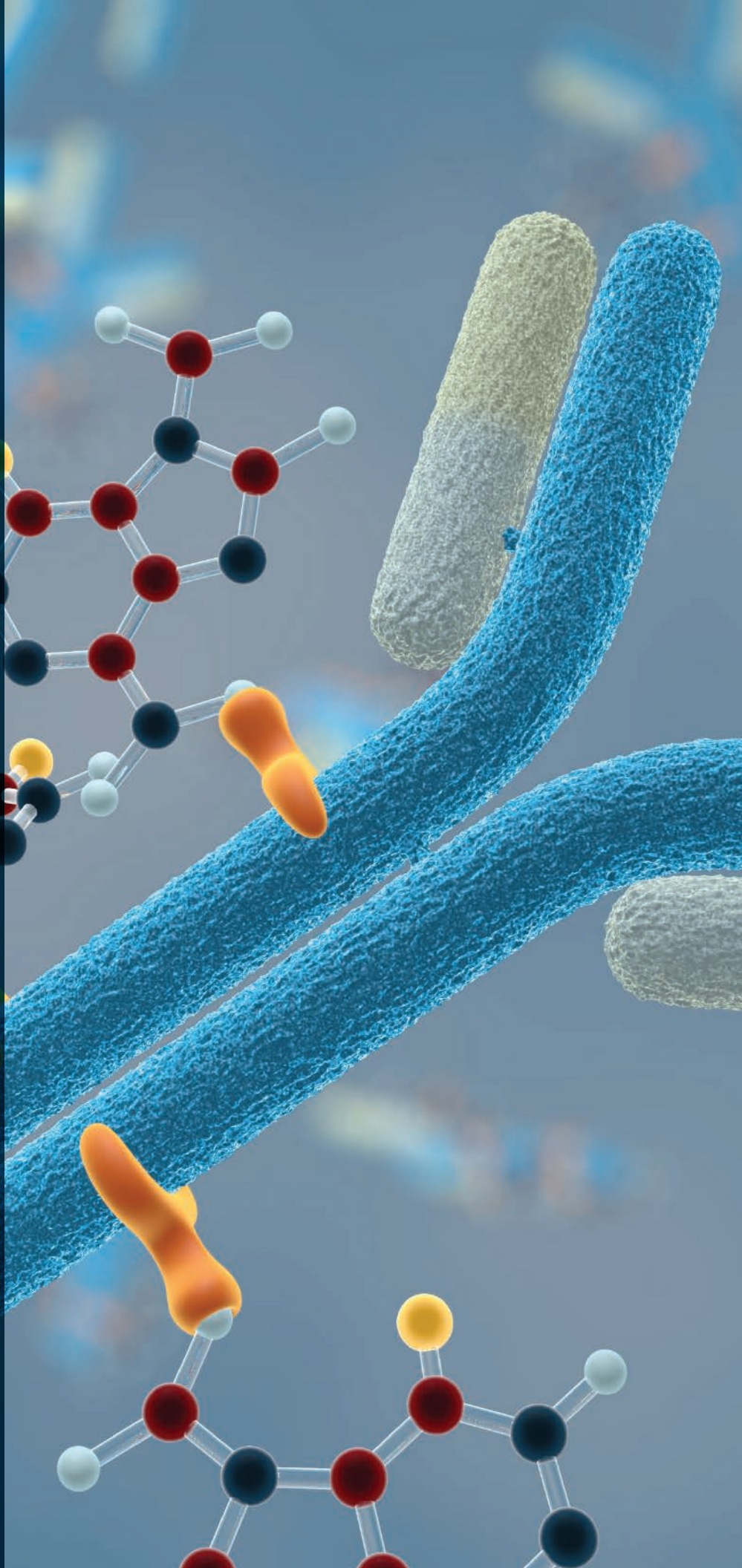
Biologics Analytical Characterization Compendium

GUIDE TO INNOVATION FOR ANALYSIS OF BIOTHERAPEUTICS

SCIEX



Innovation to Speed the Routine and Simplify the Challenging

With the number of new biologics being approved each year quadrupling since 2004-2008, biotherapeutics are the fastest growing segment of the pharmaceutical market.[1] Looking back at the past ten years, SCIEX has prepared for this market shift by collaborating with scientific experts in academia, pharmaceuticals, and global companies to develop and shape innovative technologies, such as CESI-MS, SWATH® Acquisition, SelexION® Differential Mobility Spectrometry, and BioPharmaView™ Software. These breakthroughs enable researchers to perform routine biologics analyses faster, and provide next level tools to answer the difficult questions easier.

In this Compendium, we demonstrate how technologies like Mass Spectrometry and Capillary Electrophoresis can be used to carry out routine, yet comprehensive, protein characterization that leads to results in hours, not days. Moreover, when the molecules and workflows become more complex, these same technologies can help overcome analytical challenges while providing the detailed information needed to protect the pipeline. You will learn how workflows are simplified and time to market for new therapeutics can be reduced using established and innovative methods.

This is an exciting time for large molecule drug discoveries and as the market continues to grow, researchers can be confident SCIEX will lead in biotherapeutic analysis.

SCIEX global associates and sites

- 1,500+ associates worldwide
- 260+ hold Ph.D.s or other advanced degrees
- 14 operating sites
- Global sales and service teams
- 7 global demo labs

[1] http://www.bioworld.com/content/biologics-share-medicine-chest-grows-and-so-do-pricing-concerns

Biologics Analytical Characterization Compendium

Contents

The Future of Drug Development is Today

Since the 1982 approval of Eli Lilly's recombinant human insulin, Humulin, biotherapeutic drug development has steadily grown into a global market valued at $140 billion in 2013, increased from $25 billion in 2001[1] (Table 1). Biologics or biotherapeutics make up a class of drugs including proteins and peptides, which are generally produced in living organisms and used for treatment of a wide variety of diseases including cancer, diabetes, and rheumatoid arthritis. These products also provide genuinely new strategies against infectious agents and orphan diseases alike. There are currently over 200 approved biotherapeutic drugs on the market[2] (in the US and EU) and hundreds more potential products in clinical trials or awaiting regulatory approval, a strong indication of their continuing value. The biologics pipeline is comprised of an extensive array of products with monoclonal antibodies (mAbs) representing the most broadly developed and profitable class (Figure 1).[2] Currently, mAb-based biotherapies account for 50% of the top 100 drugs[3] and are projected to maintain a dominant position in the pharmaceutical market over the coming years.

The biotherapeutics pipeline

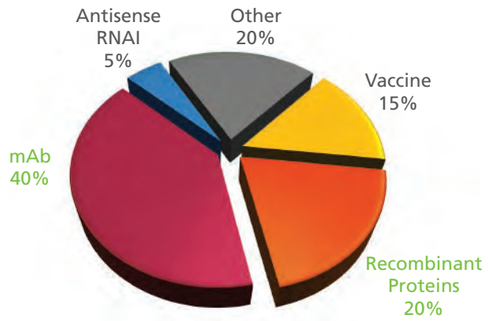


Figure 1: The biotherapeutics pipeline encompasses a diverse array of products that includes monoclonal antibodies (mAbs), recombinant proteins, vaccines, gene therapies, antibody-drug conjugates and FC fusion proteins, peptides, and antibody fragments.[3]

The continuing growth of biopharmaceuticals can be attributed to their many advantages over small-molecule therapies including minimal safety/toxicity issues, well-understood mechanisms of action, and high target specificity. The challenge of developing a biotherapeutic, such as a biologic protein, stems from the structural complexity of these large molecules and their production processes. Because biologics are manufactured in living cellular systems, they often undergo modification as a result of growth/media conditions, the bioprocessing environment, or purification and/or formulation. Some of these modifications are primary sequence variants, while others are post-translational modifications (PTMs) to the protein sequences (Figure 2). This structural heterogeneity is dynamic and can influence the protein's overall conformation with the potential to change its clinical efficacy. Advances in recombinant DNA technology, as well as in fermentation and purification processes have provided robustness in generating large amounts of protein-based therapies. Typically, biologics are produced in mammalian cell systems like Chinese hamster ovary (CHO) cells using carefully controlled growth and environmental conditions. However, cell-culture can be unpredictable, and even minor adjustments to the manufacturing process, like changes in the culture media or even in the purification procedure, can alter a molecule's structure or stability, and therefore its safety and efficacy. Consequently, implementation of chemical manufacturing controls (CMC) and analytical characterization processes at each stage of manufacturing is necessary to ensure product biocomparability.

As the biologics market continues to grow, there is a significant need for established analytical and bioanalytical methods that can help improve discovery and development. SCIEX offers technology including capillary electrophoresis (CE) and Mass spectrometry (MS) to help facilitate these improvements.

Capillary electrophoresis (CE) is a technique commonly used to assess purity and heterogeneity of biologics. In principle, CE separates molecules based on differences in their charge by generating an electric field across a capillary into which sample has been introduced. The voltage applied facilitates differential migration of analytes through a capillary and across a detection window where their absorbance or fluorescence can be measured. In practice, conventional gel electrophoresis techniques like SDS-PAGE and isoelectric focusing (IEF) have been replaced with capillary-based methods (CE-SDS and CIEF) using the SCIEX PA 800 Plus platform because of its ability to generate highly reproducible results in an automated, quantitative fashion. In addition, oligosaccharide characterization can similarly be performed allowing for ultra-high resolution separation of glycoforms associated with protein biologics. Coupling high resolution separations with validated methodology and powerful software analysis on the PA 800 Plus has been providing critical information across development and quality control processes in the biopharmaceutical sector. SCIEX CESI technology couples the power of CE with the detection capability of electrospray ionization mass spectrometry (MS). Utilizing flow rates as low as 10 nl/min, CESI minimizes ion suppression, thereby enhancing overall sensitivity at the MS when performing key assays, including peptide mapping and characterization of intact biologics.

Total number of approved biopharmaceuticals marketed in EU/US	212
Number of biotherapeutics approved in 2010-2014	54
• mAbs	17
• Hormones	9
• Blood-related proteins	8
• Enzymes	6
• Vaccines, fusion proteins and filgrastims	4
Total cumulative sales in 2013	$140 billion
Highest selling product in 2013	Humira (adalimumab), $11 billion
Total mAb sales in 2013	$69.8 billion
Highest grossing target indications in 2013	• Inflammatory/autoimmune disorders ($41 billion) • Cancer ($26 billion)

From *Biopharmaceutical Benchmarks 2014*[2]

Table 1: Biopharmaceutical industry metrics (2010-14)

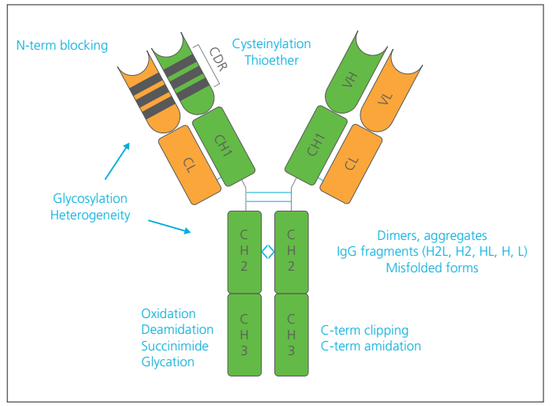


Figure 2: IgG1 structural heterogeneity. Therapeutic antibodies (monoclonal antibodies, mAbs) produced using recombinant DNA technologies are generally complex, heterogeneous and subject to a variety of enzymatic or chemical modifications during expression, purification, and long-term storage. These modifications are listed here in blue. The mAb pictured here is composed of two heavy chains (H) that contain three different constant regions (CH1, CH2, CH3) and one variable region (VH). The mAb also contains two light chains (L) that contain a constant (CL) and a variable region (VL).[6]

MS technology is being widely used to characterize proteins due to exceptional sensitivity, selectivity, and specificity, positioning itself as a superior method for profiling protein sequence, PTMs and other structural attributes of biotherapeutics.

Recent innovations in source electrospray ionization (ESI) has improved detection sensitivities.[4] Expanded mass ranges can now accommodate very large proteins, and improved quantitation as a result of state-of-the-art detector design has enabled increasingly smaller detection limits approaching the femtomole range.[1] Additionally, powerful software analysis has streamlined the management of overwhelmingly large data sets generated in unbiased, comprehensive sample analysis. Together, these analytical advances have triggered an emerging dominance in the use of CE, and MS by the biopharmaceutical industry.

SCIEX has designed this compendium to support investigators as they navigate the growing array of options for comprehensive product characterization to in-depth analysis. We highlight several SCIEX technologies and applications that emphasize efficiency, while still providing the precision and accuracy necessary for rapid decision-making regarding a product's quality. Each section identifies solutions that accelerate biotherapeutic characterization through the use of automated sample preparation and analysis workflows, and optimized separation methods. These sections focus on analytical areas that are critical for product development, and are segmented in a similar manner as the basic Biologics Workflow:

- Biologics Research
- Development and Quality Control
- Bioprocessing

To succeed, many of these applications rely on hybrid triple quadrupole/time-of-flight instruments, such as the TripleTOF® 5600+ and 6600 Systems, which are particularly capable of identifying and quantifying unknown analytes while easily handling the higher mass ranges of proteins. Historically, users have performed multiple experiments to obtain accurate mass MS and MS/MS information, but now the TripleTOF System's rapid acquisition rates and high-speed processing provide the high resolution necessary for creating detailed peptide maps, confirming sequences, and accurately identifying modifications in a single analysis.

In the **Biologics Research** section, applications in four areas—molecular weight determination, peptide mapping, disulfide bond assignment, and antibody drug conjugate analysis—explore next-generation analytical technologies that create a deeper view of protein structure. The key to accelerating these processes lies in automating data processing and decreasing data complexity. To streamline the evaluation of large data sets, BioPharmaView™ Software automatically computes protein structural features, such as intact molecular weight, antibody-drug ratios, glycoforms, and terminal modifications, generating a global overview of any lot-to-lot changes. The software's smart-filtering capacity efficiently deciphers peptide-mapping data, shrinking analysis time to just a few hours. By enhancing selectivity, the orthogonal chromatography techniques of differential mobility spectroscopy (DMS) and capillary electrophoresis electrospray ionization (CESI) can reduce spectral complexity resulting from background interferences, simplifying the resulting data and its processing—to give an all-inclusive structural overview.

In the **Development and Quality Control** section, top-down sequencing and data-independent acquisition strategies are highlighted as accurate methods for identifying and characterizing product variants arising from charge heterogeneity, changes in primary sequence, and other critical quality attributes. Dynamic database searches supported by informatics packages, such as ProteinPilot™ Software, can search for hundreds of PTMs and sequence variants simultaneously, flagging variants in much less

time, and giving a high quality snapshot of the current state of a biologic. Additionally, because carbohydrate moieties can significantly influence a biotherapeutic's safety and efficacy and have been difficult to characterize, we give special attention to methods that rapidly evaluate glycan content in the section **Glycosylation and Glycan Analysis**. Responding to the increasing demand for high-throughput N-glycosylation profiling, SCIEX has developed a workflow that condenses time-consuming, sample-prep steps into a fully automated liquid handling format. Other advancements include boosting glycan isomer separations using DMS- and MRM-based methods to isolate hard-to-separate species—supplying a more thorough understanding of critical attributes.

In the **Bioprocessing** section, the focus is on comprehensive, unbiased acquisition methods, such as SWATH® Acquisition, that enable the detection of extremely low levels of host cell protein (HCP) contaminants from amongst an excess of purified product. In addition, high-resolution separations using CESI enable sensitive detection of these low-level impurities.

The compendium culminates with a section on **Biosimilar Comparability**, where SCIEX explores ways to simplify the critical task of determining product comparability, and comparing a biosimilar to an originator. The inherent structural heterogeneity of proteins makes direct comparisons to biosimilars traditionally time-consuming and complicated. The BioPharmaView Software can automatically evaluate high-resolution mass spectrometry (HRMS) data for molecular mass and PTMs, quickly highlighting product heterogeneities–with just a single analytical tool.

All areas of biotherapeutic development–from discovery throughout development to the final quality controls– require bioanalytical approaches that can strategically remove long-standing bottlenecks and offer efficient, streamlined methodology. Biotherapeutic development also strongly benefits from reproducible and reliable data collection, an essential component of any application used to create a consistent and accurate picture of a biotherapeutic's complicated structure. Powered by cutting-edge SCIEX instrumentation and software, the applications and approaches explored on the following pages are designed to save time and labor, while generating reliable structural data that can be used to confirm the safety and efficacy of any biotherapeutic product.

Prepared by Laura Baker, Ph.D., Science and Technical Writer

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[3] Van Arnum, P. "Moving to the Next Level in Biomanufacturing." *Pharmaceutical Technology.* April 02, 2010; 34(4). Retrieved at: *http://www.pharmtech.com/moving-next-level-biomanufacturing.*

[4] Cotte-Rodriguez, I., et al. "Introduction to Protein Mass Spectrometry." *In Guodong Chen (Ed.) Characterization of Protein Therapeutics using Mass Spectrometry.* 2013; Springer, New York.

[5] Lew, C., et al. "Rapid Sample Preparation and Analysis of Monoclonal Antibody N-Glycans by Magnetic Bead Technology and CE-LIF." *SCIEX technical note.* 2015; Document number RUO-MKT-02-1896.

[6] Johansen, E., Shin, B-H., and C. Hunter. "Simultaneous Quantitative and Qualitative Analysis of Proteolytic Digests of Therapeutic Monoclonal Antibodies Using a TripleTOF® System." 2013. SCIEX technical note. Document number 7460213-01.

INTRODUCTION

Routine and Enhanced Intact Mass Analysis Without Compromise on TripleTOF® Mass Spectrometry Platforms

Eric Johansen and St John Skilton
SCIEX, USA

Key benefits:

- High Dynamic Range of TripleTOF Instruments capture both high-abundance and low-abundance molecular isoforms.
- Balance of processing power and simplicity: many processing parameters are available to the user and can be customized based on the heterogeneiety and complexity of the molecules. The interface is simple and allows display of the right amount of detail with visual simplicity for the analyst.
- BioPharmaView™ Software brings routine molecular weight determination by mass spectrometry to all laboratories.

Introduction

Any successful pharmaceutical organization aims to get the best possible product quality in the shortest possible time. From early development, to process optimization, or in QC assays, the ability to demonstrate intact molecular weight routinely and accurately is important. Time of Flight (TOF) systems offer unrestricted mass range, can operate at high resolution continually, and have a wide dynamic range to simultaneously detect low and high abundance isoforms. These attributes have made TOF MS platforms the analytical system of choice for biotherapeutic development and production . In this study, we demonstrate the capabilities of the SCIEX TripleTOF 6600 system, which introduces the next generation analytical technology for intact mass analysis of biologics.

Results and Discussion

One of the most important characteristics when selecting a mass spectrometry platform for intact protein determination is the interscan linear dynamic range. TripleTOF Platforms do not sacrifice dynamic range for resolution, maintaining a dynamic range of 4 to 5 orders of magnitude. This allows the detection of a wide variety of low abundance isoforms at the same time as major isoforms, with no loss of fidelity.

Deconvolution and Addressing High Mass

It is impossible to directly measure accurate mass based on the carbon 12 isotope of large, multiple-charged ions when the dynamic range between and the isotopes of lowest and highest intensity are beyond the limits of detection. For larger, heterogenous species, generally 15 kDa or above, average molecular weight is often reported. Almost all software deconvolution tools today use a system of iteration to determine when the modeled peak shape fits the raw data, and compares the end result to the original spectra to determine the number of optimal "iterations." Additionally, software deconvolution has become critical to making the process routine and minimizing the parameters to optimize. Overall, greater application of smoothing, background subtraction, and a large number of iterations will lead to a more simplified visualization. This is likely to help the reviewer, but there can be a risk of obscuring low abundance isoforms. In this application we demonstrate the capabilities of SCIEX TripleTOF 6600 system in offering both levels of information without compromise. This capability also means that the user does not have to adjust resolution settings, or repeat analysis because all of the data is already available for processing.

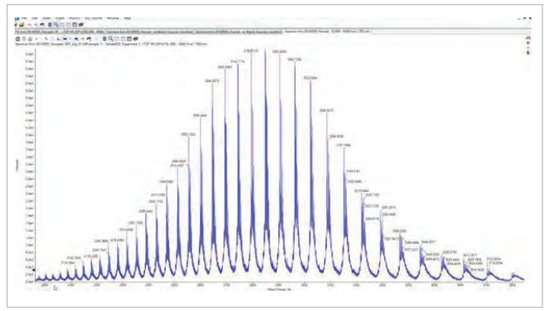

Figure 1: Intact Electrospray Ionisation mass spectrum of a commercial IgG1 over a wide m/z range. The multiplicity of charge states makes this data challenging to process visually.

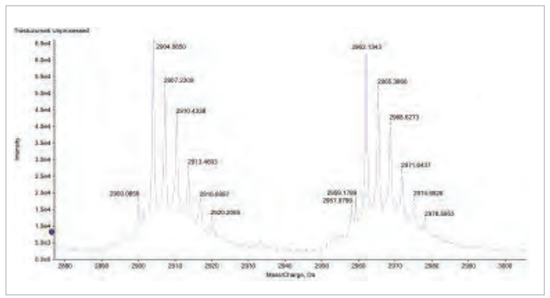

Figure 2: Zoomed view of the Intact IgG1. All major isoforms are visible, as well as a number of smaller isoforms.

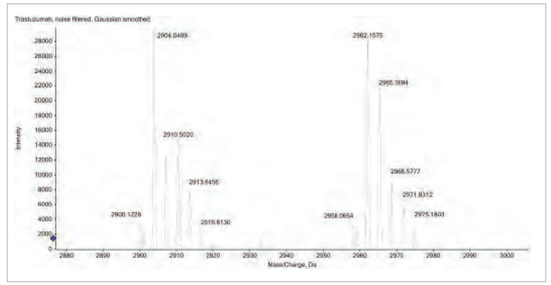

Figure 3: 'Heavily' processed intact IgG1 data to simplify the spectrum to the major species and separate the peaks as far as is amenable. This data processing is not generally used for average molecular weight reporting, but may be used to simplify visualization. It is clear that the relative ratios of some peaks are different from the unprocessed raw data.

Figures 1 and 2 show the raw data for a monoclonal antibody, with an intact molecular weight measurement of ~148 kDa. Figure 1 shows the mAb detected over a wide m/z range, with the multiplicity of charge states. Figure 2 is the zoomed in view of the non-processed raw spectra, displaying both the major isoforms as well as many of the minor species. The peak ratios are maintained, and relative proportions of the isoforms can be estimated.

Figure 3 shows the same spectrum with baseline subtraction and Gaussian smoothing parameters applied. The heavily processed view is greatly simplified by comparison to the unprocessed view, as well as displaying a baseline separation. Although this might offer a simpler visual data representation, it runs the risk of omitting some minor details. However, under both conditions, the same average molecular weight determination can be made without recourse to changes in instrument settings (Figures 4-5). The data in this study was all acquired within the original run and without compromising quality. An organization can feel confident that they are capturing low-level isoforms with the wide dynamic range of the SCIEX TripleTOF 6600. This additional dynamic range will allow an organization to see deeper into the structure of each potential therapeutic molecule at the intact mass level and allow additional screening capabilities before needing to resort to time-consuming peptide mapping techniques.

This assay can be linked to multiple separations techniques and the processing automated using BioPharmaView™ Software (Figure 6), allowing for fast, easy, and reproducible determination of molecular weight of both high and low abundance protein isoforms.

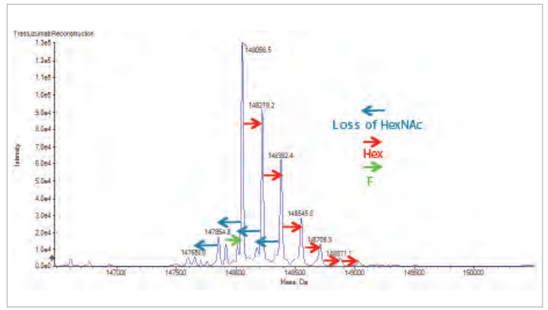

Figure 4: Deconvolution spectrum of the unprocessed data from Figures 1-2, showing the average masses of detectable isoforms of trastuzumab. Note that many low-level isoforms are easily detectable.

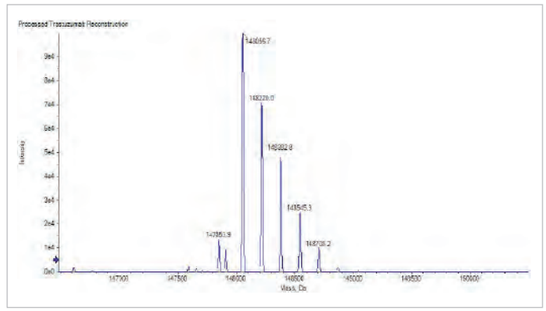

Figure 5: Deconvolution spectrum of the heavily processed data from Figure 3, showing the average masses of many of the detectable isoforms of trastuzumab. Note that some of the low level isoforms are obscured by processing.

Figure 6: Deconvolution of the unprocessed raw data using BioPharmaView Software is shown in the bottom right hand pane of this screenshot. This automated solution allows for batch sample processing and sample comparisons, accelerating your analysis time.

Enhancing Characterization of Antibody-based Biologics Using Differential Mobility and Mass Spectrometry

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Introduction

An important class of emerging biologics currently being developed are antibody drug conjugates (ADC). Due the large size of these molecules, and their heterogeneity, detailed characterization of the material is required. During this characterization, multiple analytical approaches are used, including intact, heavy- and light-chain MW determination, and amino acid sequencing. For analysis of some of the high molecular weight species (intact or reduced), LC frequently results in co-elution of the various forms, which can lead to overlapping deconvolution. Differential mobility separation (DMS) is a technique that can separate ions in the gas phase, prior to MS analysis and can complement the LC separation. Since parameters such as size, conformation and dipole moment have been show to play a role in the separation of species in a DMS cell, this technique could be used to provide additional selectivity during the characterization of an ADC, simplifying the data processing.

Experimental

- Sample Preparation: IgG1 was cleaved at the hinge region to generate F(ab') and Fc fragments enzymatically.
- DTT reduction is achieved by incubation at 37°C for 30min with 10mM DTT at pH 7.5 with Ammonium Bicarbonate.
- IgG1 was also enzymatically digested with trypsin to provide signature peptides based on standard protocols.
- HPLC Conditions: A Shimadzu Prominence-XR LC system with Agilent Poroshell SB 300 C8 (1x75mm) at 70°C with a gradient of eluent A water/acetonitrile (98/2) + 0.1% formic acid and eluent B water/acetonitrile (5/95) + 0.1% formic acid was used at a flow rate of 400µL/min. For peptide analysis, a Phenomenex Luna C18 (2x50mm) was used at 45°C with a 5 min gradient (0-45% organic) at 450µL/min
- MS/MS Conditions: A SCIEX Turbo V™ source and Electrospray Ionization (ESI) probe was used. A TripleTOF® 5600 LC-MS system and a QTRAP® 5500 LC-MS System, both equipped with a SelexION® DMS Technology Device (Figure 1).

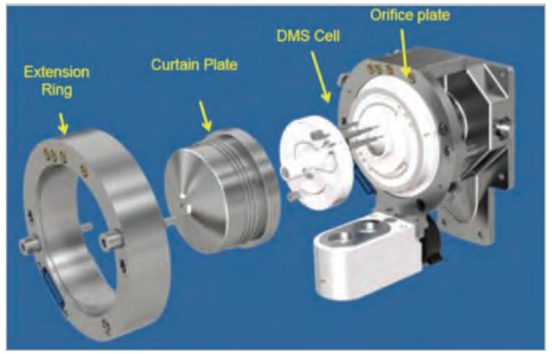

Figure 1: Components associated with SelexION Technology, Cell dimensions are 1 x 10 x 30 mm (gap height x width x length).

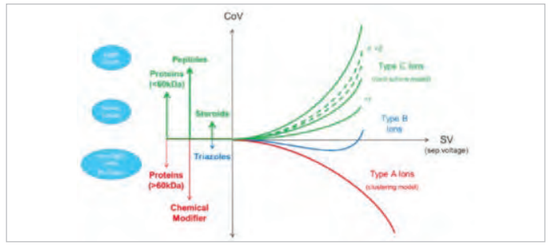

Figure 2: Typical behavior of various species in DMS cell as a function of separation voltage (SV) and compensation voltage (CoV).

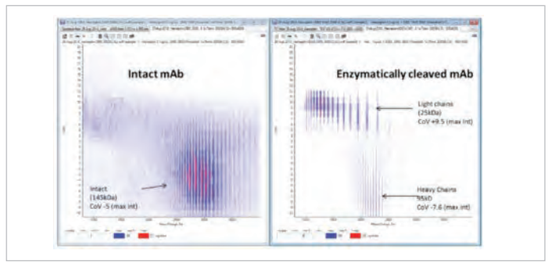

Figure 3: Separation of an intact mAb and enzymatically cleaved mAb. SV was set to 3500V and all data is averaged over the elution profile of the analyte (LC gradient 3 min). As can be seen, the light chain, heavy chain, and intact mAb can be separated from one another based on their unique CoVs.

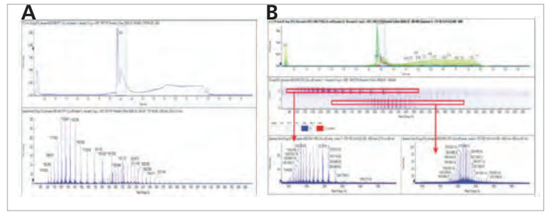

Figure 4: Analysis of enzymatically cleaved mAb with (A) all DMS cell parameter turned off (i.e. SV=CoV=0) and (B) in CoV mapping mode with SV set to 3500V. When DMS cell is activated, it is possible to get simplified mass spectrum for both the Fc as well as the Fab region even under co-eluting conditions (bottom right).

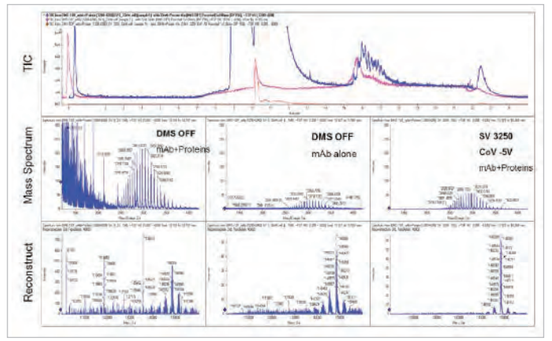

Figure 5: Effect of DMS on mass spectra deconvolution of mAb data in the presence of other protein species in solution. When the DMS is OFF, lower m/z ions contribute to additional chemical noise. When the DMS is activated, only mAb signal is detected, thus reducing noise contribution in the deconvolution and simplifying data processing.

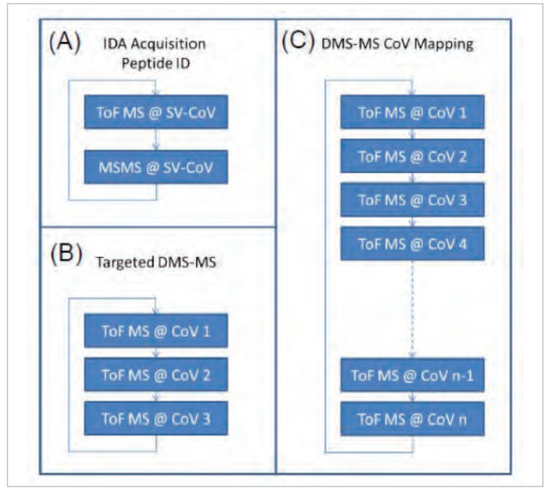

Figure 6: LC Acquisition workflow typically used with DMS to support characterization of ADC. (A) The IDA acquisition workflow is used to improve peptide analysis by reducing chemical noise associated with peptide detection while automatically selecting precursor ions for MSMS analysis. Both the MS and MSMS data are collected at same SV/CoV values. (B) Targeted DMS-MS analysis to improve spectral quality of associated with subunit of mAB (heavy and light) and intact mAb. Typical target CoV value would be CoV of +10, +5 and -5 all at the same SV value. (C) DMS-MS CoV Mapping on LC time scale. This provides a wide range of CoV values to generate 3D map of the ions behaviors at a specific SV value. The data displayed in Figure 3 and 4 were generated using this acquisition mode.

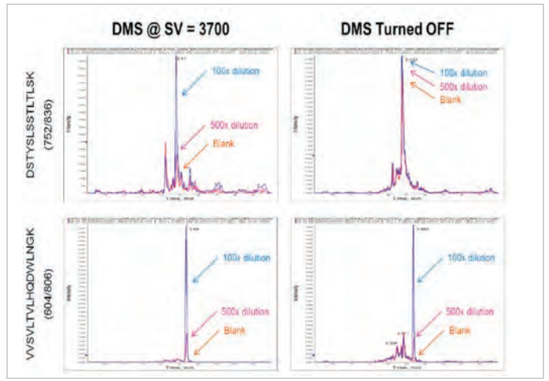

Figure 7: Improving detection and quantitation of digested mAb signature peptide with DMS under rapid LC analysis (5 min gradient). When DMS is active, the matrix blank and low level sample are readily distinguished. When the DMS is OFF (or removed) the signature peptide exhibits significant interferences at the same R.T. or near the elution time of the peptide. Using the DMS ensures more selective detection and simplifies reliable peak integration for quantitation.

Results

The DMS cell provides a means of separating ions at atmospheric pressure, ahead of any mass analysis step, thus in many cases, improving ion selection prior to MS/MS analysis. The separation capability offered by differential mobility are affected by several parameters associated with the ions, such as size, conformation, dipole moment and interaction with chemical modifier (gas vapor of organic solvent) added to the transport region. For any ion, as the separation voltage (SV) is increased it is possible to adjust the compensation voltage (CoV) to ensure proper transmission of the analyte for mass detection. Figure 2 shows the typical behavior observed for various analyte types under different conditions. Of particular interest in the analysis of ADCs is the possibility to ensure discrimination of ions that could cause interference in the detection of analyte and lead to a more complex data deconvolution scheme. When performing analysis on a DMS enabled TripleTOF® 5600 LC-MS System, one could acquire data in one of 2 modes; 1) CoV mapping or 2) target CoV values. In CoV mapping mode, the instrument is set to acquire data over a wide range of CoV values, all at the same SV value. This can be performed on LC time scale, thus providing a complete view of all species and their behavior at the selected SV value. Figure 3 shows a CoV map for an intact mAb sample (A) and for a mAb sample that was enzymatically cleaved at the hinge region to generate the Fc and Fab chain (B). As can be seen, the DMS can easily separate each of these species, which leads to simplified mass spectral data.

Conclusions

- The implementation of differential mobility (DMS) as a "front end" separation prior to mass spectrometric detection provides a generally deployable tool for pharmaceutical organizations that need to be conscious of method deployment through all departments or with external collaborators.
- Implementation of DMS in the source region also reduces the dependence on complex software for data processing because data can be processed with existing software packages. This allows novice users to access the technology immediately.
- The general utility of Differential Ion Mobility Separation is evident from the wide variety of biomolecules studied and separated.
- The ability to interface all types of mass spectrometry platforms makes this tool applicable to all stages of production, development, and clinical stages of biotherapeutic development from characterization to bioanalysis.

BIOLOGICS RESEARCH

Automated Drug-to-Antibody (DAR) Calculations for Greater Efficiency in Analyzing Antibody Drug Conjugates (ADCs)

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Key Benefits

Software and hardware tools to facilitate the analysis and review of complex, high molecular weight species such as ADCs have been developed by SCIEX.

Large molecules such as monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs) can be separated from contaminants such as free drug, linker, and peptides/small protein fragments by Differential Ion Mobility with SelexION® Technology.

Data from species that have been selectively passed through the SelexION device can be reviewed easily within BioPharmaView™ Software. Each separate compensation voltage (CoV) is represented as a discrete experiment, allowing for simplified data viewing.

The routine use of differential ion mobility is accessible to scientists at all stages of biopharmaceutical development to help tease out differences that would otherwise remain obscured by matrix or other contaminants.

Introduction

In 2013 the US FDA approved the antibody drug conjugate Kadcyla as a new therapy for patients with late stage metastatic breast cancer.[1] This was not the first molecule in its class to be approved, but such is the importance of these biotherapeutics that it was reviewed under the FDA's priority review program.[1] Trend analysis of novel new drug approvals by the FDA showed that in 2014, 41 novel drugs were approved. This was more than the average annual approvals over the last decade.[2] As this trend continues, and the complexity of biopharmaceutical molecules continue to increase, developers of ADCs need to find better ways of coping with their complex datasets. This Technical Brief outlines a new tool from SCIEX that facilitates the rapid assessment of drug-to-antibody ratio (DAR) for ADCs.

Results

Using TripleTOF® technology, with separations systems such as CESI, and MicroLC, along with data processing in BioPharmaView Software, an analyst can provide a DAR readout within seconds. Calculations using peak areas that may have previously taken hours to complete are now ready to report instantly. The readouts can be obtained by an analyst with no expert training. In this way, BioPharmaView Software facilitates method development in less time, and provides higher sample throughput. Faster review helps an organization develop a robust and reproducible DAR method.[4] Without an automated solution, exporting the data to separate spreadsheets and setting up calculation mechanisms is time consuming and adds multiple steps that increase risk of human error. For example, an analysis where there are four chromatographic peaks, each requiring 15 minutes of analysis for DAR, and 10 samples to analyze, would require about 10 hours. Using new functionality in BioPharmaView™ Software reduces this analysis time to approximately 10 minutes, including reporting the data out to a document ready for review.

Figure 1: An automated DAR calculation for a biotherapeutic in development within the BioPharmaView Software. The screenshot shows a tabular readout of the DAR (left), a bar graph in the center, and a chromatographic readout of the identified species on the right.

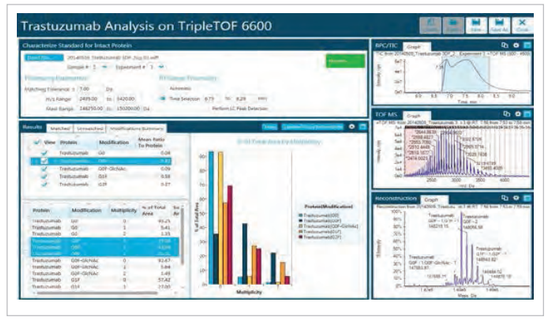

Figure 2: Automated Functionality to show relative proportions of modifications present on intact trastuzumab antibody.

Figure 1 shows the ESI-MS spectrum of a trial intact lysine-linked ADC based on an IgG molecule. The screenshot shows how an analyst can simply read out a DAR calculation from an intact molecule, which is ready to send to a report immediately. In the top left hand panel is a tabular readout of the DAR with the assigned number of attached drugs clearly shown and a 'mean ratio' calculated. The corresponding visual display is in the central panel, with a bar graph denoting how many drugs are attached. In this example, the drug-to-antibody ratio distribution is between 3 and 10. In the three right hand panels are the chromatographic, raw, and deconvoluted data respectively, from top to bottom. In the raw data in the top panel the blue area indicates which (chromatographic) peak area has been used to make the calculations. Using a mouse drag, the user can simply select different chromatographic regions to work out retention time variability of isoforms present in a sample. The graphs are updated automatically.

The same functionality can also be applied to other modifications, such as glycoforms or terminal modifications. In Figure 1, pyroglutamic acid conversion is calculated as one of the assigned modifications. In Figure 2, a number of glycoforms are tracked simultaneously, showing all the modifications that are assigned automatically by BioPharmaView Software. Note that the user may also filter the results according to their needs and display only those modifications which they need to report. A number of filter criteria are available to the user which may be used to provide reportable information on specific quality attributes.

Discussion

Early in development, analysts are tasked with providing rapid feedback to their synthetic chemists on how well a conjugation strategy may have worked. This allows the synthetic chemists to select optimal methods. The faster the feedback, the faster development can proceed. Researchers working in this field have identified DAR as "one of the most important quality attributes of an ADC" because this "determines the payload that can be delivered to the tumor cell and can directly affect both safety and efficacy".[3]

During later development and subsequent production, analysts need ways to rapidly confirm that the product retains the expected characteristics. Therefore the ability to respond rapidly with an accurate readout of DAR allows potential problems to be identified early and allows development to become more efficient. If DAR calculations are used as part of release assays, rapid and robust measurements can help an organization maintain the integrity of a product. The task can be complicated by the heterogeneity of the constructs, the number of impurities (or fragments if the synthesis is still at an early stage of optimization), difficulty in chromatographic separations, or interfering compounds such as those found in formulation.

Conclusions

SCIEX has continued to innovate to allow analysts to quickly and accurately perform their scientific work, and save hours of time. For a representative sample set, the savings amount to an entire working day, allowing a scientist more time to devote to more productive scientific questions. The rapid, simple application of the automated tool in BioPharmaView Software facilitates the analysis of Intact ADCs to allow researchers to:

- Immediately determine the intact molecular weight of the construct
- Obtain Drug-Antibody Ratio (DAR)
- Adjust processing parameters instantly
- Determine the range of the number of drugs linked
- Provide a general purpose tool that provides information on modifications of intact species.

For complex biologics species such as ADCs, SCIEX provides a toolkit that provides analysts with instant, automated readouts of quality attributes, irrespective of the mode of separation, or the presence of an ion mobility device.

References

[1] *http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm340704.htm*

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[3] Wakankar et al (2011); MAbs. 2011 Mar-Apr; 3(2): 161–172. Published online 2011 Mar 1. doi: 10.4161/mabs.3.2.14960. Analytical methods for physicochemical characterization of antibody drug conjugates. Aditya Wakankar,2 Yan Chen,1 Yatin Gokarn,2 and Fredric S Jacobson; *http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3092617/*

[4] *http://sciex.com/Documents/posters/271_antibodyDrug_cong_char.pdf*

BIOLOGICS RESEARCH

Analysis of Glycosylated Antibody-Drug-Conjugates by TripleTOF® High-Resolution System and BioPharmaView™ Software

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Introduction

Antibody drug conjugates (ADC's) are an important class of biotherapeutic compounds delivering a targeted, usually cytotoxic, drug selectively to the target cell. Lysine –linked ADC's can be very complex with multiple payloads conjugated to the same biotherapeutic protein. The drug-antibody-ratio (DAR) needs to be characterized and monitored from batch-to-batch, to ensure safety and efficacy of the biotherapeutic. An analysis time bottleneck in a conventional set-up would be the deglycosylation step, which is usually required to eliminate complexity in the sample. Here we present data acquired from a non-treated ADC with multiple payloads, and N-glycosylation intact. Despite the complexity, reproducible analysis and processing was achieved. Differential ion mobility was used to further reduce the complexity and the background interferences.

Intact protein analysis for antibodies and ADCs is a rapid method for global observations of changes in product from lot-to-lot. Intact protein analysis is used to determine the mass of the complete protein product, (i.e. to discover possible clipping or truncation), glycosylation heterogeneity, and to assess the DAR of ADCs. These aspects should be vigorously controlled, as biological therapeutics are produced in cells, and glycosylation patterns may vary due to changes in production. The drug conjugation efficiency should also be assessed to ensure safety and efficacy.

Reproducible, reliable and trustworthy data and processing are the most important factors to assess the information from intact protein analysis.

Lysine linked ADC's are a common class of ADCs, with a high number of possible conjugation sites in the structure (IgG amino acid sequence typically has more than 70 Lys –residues). Monitoring and characterization of the DAR calls for strong data quality and confidence in data processing, with automated calculations to allow fast decision making on the quality of the product. The tools allowing for good quality spectra and automated batch processing in the biopharmaceutical industry allow not only for the sample preparation time to be reduced, but also to allow the response time to the synthetic chemist department to be reduced. Increasing the speed of the analysis would also allow for the opportunity to look at more synthesis options in the same timeframe, leading to faster synthesis method optimization.

Total analysis time depends on the sample preparation; the simpler this can be made, the more samples can be analyzed in a reasonable time frame. To reduce the sample preparation time, high data quality and orthogonal separation methods can be used to evaluate product complexity. Differential ion mobility has been shown to effectively reduce the data complexity and to separate features of different sizes and mobility. Here, the interferences in the spectra were separated from the ADC of interest.

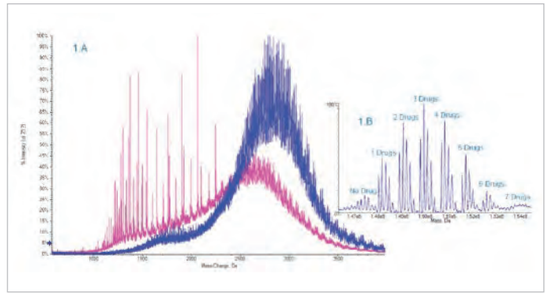


Figure 1: (A) comparison of raw spectra from the chromatographic system 1: reverse phase separation. In Blue: spectra with SelexION® technology cell optimized for the transmission of ADC ions. In Pink: no differential mobility separation applied. (B) Re-constructed spectrum of the SelexION technology-acquired data, showing detection of multiple forms of drug conjugation from DAR 0-6.

Experimental

Lysine-linked ADC was kindly provided by Sanofi-Vitry (France). The amino acid sequence and drug moiety structure are proprietary information.

The ADC sample was analyzed using TripleTOF® 5600 coupled to Shimadzu Nexera UHPLC, by two different chromatographic set-ups:

1. Agilent SB C-8 (5µm 1mmx75mm). Gradient with 20 min run time, 200 µL/min flow rate. Solvents A: 0.1% formic acid (FA) water; B: 0.1% FA Acetonitrile.

2. Waters Acquity UPLC BEH 200 SEC (1,7µm 4,6mmx300mm). Gradient with 35 min run time, 300-400 µL/min flow rate. Solvents A: Ammonium formate 25mM, 1% FA; B: Acetonitrile. Column temperature 60 °C in both cases. 5-10µg of ADC loaded on column per run.

SelexION DMS technology was used with chromatographic system 1. Data was acquired with a TOF-MS scan over the mass range (m/z = 400-4500). Peak reconstruction was performed using BioPharmaView™ Software.

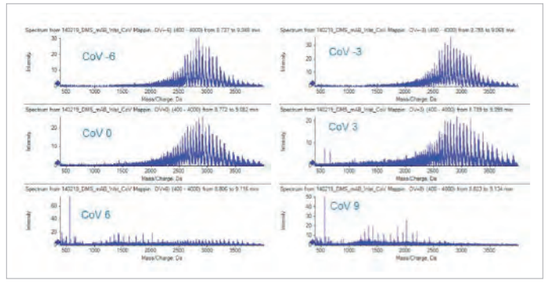


Figure 2: Optimization of the SelexION technology compensation voltages (CoV) on a monoclonal antibody (mAb). Panes have been marked with the applied CoV (here shown from -6 to 9). The separation voltage was optimized to 3500V. For large proteins such as mAbs, the transmission optimum was found with negative CoVs; for the smaller proteins (Ab light chain) and interferences, the transmission optimum was found with positive CoVs.

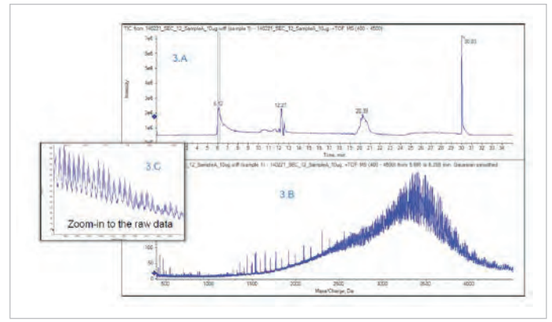


Figure 3: Chromatographic system 2 (SEC separation) total ion chromatogram (3A) and the obtained ADC raw spectrum (3B) and a zoom-in of the spectrum (3C) with the charge states highlighted found with positive CoVs.

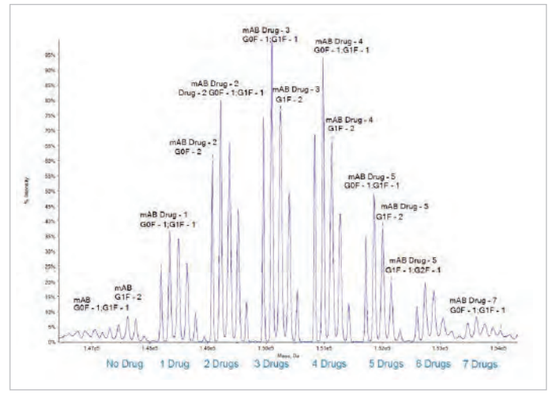


Figure 4: BioPharmaView Software reconstruction of the data presented in Figure 3 Drug load was up to 7 per antibody moiety, with all the different drug conjugations demonstrating the same glycosylation pattern of combinations of G0Fs to G2Fs.

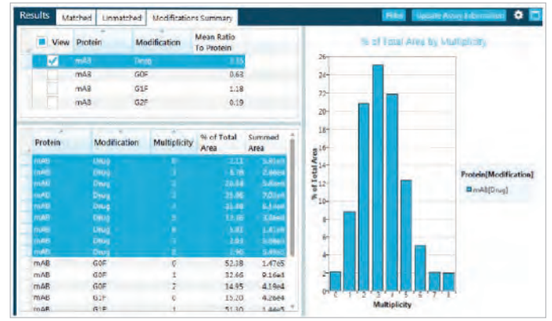


Figure 5: Automated DAR calculations for the non-deglycosylated ADC. The reconstruction area is given as a value, and also as a percentage of the total area per each multiplicity of conjugation. The multiplicity is represented as columns and the final DAR ratio is calculated for each protein glycosylation form.

BIOLOGICS RESEARCH

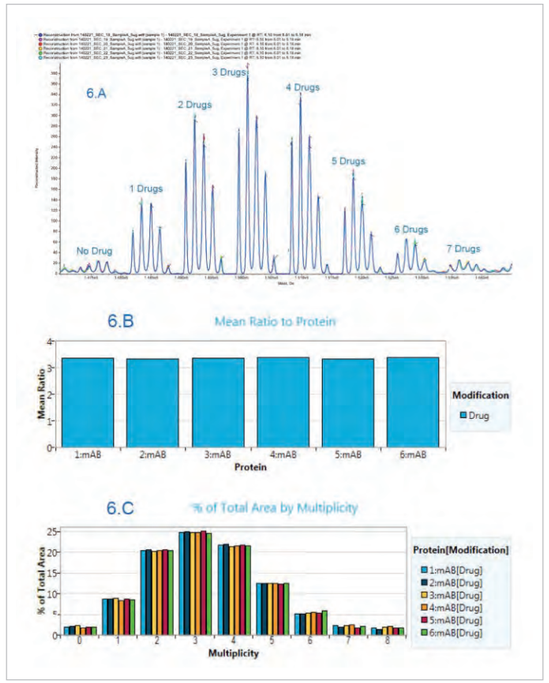


Figure 6: Reconstruction of analysis of six injections of the non-deglycosylated ADC: (6A) the overlay of six reconstructed spectra, (6B) the DAR ratios and (6C) the multiplicity represented as columns for the six injections, all showing excellent reproducibility.

Results

The use of SelexION differential mobility separation technology reduced background interference to allow for cleaner spectra and better processing when coupled with C8-RP chromatography (Figure 1). The signal from the light chain (MW around 25kDa) and the formulation background interference was separated from the signal of the ADC (MW around 150 kDa) by separation voltage (SV) of 3500 V and compensation voltage (CoV) of 3. The SelexION parameters were optimized by multiple injections to ramp through compensation voltages from -12V to +12V. The lower molecular weight features showed a transmission optimum at the higher end (6-12V) and the higher molecular weight features showed their transmission optimum at lower CoV values (-3 - 0), allowing for separation of the different species from one another (Figure 2).

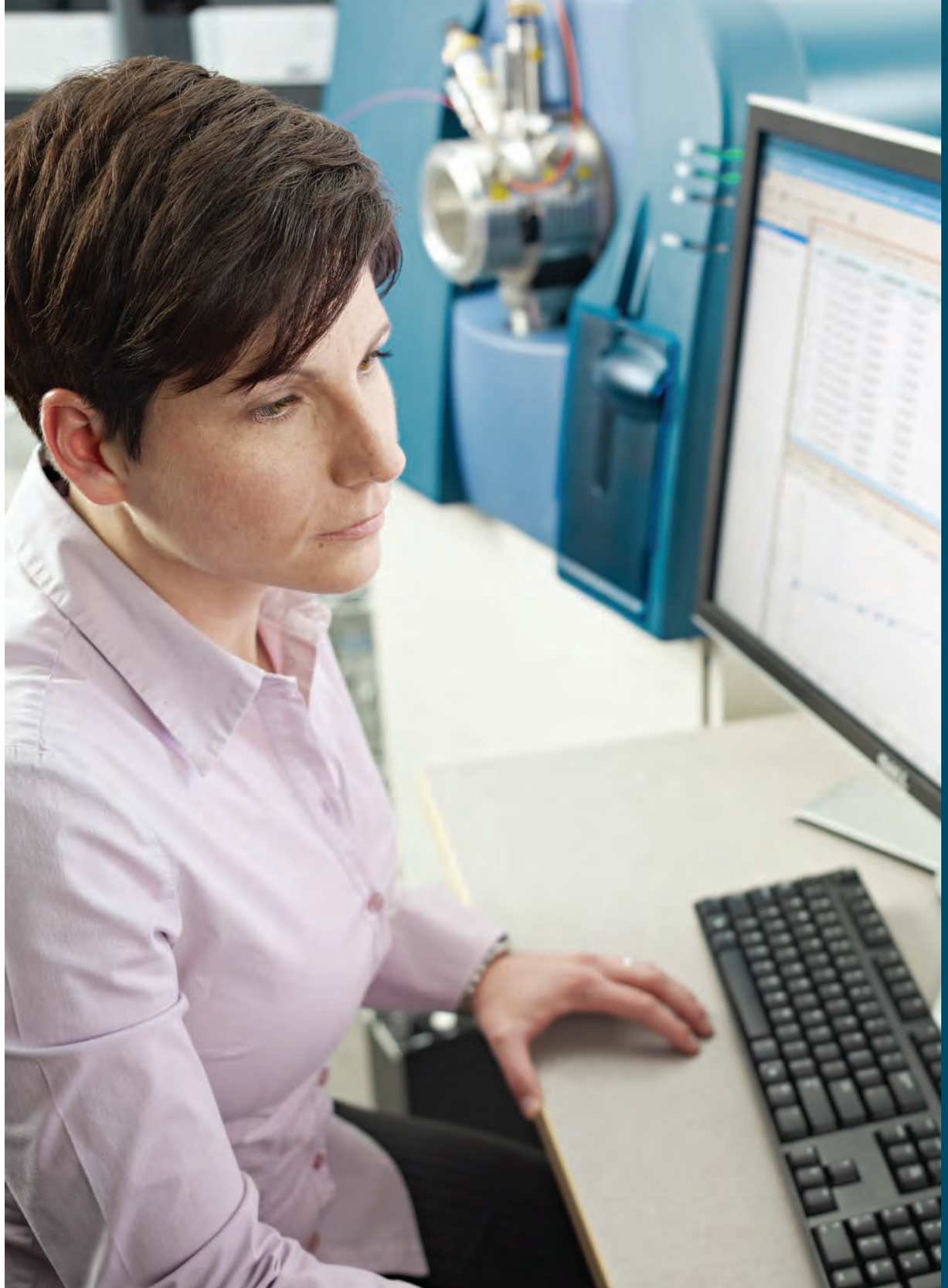
The second chromatographic set-up, with size exclusion type separation, allowed for reconstruction quality spectra to be acquired in normal mode with the TripleTOF system (Figure 3).

Data processing was achieved in BioPharmaView Software which employs maximum entropy reconstruction, here 100 iterations employed (Figure 4). Non-deglycosylated ADC was identified to carry up to 7 payloads per antibody (Figures 4 and 5). The glycosylation pattern showed three glycan structures to be the most significant ones (combinations of G0F-G0F, G0F-G1F, G1F-G1F, G1F-G2F, G2F-G2F; Figure 4). The raw data was complex, carrying heterogeneity from both, the drug conjugation and the glycosylation.

In addition to reconstruction, BioPharmaView Software performs automated DAR calculations (Figure 5). The calculations for the samples shown in Figure 4 are shown in Figure 5. The reconstruction area for each conjugation form is given as a value in the software, and also as a percentage of the total area per each multiplicity of drug conjugation. The multiplicity is represented as columns and the final DAR ratio is calculated for each glycoform. Finally, for batch processing of multiple samples, the software demonstrates a high level of reproducibility in both analysis and reconstruction process (Figure 6).

Conclusions

Complex ADC's can be reproducibly analyzed without the time consuming sample preparation to deglycosylate the protein. The reconstruction with BioPharmaView Software enabled both the DAR to be calculated and the glycosylation pattern to be monitored within a single sample and single analysis for this complex biotherapeutic. The sample analysis time was reduced to obtain higher throughput by excluding sample pretreatment time, combining shorter analysis time with SelexION technology separation, and by enabling fast data-analysis batch-processing by BioPharmaView Software.



BIOLOGICS RESEARCH

Routine Peptide Mapping Workflow for the Rapid Characterization of Biologics

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[1]SCIEX Concord, ON, Canada; [2]SCIEX Foster City, CA, USA; [3]SCIEX Seoul, South Korea

Introduction

In the fast growing biopharmaceutical development space, mass spectrometry has become an important analytical tool in the characterization of biologics. During the development of novel protein biologics, LC-MS characterization needs to be performed to provide detailed structural information in order to address increasingly challenging regulatory expectations. LC-MS can be used to localize and structurally characterize sites of both biological and artifactual modifications (e.g. acetylation, oxidation, deamidation, etc.). However, manual data analysis is inefficient, and typically requires significant levels of experience and expertise. Here, we describe LC-MS workflows using the SCIEX TripleTOF® family of instruments and an advanced, easy to use software solution, BioPharmaView™ Software, that allows for highly efficient characterization of biologics. In this work, we present a completed peptide mapping workflow for characterization of biologics and routine QC analysis with a focus on monoclonal antibodies (mAb).

Materials and Methods

Samples:

Intact mAb Mass Check Standard was purchased from Waters Corporation, 34 Maple Street Milford, MA 01757.

Chromatography:

A Shimadzu Prominence XR system with a Thermo C18 Column, 2.1 x 150 mm, 3 µm at 35 °C was used for separating mAb tryptic digest mixtures. The samples were eluted in a linear gradient from 4-35% acetonitrile and 0.1% formic acid in 45 min at a flow rate of 0.4 mL/min.

Mass Spectrometry:

A SCIEX TripleTOF 5600 system equipped with a TurboIonSpray® ion source was used for data acquisition.

Software:

Analyst® TF 1.5 Software was used for instrument control and data acquisition using Information Dependent Acquisition (IDA). BioPharmaView Software 1.0 was used for peptide mapping analysis and the QC workflow.

Key Benefits

- BioPharmaView Software provides a complete peptide mapping workflow for biologics with a simple user interface.
- The software, in combination with the fast scan speed and high resolution of the TripleTOF system, provides a powerful and easy to use solution for
  - – characterization of biologics
  - – routine QC analysis of biologics

Results

BioPharmaView Software is structured around five key tasks: (1) entering assay information, (2) characterizing standards, (3) batch processing, (4) reviewing results, and (5) generating reports (Figure 1).

The workflow begins with the user entering the information about the protein which is then stored in the project. Once the assay information is entered, the software will subsequently process a standard that has been enzymatically digested to evaluate potential target peptides in the LC-MS profile. The software automatically determines the accurate masses of the potential target peptides and assesses their applicability to the protein of interest. After the processing is complete, the user can sort the result table and review the spectra of the peptides of interest. The user can then choose to update the characterized information of the matched peptides and the processing parameters if desired. After characterizing the biological standard and setting up the comparison criteria, the user can then set up sample and control batches for routine QC analysis. Finally the user can generate a fully customizable report of the QC analysis. Each step is discussed in more detail below.

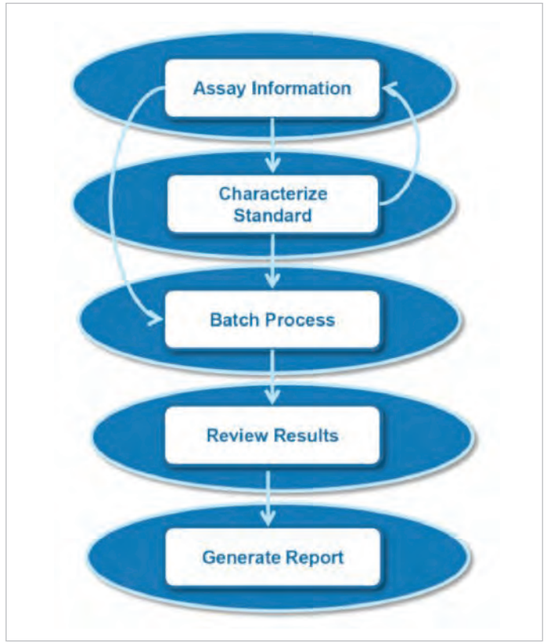

Figure 1: The steps in BioPharmaView Software are shown for characterizing a biologic standard, and then using that information for subsequent automated batch analysis of that biologic.

Assay Information:

Assay information is a central place to store knowledge about the therapeutic protein which can be updated as more about the protein is learned. It contains three tabs: Sequence Features, Intact Protein, and Peptide Mapping. Sequence Features allows users to enter the protein sequence and any known modifications through a user friendly, searchable modification library (Figure 2). The software can calculate the molecular weight (MW) of the intact protein, or a partial sequence of interest. The Intact Protein tab features a summary of the intact protein MW. The Peptide Mapping tab features alkylation, enzymatic digestion, maximum cleavage, and the generation of a digested peptide list.

Characterize Standard:

The user is able to characterize the standard data by simply choosing a file and clicking "Process". The simplified processing parameters and appropriate default settings make it easier for the user to get started and focus on the results. The target peptides are compared with the *in silico* digested peptides based on a user-defined mass to charge tolerance. The processed results are summarized in a tabular format that allows the user to easily navigate and visualize the various aspects of the data (Figure 3). The user has the option of overlaying the XIC with the TIC to aid in the navigation of the chromatography.

Using the tabular result, the user is able to easily navigate among the spectra to confirm the results. The MS/MS spectral information provides further sequence confirmation of the peptide. Once the review is complete, the user can update the characterized peptide information to the central assay information, i.e., peptide matched, intensity, and corresponding retention time. To set up a QC batch, the user can also define pass/fail criteria based on RT, intensity, sequence coverage and unique peptide matched. All information is saved in the project to be used for future QC analysis.

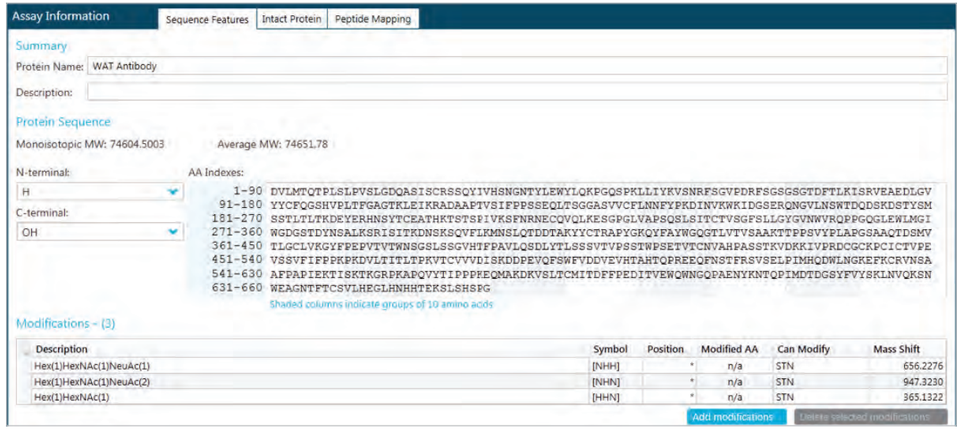

Figure 2: Information for the Biologic is entered into the Assay Information section of BioPharmaView Software.

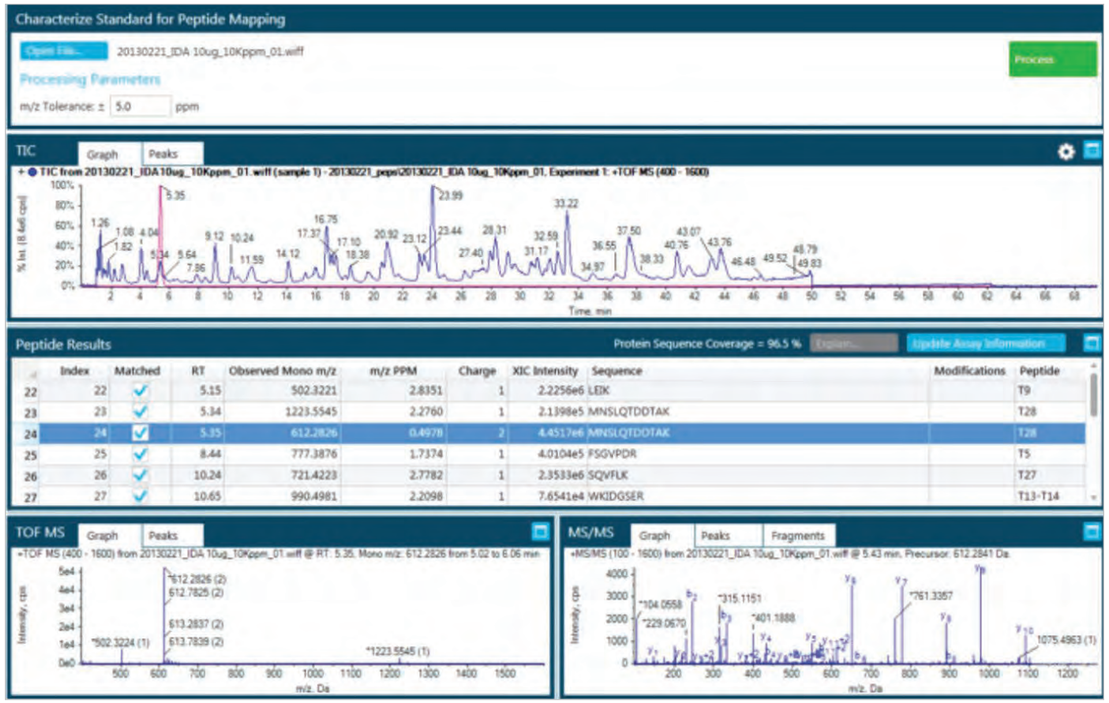

Figure 3: The TIC is shown in the top pane in blue. The XIC of the selected peptide is overlayed in pink, with corresponding MS1 spectra (bottom left) and annotated MS/MS spectra (bottom right). Allowing for a comprehensive view of each selected peptide.

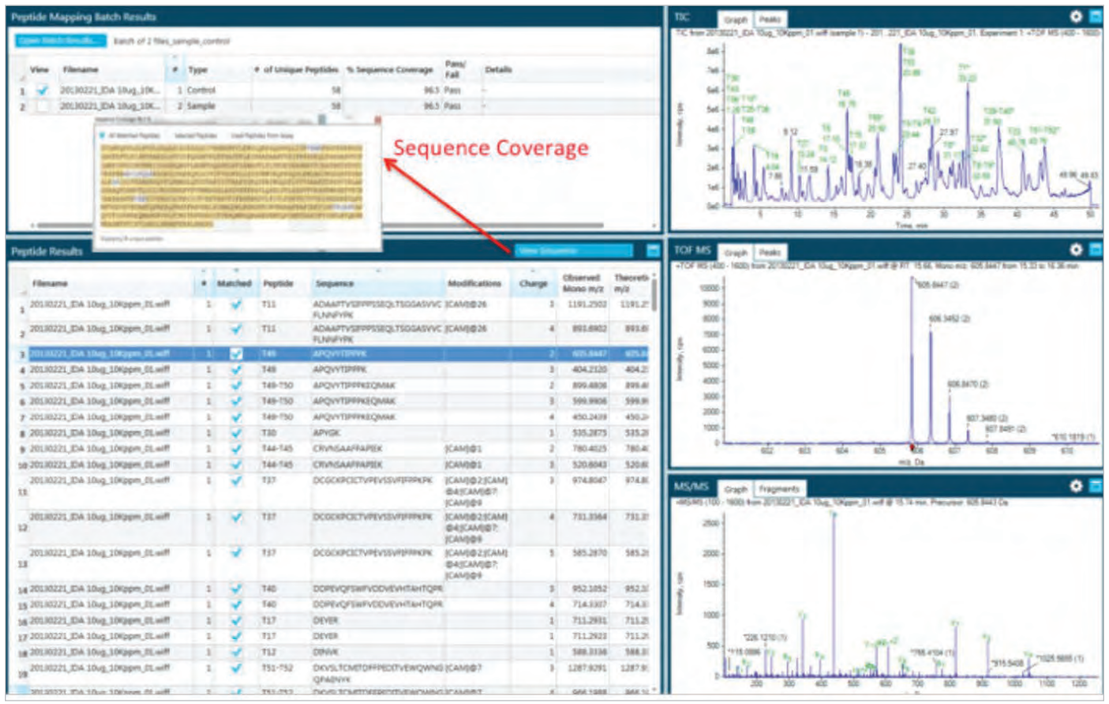

Figure 4: Batch results screen allows for a quick view of overall sequence coverage, labeled TIC, MS1, and MS/MS data for each sample analyzed.

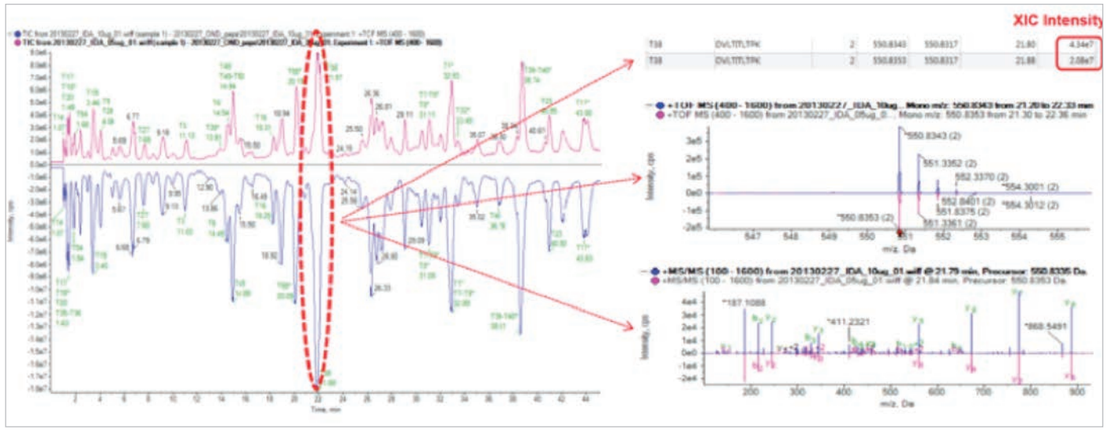

Figure 5: Mirror plots at the chromatographic and spectral levels enable easy comparison of samples with standard.

Batch Process:

In the software, users can perform two types of batch processes: a sample-only batch and a sample-control comparison batch as a QC analysis. Overlaid TIC graphs can be used to pre-screen QC sample files prior to batch processing.

Review Results:

The result review contains three types of information: a high level batch summary, detailed peptide results, and graphical views (Figure 4). The batch summary shows QC results and whether each sample passes or fails based on criteria defined in the assay information. The peptide result table reveals details including each peptide matched, sequence, charge state, PPM difference and RT and intensity information. The corresponding graph information allows users to review the TIC, TOF MS spectrum, and MS/MS spectrum along with the peptide information.

The TIC of both the sample and the control can be displayed as a mirror plot to allow users to examine and compare data visually (Figure 5). If there are differences in the TIC region of interest, the TOF MS and MS/MS spectra can be compared using mirror plots as well. In addition, XIC intensities of matches are reported in the peptide results table and the relative quantitation can be compared.

Generate Report:

After the batch process is complete, you can create and generate QC reports (Figure 6). The user-changeable QC report template includes batch summary information, detailed peptide results, and graphical XIC, TOF MS, and MS/MS spectra for each of the matched peptides.

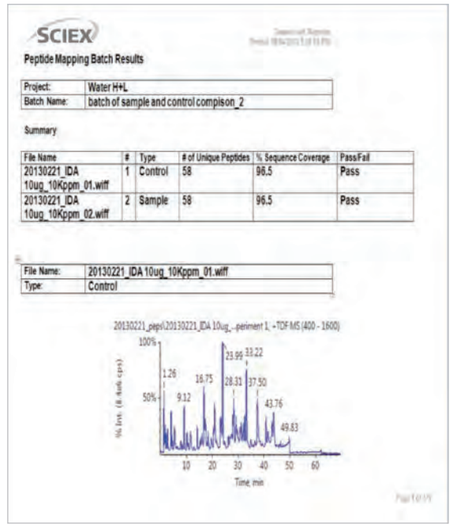

Figure 6: Example of one type of report view.

Conclusion

BioPharmaView Software provides complete characterization and analysis of biologics. The basic steps include assay development, characterization of standards, batch processing, result review, and report generation. When used in combination with the fast scan speed, high mass accuracy, and high resolution of the TripleTOF system, the software provides a powerful and easy to use solution for the characterization of biologics and routine QC analysis.

BIOLOGICS RESEARCH

Automated High Throughput Trypsin Digests for Highly Reproducible Peptide Map Analysis

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Introduction

The extended capabilities of LC-MS characterization for biotherapeutics have put increasing strain on throughput and efficiency. Many processes still depend on manual sample handling, and proteolytic digestions that tie up valuable scientific resources. The added risk of variability in pipetting, digestion time, or time each sample is exposed to reduction/alkylation reagents is high. Highly reproducible LC-MS measurements of biopharmaceuticals may therefore be at risk from processes that are not reproducible, independent of the analytical technique. LC-MS techniques are increasingly able to pick up minute variations and may lead to undesired repeat analyses or additional QC procedures and costs. Automation of default workflows can address variability and improve analytical efficiency in the modern biopharmaceutical industry.

Materials and Methods

Sample Preparation:

A Biomek® NXP Span-8 Laboratory Automation Workstation (Beckman Coulter) with Biomek Software (Figure 1) was used to perform rapid automatic trypsin digestion of 16 sample replicates of a representative monoclonal antibody (mAb). The Biomek was programmed to use octyl glucoside as a denaturant, TCEP as a reducing agent, and MMTS as the alkylating reagent. All reagents were from an in-house SCIEX trypsin digest reagent kit and were used according to the instructions in the kit. A Peltier-heater shaker ALP was used primarily for shaking incubations at different temperatures.

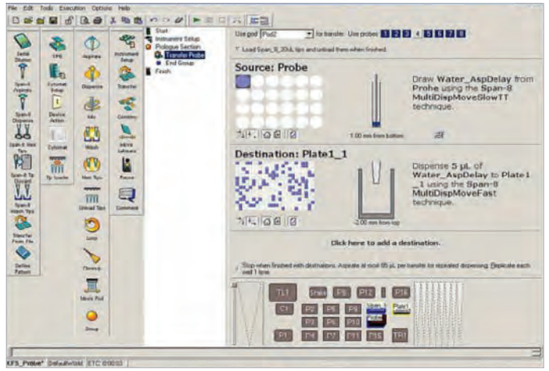

Figure 1: Biomek Software leverages Graphical Representations of Labware to simplify the automation process.

Chromatography:

Samples were analyzed using a SCIEX UPLC System and a CSH c18 column (Waters, 130Å, 1.7 µm, 2.1 mm X 100 mm). Elution gradients of 5-35% B at 250 µL/min in 30 min were run with the column at 60°C. Solvent A was 0.1% formic acid; solvent B was acetonitrile with 0.1% formic acid.

Mass Spectrometry:

All 16 mAb digests were analyzed using a TripleTOF® 6600 LC-MS system. An information dependent acquisition (IDA) LC-MS/MS method was used consisting of a high resolution TOF MS survey scan followed by 20 MS/MS scans in a second with a minimum accumulation time of 50 msec.

Data Processing:

IDA data were matched using BioPharmaView™ Software against the sequence of the mAb. Quantitation was performed using MultiQuant™ Software on a selection of high, medium, and low abundance peptides.

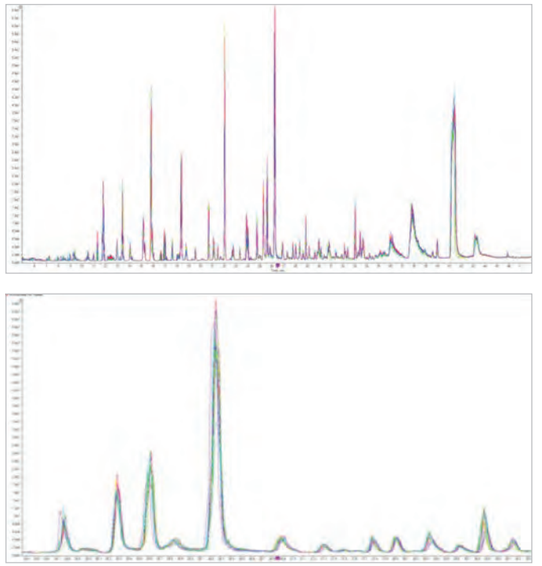

Figure 2: Chromatographic overlay of 16 technical replicates.
Top – Total Ion Chromatograms.
Bottom – Expanded time region between 24.4 and 29.3 minutes.

Light Chain		Heavy Chain	
#	% Coverage	#	% Coverage
1	100	1	98.9
2	100	2	99.3
3	100	3	100
4	97.8	4	96.8
5	100	5	99.1
6	100	6	96.8
7	100	7	97.8
8	100	8	96.7
9	97.3	9	96.8
10	96.8	10	98.0
11	100	11	100
12	100	12	95.8
13	97.3	13	94
14	100	14	97.6
15	100	15	99.3
16	100	16	96.4

%CV of 9.95 was obtained for the 16 peak areas.

Table 1: Peptide Map Qualitative Reproducibility Data for 16 Digest Replicates.

Figure 3: BioPharmaView Software results for the analyzed mAb. The labeled TIC is shown in the top pane in blue. The XIC of the selected peptide is overlayed in pink, with corresponding MS1 spectra (bottom left) and annotated MS/MS spectra (bottom right). Allowing for a comprehensive view of each selected peptide.

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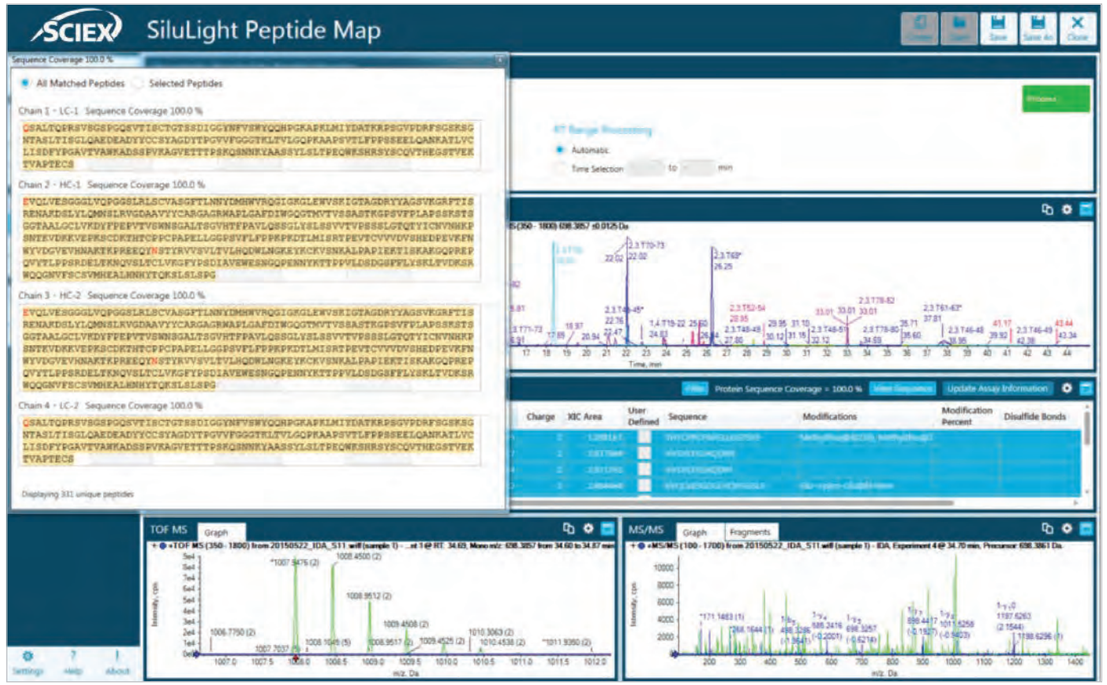

Figure 4: BioPharmaView Software results for mAb peptide mapping showing protein sequence coverage and XIC's for all of the individual matched peptides.

Results

The reproducibility of the trypsin digestion was assessed visually with the chromatograms, as well as qualitatively and quanti-tatively using BioPharmaView and MultiQuant Software.

The top of Figure 2 shows the chromatographic overlay of the 16 mAb digests, while the bottom shows an expanded region between 24 and 29 minutes. As shown in the figure, very good chromatographic reproducibility was obtained with only a small variation observed throughout the entire run.

BioPharmaView Software was used to map the peptides to the mAb sequence. Using this software, the sequence of the mAb was digested in-silico with trypsin and searched with 0-4 deamidations per chain, 0-4 oxidized methionines per chain, and with G0, G1, G2, as well as their fucosylated counterparts assigned to the correct amino acid in the heavy chain. The cysteine alkylating agent was MMTS. Sequence coverage was very high for all 16 runs. Figure 3 shows the Peptide Map and Extracted Ion Chromatogram (XIC) analysis for the doubly-charged ion eluting at 34.7 minutes with m/z 698.3857. Also shown are the TOF MS and MS/MS spectra for this ion. The middle pane shows the peptide sequence match for this peptide with 0.7 ppm mass accuracy.

Clicking the "View Sequence" button from the Peptide Results pane shows the protein sequence coverage as shown in Figure 4.

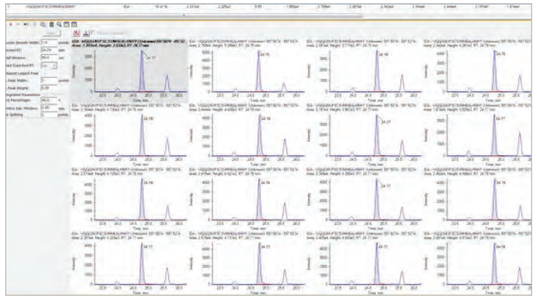

Figure 5: Low abundance peptide XIC data for all 16 replicates. A %CV of 9.95 was obtained for the 16 peak areas.

Here, 100% sequence coverage is observed. The XIC's of all, individual, matched peptides are shown in the chromatogram section behind the sequence coverage.

The sequence coverage data for each of the 16 digests is organized in Table 1. As shown in the table, highly reproducible coverage maps are obtained from all 16 replicates. Data for all different types of peptides were highly similar. The small variation in Heavy chain sequence coverage was entirely attributable to one dipeptide and one tetramer peptide with m/z that were too low to be detected by the method (not within the scan range), but were sometimes detected as a low-level missed cleavage peptide.

Row	Component Name	Num. of Values	Mean	Standard Deviation	Percent CV
1	ALPAPIEK	16	1.67E7	1.171E6	6.97
2	YVDGVEVHNAK	16	1.056E5	7.73E3	6.37
3	WQQGNVFSCSVMHEALHNHY	16	1.19E6	2.325E3	9.95

Table 2: MultiQuant Software Analysis of 16 Digest Replicates.

The digest reproducibility was also assessed quantitatively using MultiQuant Software. For any type of MS-based chromatographic quantitation, MultiQuant Software will automatically integrate, calculate peak areas, Mean, Standard Deviation and Percent CV for any selected group. The Software can also be validated for 21CFR11 compliance.

Table 2 summarizes the MultiQuant analysis. In this case, it was the peak area of the XIC from three different peptides across all 16 digest replicates. These three peptides were chosen as they represent high abundance (Row 1), medium abundance (Row 2, chosen from the middle of the list of all peptides sorted by XIC area), and a low abundance peptide (Row 3). The raw XIC data from all 16 replicates of the low abundance peptide is shown in Figure 5.

Conclusion

Qualitatively, each of 16 digests had 96.8% or higher coverage of the light chain and 94% or higher coverage of the heavy chain with slight variation in detection of very small peptides that were below the acquisition m/z range in the mass spectrometry IDA method. Quantitatively, the chromatographic peak areas from low, medium and high abundance peptides were within 10% CV.

Automated digestion, data acquisition and data analysis work in concert to provide robust and reproducible relative intensity results to benefit an organization in a number of ways:

- Streamlines peptide mapping sample preparation.
- Increases efficiency and reduces time and money costs associated with human error in sample prep and pipetting measurements.
- Automated sample prep increases analytical throughput.
- Saves time for scientists to do science.

Fast, Efficient, Disulfide Bond Mapping Using BioPharmaView™ Software

Annu Uppal[1], Dilip K Reddy[2], Shirish Patel[2], Anita Krishnan[2], and Manoj Pillai[1]

Introduction

Accurate disulfide bond mapping is essential for correctly establishing structure-function relationships as well as for monitoring the structural integrity of recombinant monoclonal antibodies (mAbs) throughout their production. Inappropriate disulfide bonds can affect a mAb's stability, potency, aggregation, and may also signal errors in the cell culture or purification process. By following a mAb's disulfide patterns over time, manufacturers can quickly detect production problems and then correct them as early as possible.

Correctly assigning disulfide bonds in a mAb can be challenging and time consuming due to the heterogeneity, large size, and multiple cysteine residues found in these biomolecules. Traditional approaches for disulfide mapping are based on fast liquid chromatography-mass spectrometry analysis; however, these methods can be inefficient and usually involve digestion with multiple enzymes, tedious data processing, and intensive manual inspection of chromatograms for the identification of any possible disulfide linkages.

As the biotherapeutics industry develops and expands, there is an urgent need for software tools that can rapidly facilitate and accelerate the higher-order structural characterization of biopharmaceutical products. To meet these needs, SCIEX has developed BioPharmaView™ Software, a data processing suite that can reduce the complexity of the massive data sets generated during large macromolecule analysis. BioPharmaView Software uses rapid processing tools to accelerate critical characterization assays–such as peptide mapping and disulfide bond identification–by automating peak assignments, simplifying data processing, and streamlining the reporting process.

To identify and sequence peptides, BioPharmaView Software automatically scores b- and y- ion annotations; and then the highest scoring experimental peaks are compared to a list of theoretical masses automatically generated by the software. The peak assignment process is further enhanced by predicting the theoretical fragment ion masses for non-reduced, disulfide-linked peptides prior to comparison with experimental data. Including other criteria in the ion selection process–such as MS/MS scoring, multiple charge states, and a retention time (RT) filter–can also help reduce the time needed for peptide mapping experiments. This enables manufacturers to meet regulatory requirements more quickly during the production and marketing of a new biotherapeutic product.

In this article, we successfully developed an efficient and automated workflow that comprehensively identified every disulfide linkage in the Fab region of a mAb. By using BioPharmaView Software to process accurate mass data sets obtained on a hybrid triple quadrupole time-of-flight (TOF) mass spectrometer, we were able to identify the location of five disulfide linkages in the Fab region of a mAb. The use of the high-speed, TripleTOF® 5600 LC-MS System further contributed to time-savings during disulfide analysis by permitting accurate mass MS and MS/MS information to be collected simultaneously, providing the high-resolution data necessary for differentiating closely related species and confirming structural assignments, all at the fast chromatographic speeds needed for efficient analysis.

Materials and Methods

HPLC Conditions

Samples of a mAb raised against lupine seeds were digested with trypsin under both reducing and non-reducing conditions. Native and reduced tryptic digests were separated using a Shimadzu UFLCXR system equipped with a Kinetex C18 column (Phenomenex, 2.1 x 100 mm, 3 µm). Solvent A consisted of 2% acetonitrile and 0.1% formic acid, and solvent B consisted of 98% acetonitrile with 0.1% formic acid. Samples were injected and analyzed under high-flow conditions (0.2 mL/min).

Mass Spectrometry

LC-MS/MS analysis of peptides separated under high-flow conditions was completed using a TripleTOF 5600 System coupled to a DuoSpray™ Ion Source. A generic information dependent acquisition (IDA) workflow was employed as follows: 1) an MS scan was acquired in high-resolution mode using an accumulation time of 250 ms per spectrum; 2) followed by the acquisition of 20 MS/MS scans of 50 ms each; 3) after peak selection, each previously acquired ion was placed on a dynamic exclusion list for 5s. Rolling collision energy and a collision energy spread of 5 V were used.

Data Processing

Experimental accurate mass data was compared to a list of theoretical peptide masses generated within a pre-defined mass error tolerance for the automatic identification of disulfide bond pairings using BioPharmaView Software. Peptide sequence information and any corresponding post-translational modifications were assigned to each peak during the analysis. Additional criteria in the peptide ion selection process, such as MS/MS scoring, inclusion of multiple charge states and retention time (RT) filtering, were used to ensure the correct assignment of disulfide bonds.

Results

To investigate the pattern of disulfide bonds in the Fab region of a mAb, native and reduced tryptic peptides were analyzed using the TripleTOF 5600 System, a high-speed hybrid instrument that acquires accurate mass MS and MS/MS data simultaneously using an IDA workflow. This high-resolution accurate mass information provides increased depth to the structural characterization process and permits researchers to differentiate more easily between closely related species and to confirm structural details more quickly. BioPharmaView Software was able to assign identity to each disulfide-containing peak using the high-quality, high-resolution structural information acquired through IDA experiments on the TripleTOF System.

The comparative profile of the reduced and non-reduced digest (Figure 1) predominantly mirror each other; however, there are unpaired peaks in the non-reduced and native profiles, which indicate the presence of disulfide-linked peptides and reduced peptides that were prior disulfide partners, respectively (Figure1).

In total, five disulfide pairs in the mAb Fab region were identified: two intra-light-chain pairs (C194-C134 and C23-C88), two intra-heavy-chain pairs (C22-C96 and C150-C206), and one inter-chain disulfide bond between the heavy and light chain (C226-C214).

Included below are three representative examples of accurate-mass MS spectra and the corresponding MS/MS data that were used to characterize disulfide bond pairs from different regions of the Fab fragment:

- A disulfide bond located within the light chain (C194-C134, Figure 2)
- A disulfide located in the heavy chain (C22-C96, Figure 3)
- A disulfide located between the light and heavy chain (C226-C214, Figure 4)

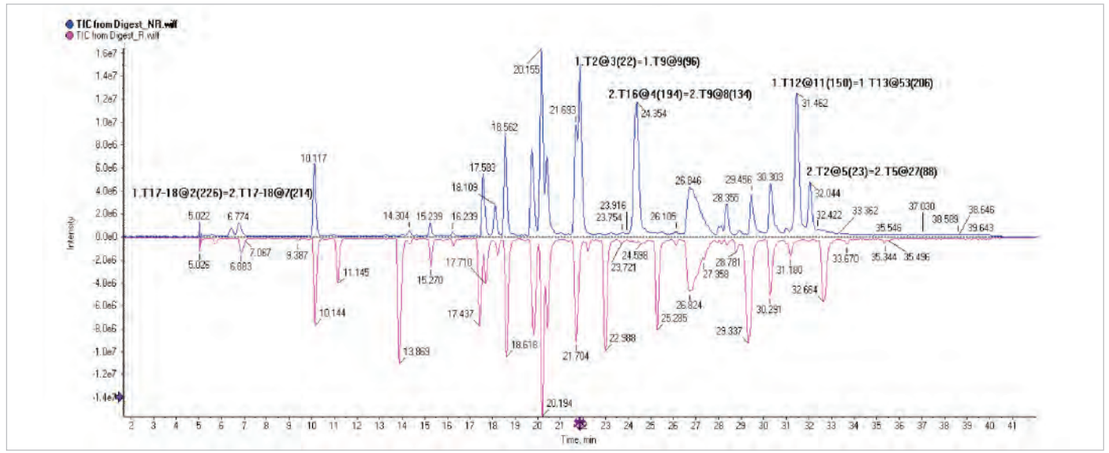

Figure 1: The comparative profiling of tryptic peptides from a mAb under reducing (pink trace) and non-reducing (blue trace) conditions is shown. Non-reduced peaks that do not align with a peak in the reduced trace indicate the presence of disulfide-linked peptides.

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Figure 2: Automated identification of the disulfide bond C194-C134 in the light chain of the Fab. An extracted ion chromatogram (XIC) trace displays peaks obtained during TOF MS analysis of the tryptic digest (upper panel). BioPharmaView Software automatically identified four charge states for the C194-C134 disulfide-containing peptide with a good MS/MS score (middle panel, +3, +4, +5, and +6). A high-resolution TOF MS spectrum of the disulfide-containing peptide is displayed (lower left panel), and the corresponding MS/MS spectrum shows the fragment ions for that peptide (lower right panel).

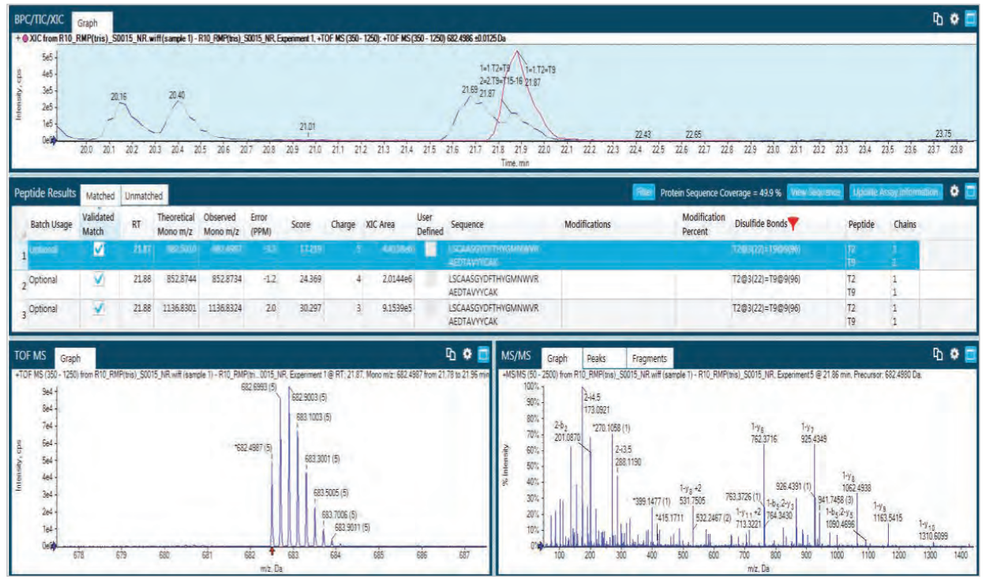

Figure 3: Automated identification of the disulfide bond C22-C96 in the heavy chain of the Fab. An extracted ion chromatogram (XIC) trace displays peaks obtained during TOF MS analysis of the tryptic digest (upper panel). BioPharmaView Software automatically identified three charge states for the C22-C96 disulfide-containing peptide with a good MS/MS score (middle panel, +3, +4 and +5). A high-resolution TOF MS spectrum of the disulfide-containing peptide is displayed (lower left panel), and the corresponding MS/MS spectrum shows the fragment ions for that peptide (lower right panel).

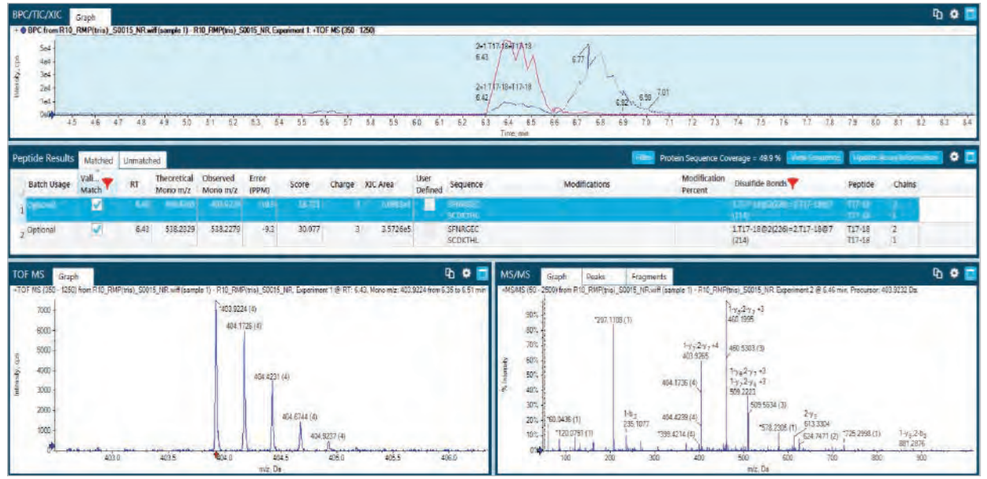

Figure 4: Automated identification of the disulfide bond C226-C214 between the heavy and light chain of the Fab. An extracted ion chromatogram (XIC) trace displays peaks obtained during TOF MS analysis of the tryptic digest (upper panel). BioPharmaView Software automatically identified two charge states for the C226-C214 disulfide-containing peptide with a good MS/MS score (middle panel, +3 and +4). A high-resolution TOF MS spectrum of the disulfide-containing peptide is displayed (lower left panel), and the corresponding MS/MS spectrum shows the fragment ions for that peptide (lower right panel).

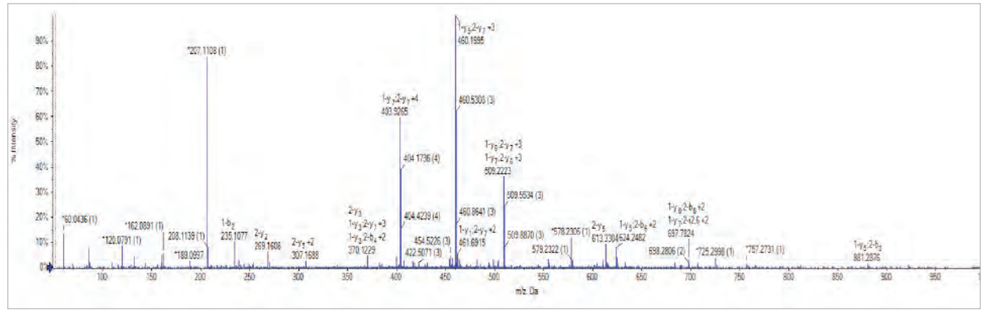

Figure 5: MS/MS annotation of Disulfide linkage C226-C214. The b- and y- ions of individual as well as bonded peptide provides the high level of confidence in identification and localization of linkage.

The extracted chromatograms or base peak chromatograms (upper panels, Figures 2-4) highlight the relevant species that were evaluated for disulfide bonding. In the middle panels, peak characteristics–such as RT, theoretical and observed m/z, MS/MS spectra scores, and the predicted charge-state–are catalogued in a table. Because of the high quality of the MS and MS/MS data, multiple charge states can be identified for each peptide, which builds significant confidence in the disulfide-bond assignments.

BioPharmaView Software automates the peak assignment based on the MS information, and uses the MS/MS fragment ion information for scoring. The scoring algorithm is based on b & y fragment ions annotation, mass accuracy, along with many other parameters. The user has the flexibility to set the threshold value of the score for auto-validation of the data, thereby providing an automatic way for the software to identify the higher quality matches (middle panels, Figures 2-4). The coverage of individual b and y ions with or without the disulfide bond for disulfide linkage C226-C214 is shown in Figure 5 & Table 1, which is usually sufficient for high level of confidence in identification and assignment of the disulfide linkage position.

Conclusion

An automated and efficient peptide mapping workflow in BioPharmaView Software was used to successfully locate and identify all disulfide-containing linkages in the Fab region of a mAb. To pinpoint the location of the five disulfide-containing peptides, the profiles for reduced and native tryptic peptides were compared in PeakView® Software, and non-aligning peaks were further sequenced and structurally characterized in BioPharmaView Software. BioPharmaView Software automatically evaluates multiple factors before validating the identity for each peptide–such as b and y ion annotation, MS/MS scoring, multiple charge states, retention time, and mass accuracy. In aggregate, this workflow for disulfide-bond analysis benefits from the combination of high-quality accurate-mass MS and MS/MS data obtained simultaneously on a high-resolution TripleTOF System and the remarkable automation of the peak assignment by BioPharmaView Software, thus simplifying data processing, reporting and therefore reducing the overall processing time.

Fragment	Type	Mono. m/z	Error (Da)	Charge	Mono. Mass	Nomenclature	Fragment
RGEC[*1] / SC[*1]D	b,y	384.13	0.032	2	766.24	1-y4; 2-b3, +2	RGEC[*1] / SC[*1]D
C[*1] / C[*1]DKTHL	2y	418.18	-0.004	2	834.34	1-y1;2-y6, +2	C[*1] / C[*1]DKTHL
C[*1] / SC[*1]DKTHL	y	461.69	-0.004	2	921.37	1-y1;2-y7, +2	C[*1] / SC[*1]DKTHL
SFNRGEC[*1] / SC[*1]DKTHL	y	538.23	-0.003	3	1611.68	1-y7; 2-y7, +3	SFNRGEC[*1] / SC[*1]DKTHL
NRGEC[*1] / SC[*1]DKTH	b,y	624.25	-0.006	2	1246.48	1-y5; 2-b6, +2	NRGEC[*1] / SC[*1]DKTH
SFNRGEC[*1] / C[*1]DKT	b,y	629.25	-0.010	2	1256.49	1-y6; 2-b5, +2	SFNRGEC[*1] / C[*1]DKT
FNRGEC[*1] / SC[*1]DKT	b,y	629.25	-0.010	2	1256.49	1-y7; 2-i2,5, +2	FNRGEC[*1] / SC[*1]DKT
NRGEC[*1] / SC[*1]DKTHL	y	689.80	-0.004	2	1377.58	1-y5; 2-y7, +2	NRGEC[*1] / SC[*1]DKTHL
SFNRGEC[*1] / SC[*1]DKTH	b	741.30	-0.005	2	1480.58	1-y7;2-2-b6	SFNRGEC[*1] / SC[*1]DKTH
C[*1] / C[*1]DKTHL	2y	835.34	-0.006	1	834.34	1-y1;2-y6	C[*1] / C[*1]DKTHL
NRGEC[*1] / SC[*1]D	b,y	881.29	0.003	1	880.28	1-y5;2-b3	NRGEC[*1] / SC[*1]D
C[*1] / SC[*1]DKTHL	y	922.38	-0.005	1	921.37	1-y1;2-y7	C[*1] / SC[*1]DKTHL
SFNRGEC[*1] / SC[*1]	b	1000.36	0.001	1	999.35	1-y6;2-b2	SFNRGEC[*1] / SC[*1]
SFNRGEC[*1] / C[*1]D	b,y	1028.36	0.006	1	1027.35	1-y6;2-b3	SFNRGEC[*1] / C[*1]D
FNRGEC[*1] / SC[*1]D	b,y	1028.36	0.006	1	1027.35	1-y7;2-i2,3	FNRGEC[*1] / SC[*1]D
SFNRGEC[*1] / SC[*1]D	b	1115.39	0.004	1	1114.38	1-y7;2-b3	SFNRGEC[*1] / SC[*1]D
SFNRGEC[*1] / C[*1]DK	b,y	1156.45	-0.022	1	1155.44	1-y6;2-b4	SFNRGEC[*1] / C[*1]DK
FNRGEC[*1] / SC[*1]DK	b,y	1156.45	-0.022	1	1155.44	1-y7;2-i2,4	FNRGEC[*1] / SC[*1]DK

Table 1: The HL Chain C226-C214 bonded peptide fragment ion information is shown. The MS/MS fragment Ion assignment provided by the BioPharmaView Software helps with accurate location of disulfide bonds.

Acknowledgements

We would like thank Drs. Anita Krishnan, Dilip K Reddy and Shirish Patel of LUPIN Limited Biotech Division, Pune, India for helpful discussions during the sample analysis and data processing.

Assay of IgG Purity and Heterogeneity using High-Resolution Sodium Dodecyl Sulfate Capillary Gel Electrophoresis

Lucy Y. Liu, Chitra Ratnayake, Jeff Chapman, Narasaiah Dontha, Sae Choo, and M. P. Reddy
Beckman Coulter, Inc.

Overview

In this application note, we present the development of both a new polymer formulation and standardized methodology to assess the purity and heterogeneity of IgG and its isoforms. This methodology provides increased resolution of the IgG isoforms from typical developmental impurities like non-glycosylated heavy chain and low-molecular-weight impurities associated with the IgG light chain. The assay includes an artificially engineered IgG control with a fixed percentage of non-glycosylated heavy chain to provide assay suitability determination prior to the analysis of unknowns. All aspects of the methodology from preparing samples to automating data analysis will be discussed.

Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used analytical technique for the molecular weight estimation and purity assessment of proteins.[1] However, SDS-PAGE suffers from several limitations, including the use of neuro-toxic reagents, the manual labor-intensive methodology, and the lack of direct, accurate quantification. Alternatively, capillary gel electrophoresis (CGE) in the presence of sodium dodecyl sulfate (SDS) has been applied for automated quantitative protein analysis.[2-8] Compared to SDS-PAGE, CGE offers automation, high-speed separation, enhanced resolution, and direct, on-line quantification using UV and fluorescence detection. In SDS-CGE, the SDS is bound to all the proteins at a constant weight ratio such that the SDS-protein complexes have approximately equivalent charge densities.The constant mass-to-charge property of the SDS-bound proteins allows separation according to differences in molecular weight. As the logarithm of the molecular mass of a protein is linear with its electrophoretic mobility, the molecular weight of a protein can be estimated from a series of well-chosen standards.

Replaceable gels, comprising linear polymers in solution, are commonly used as the separation matrix in SDS-CGE. The low viscosity of the linear polymer solutions allows the replacement of the sieving matrix after each analysis, improving the assay precision and robustness. Many different polymer matrices have been applied to the separation of SDS-proteins in CGE. Demorest[9] described the use of linear polyacrylamide polymer solutions for the separation of SDS proteins and DNA. Karger[3] further described the use of a gel matrix of UV transparent polymers, such as Dextran and PEO. Since this time, several commercial products have been introduced using this approach.

We have developed a new, replaceable polymer matrix to improve the current resolving power of SDS-CGE and have developed this matrix into a commercial IgG Purity/Heterogeneity assay. The methodology involves heat denaturing a specified concentration of IgG (both reduced and nonreduced) in the presence of SDS. This application bulletin reviews the fundamental components of this assay development.

Materials and Methods

Instrumentation

All experiments were performed on the ProteomeLab™ PA 800s (Beckman Coulter, Inc., Fullerton, CA). Bare, fused-silica capillaries of 50 µm ID × 20 cm to detection were used for the separation.

Reagents

SDS-gel buffer, SDS sample buffer, SDS protein size standards, 10 kD protein internal standard, and the IgG suitability standard were all manufactured at Beckman Coulter, Inc. (Fullerton, CA). Sigma (St. Louis, MO) was the source for 2-mercaptoethanol.

The SDS gel buffer creates a physical gel of an entangled polymer network for separation of the SDS-protein complexes. The gel buffer comprises a proprietary polymer buffer formulation (at pH 8.0) with 0.2% SDS.

The SDS sample buffer is used to prepare the SDS-protein complex for the IgG assay. The sample buffer is composed of 100 mM Tris-HCl, pH 9.0, with 1% SDS.

The acidic wash solution is a high-purity reagent comprised of 0.1 N HCl. The basic wash solution is a high-purity reagent composed of 0.1 NNaOH.

Preparation of SDS-Protein Complex

When preparing the SDS-protein complex, we diluted the protein sample solution (2–40 mg/mL concentration) with SDS sample buffer to give a final concentration of 1 mg/mL. The IgG-SDS complex was reduced by adding 5% neat 2-mercaptoethanol v/v, and then heating in a 70°C water bath for 10 minutes. For a non-reduced sample, the IgG-SDS complex was first alkylated with iodoacetamide by adding 5% of a 250 mM iodoacetamide v/v solution into the protein SDS buffer mixture and then heated in a 70°C water bath for 10 minutes.

Separation and Analysis

An optimized separation method and sequence were set up for batch analysis of 24 samples at a time. For each separation cycle, the capillary was first preconditioned with 0.1 N NaOH , 0.1 N HCl, deionized water, and SDS gel buffer. All gel buffers were degassed for 2 minutes under vacuum prior to use. Samples were electrokinetically introduced by applying voltage at -5 kV for 20 seconds. Electrophoresis was performed at constant voltage with applied field strength of (-) 497 volts/cm with a capillary thermostatted to 25°C using recirculating liquid coolant. The current generated was approximately 27 µAmps. The system was programmed to automatically replenish all reagents through an increment of the buffer array after every eight cycles.

Results and Discussion

Resolving Power

To effectively resolve non-glycosylated from glycosylated IgG heavy chain, it was first necessary to formulate a gel of exceptionally high resolving capacity. A protein sizing ladder containing recombinant proteins of 10, 20, 35, 50, 100, 150, and 225 kDa was developed to assist both in the gel formulation phase to optimize the resolving capacity of the gel and to estimate the MW of unknowns. A recombinant 10 kD protein was used as an internal reference standard for mobility determination to accurately assign protein identification. Figure 1 illustrates a typical electropherogram of the analysis of the protein-sizing ladder using this method. The inset in Figure 1 illustrates a plot of the Log MW versus 1/Mobility and demonstrates good linearity of response over the standards sizing range. Figure 2 illustrates a different formulation of the protein sizing ladder in which we changed the concentration of the individual components and further spiked the mixture with an 11 kDa protein. The baseline separation of the 10 and 11 kDa proteins highlights the good resolving power in this size region. Differences in detection response are due to the concentration of the individual proteins used in the two size standard formulations.

IgG Suitability Standard

Our criteria for the development of the IgG purity/heterogeneity assay include both resolution specifications for reduced and non-reduced IgG and quantitation specifications for detecting

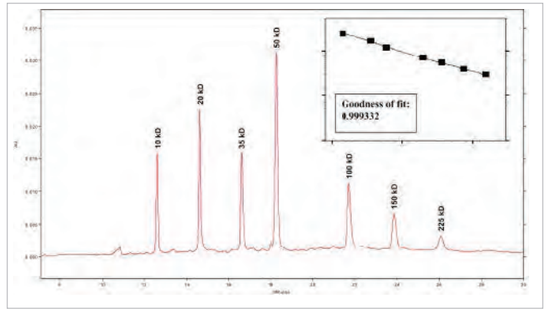

Figure 1: Resolution of the ProteomeLab SDS-Gel MW standards. The inset plot illustrates the good linearity of the sizing standards plotted as Log MW versus 1/Mobility.

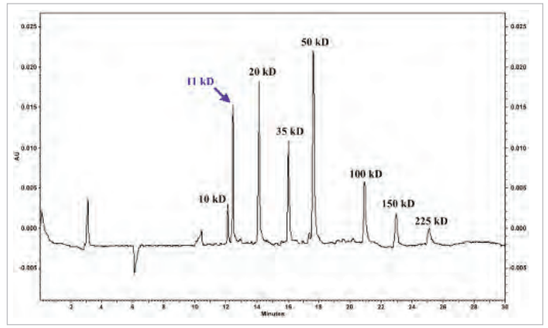

Figure 2: A different formulation of the protein size standards spiked with an 11 kDa protein.

impurities. To ensure good control in both the manufacture and implementation of this assay, we have developed an IgG suitability standard that will test both the resolution and quantitation requirements. As the percent of non-glycosylated heavy chain is an important assessment parameter, yet is variable in quantity and difficult to resolve, it became a natural component to focus upon in the creation of a suitability standard. The ProteomeLab IgG Suitability standard has been designed to contain a controlled quantity of the non-glycosylated heavy chain (9.5% of total heavy chain), which allows us to test both the resolving capacity of the gel and to ensure the quantitation variability is within an acceptable range before beginning the assay of unknowns. Figure 3 illustrates the analysis of the IgG suitability standard in both the reduced and non-reduced forms. Under reducing conditions, a very good separation between light and heavy chain is obtained, while the glycosylated heavy chain is baseline resolved from non-glycosylated (NG) heavy chain.

Assay Precision

Table 1 summarizes the results of six consecutive analyses of the reduced IgG standard. The relative standard deviation (% RSD) of both the light chain and heavy chain mobility was < 1%, while the quantitative determination of the % NG heavy chain was also < 1%. Assay precision was also evaluated with the non-reduced

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Injection	LC Mobility	HC Mobility	LC%	HC%	NG%	HC/LC
1	-0.0000448	-0.0000347	31.36	62.10	9.53	1.98
2	-0.00004482	-0.00003477	31.42	61.96	9.66	1.97
3	-0.00004478	-0.00003469	31.31	62.06	9.65	1.98
4	-0.0000448	-0.00003471	31.35	62.06	9.60	1.98
5	-0.00004489	-0.00003488	32.34	61.07	9.74	1.89
6	-0.00004488	-0.00003487	31.37	62.06	9.58	1.98
Mean:	-0.00004487	-0.00003488	31.52	61.88	9.63	1.96
Std Dev.	0.00000008	0.00000017	0.4007	0.4007	0.0729	0.0369
%RSD:	0.17	0.49	1.27	0.68	0.757	1.46

Table 1: Assay Precision for the Reduced IgG Suitability Standard Percent quantity is determined from the velocity corrected peak area of the analyte/total velocity-corrected peak area of all IgG analytes (excludes 10 kDa mobility marker).

Injection	Mobility of IgG	LMW%	Main%	NG%
1	-0.2585	6.36	85.11	9.11
2	-0.2601	6.67	84.68	9.27
3	-0.2602	6.79	84.49	9.36
4	-0.2616	6.75	84.50	9.38
5	-0.2615	6.63	84.54	9.46
6	-0.262	6.79	84.75	9.08
Mean	-0.2607	6.66	84.68	9.28
Std Dev	0.00130958	0.16	0.24	0.15
% RSD	0.66	2.4	0.28	1.62

Table 2: Assay Precision of the Non-Reduced IgG Suitability Standard

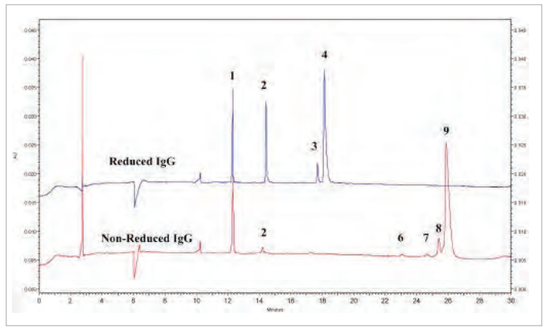


Figure 3: Analysis of both the reduced and non-reduced IgG Suitability standard using the IgG purity method. Peak 1: Internal standard (10 kDa); 2: Light Chain (L); 3: NG heavy chain; 4: Heavy chain (H); 6: Heavyheavy chain (2H); 7: 2 heavy 1 light chain (2H1L); 8: NG HC; 9: IgG monomer.

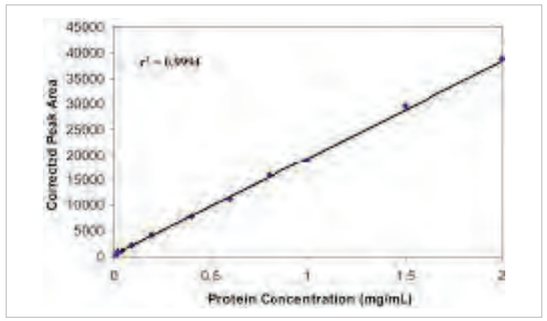


Figure 4: Linearity of detection response, plotting velocity-corrected peak area versus protein concentration over the range of 0.02 to 2 mg/mL of the 10 kDa standard.

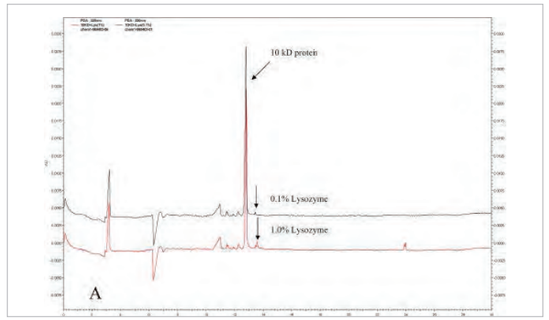
IgG (Table 2). All the impurities, such as light chain (L), heavy chain (H), heavy-heavy chain (HH), and 2 heavy 1 light chain (HHL), are well resolved from the intact antibody. The nonglycosylated heavy chain was also baseline separated from the IgG monomer (Figure 3). From six replicate analyses, the RSD value of IgG mobility is < 1% (Table 2). The % RSD of the quantity determination of the minor impurities is < 3%.

Detection Linearity

The linearity of assay detection was also investigated. Figure 4 illustrates the linearity of the detector response using the corrected peak area versus protein concentration obtained from introducing increasing concentrations of the recombinant protein mobility marker (0.02 to 2 mg/mL) into the assay. Good linearity with a coefficient of determination (r2) of 0.9994 was achieved over this concentration range.

Low-Level Impurity Detection

To demonstrate the low-level detection of impurities, we spiked the 10 kDa mobility marker with lysozyme at both 1% and 0.1% of the total loaded protein. Figure 5A illustrates the resolution and detection of this added protein at both concentration levels. The signal-to-noise ratio of 0.1% lysozyme is approximately 6:1. We subsequently introduced a second protein, the 11 kDa recombinant protein, at 0.1% into the IgG control standard. By doubling the injection time to 40 seconds, we could improve the signal-to-noise ratio to as high as 10:1 at the 0.1% level of contamination. This effect is illustrated in Figure 5B. Both examples used reducing conditions.



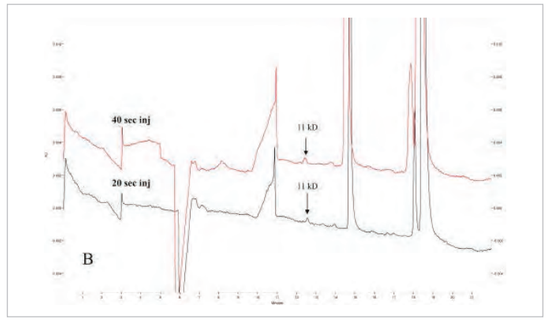


Figure 5: Panel A illustrates the detection of lysozyme spiked into the mobility marker at 1% and 0.1% levels.

Panel B illustrates an 11 kDa protein spiked into our IgG Suitability standard at 0.1%. The impact of injection time from 20 to 40 seconds is indicated.

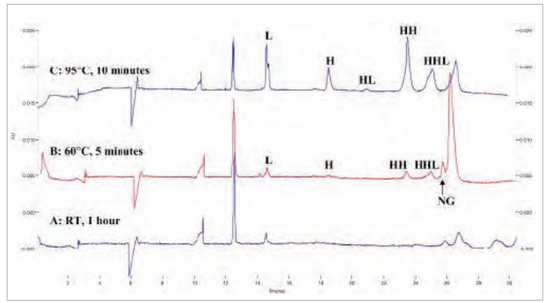


Figure 6: Separation of non-reduced IgG Standard prepared at different temperatures. A: Sample incubated at room temperature for 1 hour; B: Sample heated at 60°C for 5 minutes; C: Sample heated at 95°C for10 minutes.

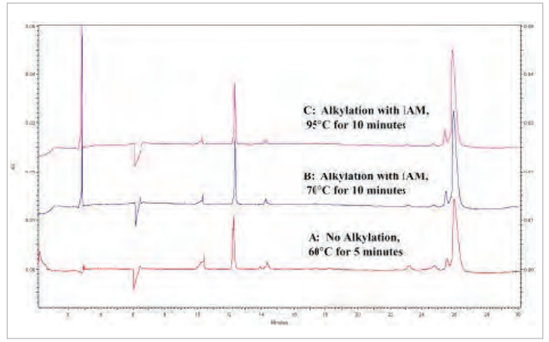


Figure 7: Separation of non-reduced IgG Standard prepared at different temperatures. A: Sample incubated at room temperature for 1 hour; B: Sample heated at 60°C for 5 minutes; C: Sample heated at 95°C for10 minutes.

Impact of Sample Preparation on IgG Fragmentation Artifacts

A 1% SDS Sample Buffer solution was used to prepare the SDS-protein complexes for CE-SDS separation. During sample preparation, heating the sample solution at high temperature is required to accelerate SDS-binding. As shown in Figure 6A, no IgG signal was observed when non-reduced IgG was prepared at room temperature for 1 hour. Figure 6B shows a separation profile of non-reduced mouse IgG treated at 60°C for 5 minutes. The whole antibody was well separated from all the impurities such as the light chain, heavy chain, heavy-heavy chain, and 2-heavy-1-light chain. However, heating a non-reduced IgG sample at high temperature may introduce fragmentation and aggregation, and thus alter the accuracy of the sample analysis. A significant increase in IgG fragment peaks was observed when the IgG sample was heated at 95°C, indicating broken disulfide bonds between both the light and the heavy chains (Figure 6C). This temperature associated fragmentation may be significantly reduced through sample alkylation procedures.

Alklyation of the non-reduced IgG sample serves to stabilize the disulfide bond. Iodoacetamide (IAM) at 12.5 mM was added into the protein sample buffer prior to heating. The sample was then treated at 70°C and 95°C for 10 minutes. Figure 7 compares the separation profile of the IgG Control Standard with and without alkylation. Better resolution was observed with the alkylated IgG sample simply because more efficient SDS binding was achieved at the higher temperature treatment. Furthermore, the disulfide bond of the IgG molecule was stabilized after alkylation, so no fragmentation was observed, even when the sample was treated at 95°C for 10 minutes.

Effect of High Salts

Since the assay uses electrokinetic sample introduction, the signal intensity and resolution of the analytes may be affected if the salt concentration in the sample becomes too high. Figure 8 shows the effect of a mouse IgG1K sample that contains 150 mM NaCl in the final sample preparation. Without desalting, using the standard sample introduction conditions, very low signal was obtained, and nonglycosylated heavy chain remained undetectable. However, after desalting the sample, the signal intensity was significantly improved, and the nonglycosylated heavy chain was well detected and separated from the glycosylated heavy chain. Generally, if the final salt concentration is below 50 mM, no desalting steps are required.

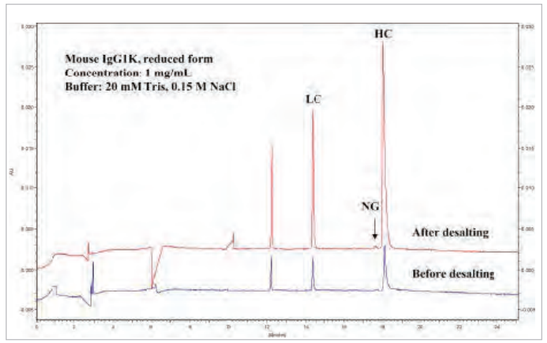


Figure 8: Effect of salt concentration on assay performance.

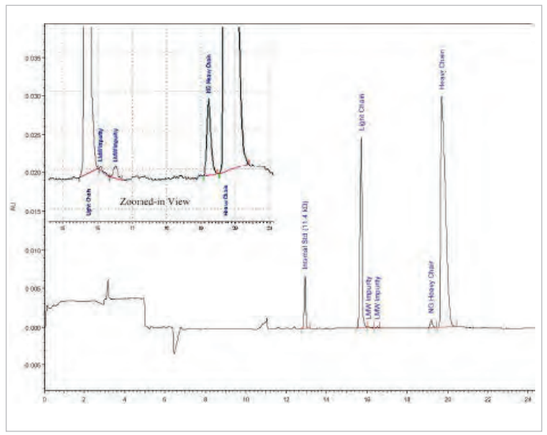


Figure 9: An electropherogram of the analysis of a typical reduced recombinant human IgG. The nonglycosylated heavy chain (NG) is well resolved from the heavy chain, while low-molecular-weight impurities are clearly discernable from the light chain. The inset simply highlights a zoomed-in region of the electropherogram.

Analysis of Unknowns

Using the methodology described in this article, we have screened several different mouse and human IgG samples. The percent of non-glycosylated heavy chain varied between the different IgG samples, ranging from 0 to 2% of the total heavy chain. Figure 9 illustrates an example of the analysis of a representative recombinant human IgG preparation taken from a bulk production at 40 mg/mL. Even though the salt present in the bulk sample was high, the dilution generated during sample preparation removed the need for sample desalting. Light chain, low-molecular-weight impurities, heavy chain, and non-glycosylated heavy chain were all automatically detected and identified by the software based on the known mobilities of these species. In this example, the non-glycosylated heavy chain represented about 1.6% of the total heavy chain, while the low-molecular-weight impurities accounted for about 0.3% of the total protein load (excluding mobility marker). The inset in Figure 9 highlights a zoomed in portion of the electropherogram, providing an indication of the stability of the baseline and the resolution of the low-level impurities.

Summary

We have developed a new, replaceable polymer matrix to improve the current resolving power of SDS-CGE, and have developed this matrix into a commercial IgG Purity/Heterogeneity assay. In doing this, we are providing a quality-controlled assay to the biotechnology industry, with specifications developed to assess the purity and heterogeneity of IgG in both a reduced and non-reduced state. This assay will detect impurities as low as 0.1% and includes an IgG control with a designated quantity of non-glycosylated heavy chain. The IgG control is used to test the suitability of the assay prior to running unknowns. With non-reduced IgG, the assay will resolve the intact IgG from: 1) IgG dimer, 2) 2 heavy chains and 1 light chain, 3) 2 heavy chains, 4) 1 light chain, 1 heavy chain, 5) 1 heavy chain, 6) light chains. With reduced IgG, the assay will resolve the IgG heavy chain, light chain, non-glycosylated heavy chain, and the lower-molecular-weight impurities that fall between the heavy and light chains, as well as higher-molecular-weight impurities that are larger than the intact heavy chain.

Acknowledgements

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DEVELOPMENT AND QUALITY CONTROL

High-Resolution cIEF of Therapeutic Monoclonal Antibodies: A Platform Method Covering pH 4-10

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Introduction

During a cIEF separation, a continuous pH gradient is formed by applying voltage across a capillary filled with carrier ampholytes (CA) whose opposing ends are submerged in acidic (anodic) or basic (cathodic) solutions. The electrical field generated drives the counter migration of hydronium and hydroxyl ions into the capillary, titrating amphoteric compounds towards their isoelectric points (pI). cIEF separation for characterization of therapeutic proteins has been increasingly adopted in recent years; pI determination adds a critical dimension to establishing identity, purity, post-translational modification and stability of therapeutic protein preparations. A significant proportion of cIEF analyses being performed in the biopharmaceutical industry involve characterization of monoclonal antibodies (mAbs). Many mAbs have charge isoforms with a pI in the basic range of the pH gradient. Basic compounds present a challenge in cIEF due to the inadequate nature of ampholytes comprising the basic region as well as decay of the whole pH gradient over time.[1,2] Addition of cathodic and anodic stabilizers to the cIEF sample solution helps overcome these obstacles.[3] Additionally, optimization of stabilizer solution volumes, focusing times, and ampholyte concentration allows for development of a single method that can be used throughout the complete pH range.[4,5,6] This strategy allows for development of a single protocol for product pipeline characterization, also referred to as a platform method. This is an important point due to the need for simplicity and versatility of method development in the biopharmaceutical industry. The result of these efforts is the development of a robust analytical technique less prone to operator error or system variation allowing for easier portability.

Methods

Preparation of mAb Samples

A 500 µL volume of a basic therapeutic mAb (5-10 mg/mL) was thawed and loaded into a Microcon Ultracell YM 10 (p/n A11530, Millipore, Billerica, MA). Following centrifugation for 5 min in a Microfuge 18 (p/n 367160, Beckman Coulter, Inc., Fullerton, CA) at 12 k rcf, the filtered volume was replaced with 20 mM Tris buffer pH 8.0. Two additional cycles of centrifugation and buffer replacement were performed and the desalted sample was aliquoted into approximately 50 µg fractions and stored at -20°C or below.

Capillaries and Reagents

A Beckman Coulter 50 µm i.d. Neutral Capillary (p/n 477441) was installed in a Capillary Cartridge equipped with a 200 µm aperture. Prior to installation, the capillary was measured so that its total length is 30.2 cm and the length from inlet to detection window is 20 cm, and from the outlet to the detection window was 10 cm.

Anolyte solutions containing 200 mM phosphoric acid were prepared as a 40 mL bulk by diluting 0.550 mL of 85% (w/v) (14.6 M) phosphoric acid (Sigma 438081) with 39.5 mL of double-deionized (DDI) water.

Catholyte solutions containing 300 mM sodium hydroxide were prepared as a 40 mL bulk by diluting 12 mL of 1 M sodium hydroxide (Sigma p/n 72082) into 28 mL DDI water.

Chemical mobilizer solutions containing 350 mM acetic acid were prepared as a 40 mL bulk by diluting 0.8 mL of glacial acetic acid (99.8%), 17.4 M (Sigma p/n 537020) into 39 mL of DDI water.

Chemical mobilizer solutions containing 350 mM acetic acid were prepared as a 40 mL bulk by diluting 0.8 mL of glacial acetic acid (99.8%), 17.4 M (Sigma p/n 537020) into 39 mL of DDI water.

Capillary cleaning solution was made as a bulk solution by dissolving 10.8 g of urea (Sigma p/n U0631) in 30 mL of DDI water. Capillary cleaning solution was mixed until all solids solubilized and then filtered through an Acrodisc 25 mm syringe filter with a 5 µm pore (Pall p/n 4199) to remove particles.

A 3 M urea-cIEF gel solution was prepared by dissolving 1.80 g of urea (Sigma p/n U1250) in 9 mL of cIEF gel (p/n 477497), mixed for 15 min and loaded into a 30 mL syringe (Becton Dickenson p/n 309650), and then filtered using a 5 µm pore Acrodisc 25 mm syringe filter (Pall p/n 4199) to remove particles. The 3 M urea-cIEF gel solution was degassed at 2,000 rcf with an Allegra™ X 15 R centrifuge (Beckman Coulter p/n 392933) and stored at 2-8°C.

Cathodic stabilizer solution containing 500 mM Larginine was made as a 50 mL bulk by first dissolving 4.36 g of L-arginine (98%) (Sigma p/n A5006) solid with 35 mL of DDI water in a 50 mL volumetric flask, then volumetric flask was shaken for 15 min to dissolve, and finally increasing the volume to 50 mL with DDI water.

Anodic stabilizer solution containing 200 mM iminodiacetic acid (IDA) was made as a 50 mL bulk by first dissolving 1.33 g of iminodiacetic acid (98%) (Sigma p/n 220000) solid with 35 mL DDI water in a 50 mL volumetric flask. The mixture was shaken for 15 min to dissolve all solids and the volume was increased to 50 mL using DDI water.

The mAb buffer replacement solution which contained 20 mM Tris was made by diluting 4 mL of 50 mM Tris buffer at pH 8.0 (p/n 477427) with 6 mL of DDI water.

Reagent	Single Sample Volume µL		3 Sample Master Mix Volume µL
3M Urea-cIEF Gel	200	x3.2	640
Pharmalyte 3-10 Carrier Ampholytes	12	x3.2	38.4
500 mM Arginine Cathodic Stabilizer	20	x3.2	64
200 mM IDA Anodic Stabilizer	2	x3.2	6.4
Synthetic Peptide pI 10 Marker	1	x3.2	3.2
Synthetic Peptide pI 7.0 Marker	1	x3.2	3.2

Table 1: Master Mix Preparation. A cIEF master mix solution was made by mixing the appropriate component volumes shown in Table 1. These volumes include a 20% overage volume to account for pipetting error. The single portion composition of the master mix contained 12 µL of Pharmalyte 3-10 (GE Healthcare p/n 17-0456-01), 20 µL of 500 mM L-arginine, 2 µL of 200 mM IDA, 1.0 µL of 1.25 mM peptide pI markers, and 200 µL of 3 M ureacIEF Gel.

	10 mg/mL mAb	5mg/mL mAb
Master Mix	240	240
Desalted mAb	5	10

Table 2: Sample Preparation. Following mixing of the 240 µL master mix, it was added to a tube containing an aliquot of desalted mAb. The complete sample solution was then vortexed, 200 µL was transferred into a PCR tube, overlaid with a single drop of mineral oil (Sigma p/n 8410-5ML) and placed in a sample vial.

Instrument Configuration

All cIEF experiments were performed on a Beckman Coulter PA 800 Protein Characterization System equipped with a UV detector and a 280 nm filter with a band pass of +/- 10 nm. The detector was set to detect direct absorbance at a collection rate of 2 Hz. The electronic filter was set at the normal setting, with a peak width setting of 16 to 25. All separations were performed at a capillary temperature setting of 20°C. The system auto sampler was set to a temperature of 10°C (Figures 2 and 3).

Conditioning Method

After initial installation of the capillary cartridge, the neutral capillary was conditioned by first performing a water rinse by submerging the inlet of the capillary into a glass vial containing 1.8 mL of DDI water and applying 50 psi of pressure from the inlet side for 2 min. The extended water rinse was followed by a weak acid wash performed by submerging the inlet of the capillary into a vial containing 1.8 mL of chemical mobilizer and applying 50 psi of pressure from the inlet side for 2 min. Finally a conditioning rinse is performed by submerging the inlet of the capillary into a vial containing 1.8 mL of Beckman Coulter cIEF gel and applying 50 psi of pressure from the inlet side for 5 min. During all the steps in the conditioning method the outlet of the capillary was placed in a waste vial.

Platform cIEF Separation Method

The mAb samples were separated using the following separation method: Prior to loading a new sample, the capillary was cleaned by submerging the inlet of the capillary into a vial containing 1.8 mL of capillary cleaning solution and applying pressure at 50 psi from the inlet side for 3 min.

Following the capillary cleaning solution rinse step, the capillary was flushed with water by submerging the inlet of the capillary into a vial containing 1.8 mL DDI water and applying 50 psi of pressure from the inlet side for 2 min. The mAb CIEF sample

Figure 1: Initial Conditions.

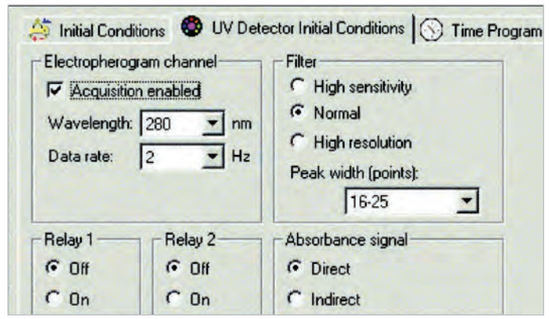

Figure 2: Detector Settings.

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solution was introduced into the capillary by applying 25 psi of pressure for 99.9 sec from the inlet side of the capillary. During the cleaning, water rinse and sample loading steps, the outlet side of the capillary was placed into a waste vial. Following sample injection, the gradient was focused by submerging the inlet side of the capillary into a vial containing 1.8 mL of anolyte and the outlet side of the capillary into a vial containing 1.8 mL of catholyte and applying a 25 kV electrical potential across the capillary for 15 min.

The focused peaks were mobilized by chemical means, during which the catholyte solution was replaced with a vial containing 1.8 mL of chemical mobilizer and applying a 30 kV electrical potential across the capillary for 20 min. Following completion of the mobilization step, data collection was stopped and a water rinse step was performed by submerging the inlet of the capillary into a vial containing 1.8 mL DDI water and applying 50 psi of pressure from the inlet side for 2 min. During the sample loading and all rinse steps in the method, the outlet of the capillary was placed in a waste vial.

Shutdown Method

The instrument was shut down and the capillary was stored on the instrument at the end of the working day using a shutdown method that first equilibrated the capillary by submerging the inlet of the capillary into a vial containing 1.8 mL of DDI water and applying 50 psi of pressure from the inlet side for 2 min. The water rinse was followed by a conditioning rinse performed by submerging the inlet of the capillary into a vial containing 1.8 mL of Beckman Coulter cIEF gel and applying 50 psi of pressure from the inlet side for 10 min. During both rinse steps in the shutdown method, the outlet of the capillary was placed in a glass vial designated for waste. The last step in the shutdown method included turning off the UV lamp and submerging each end of the capillary in a vial containing 1.8 mL of DDI water.

Buffer Tray Set-up

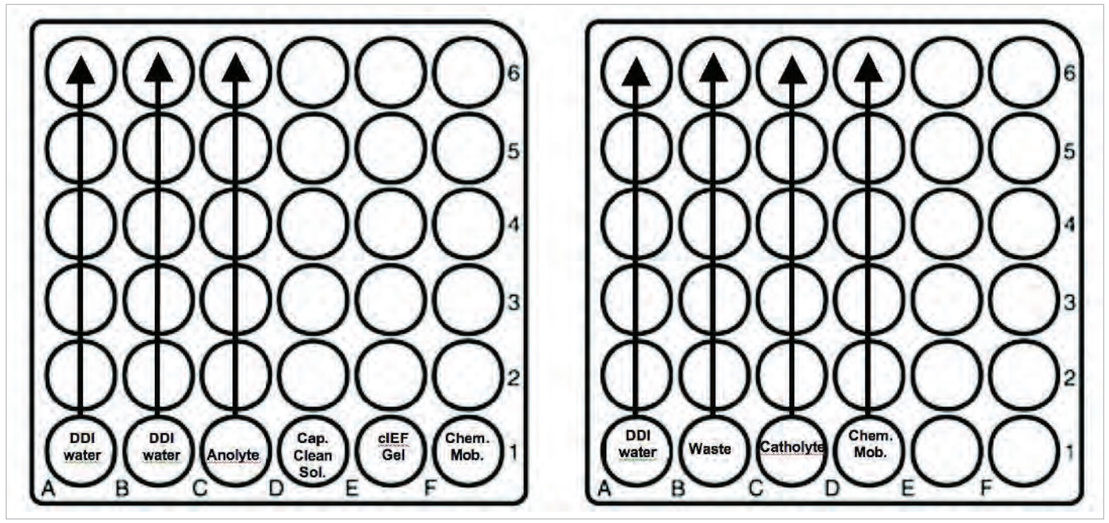
Glass vials containing 1.6 mL of cIEF separation reagents were placed in the buffer trays prior to separation in the order illustrated in Figure 3. The capillary cleaning solution is designated by the abbreviation "Cap Clean Sol." and chemical mobilizer by "Chem. Mob." Arrows indicate the reagents that are incremented by the Basic pH Gradient cIEF Separation Method and the direction of incrementing.

Results

pH Gradient Range and Linearity

The effective separation range and linearity for the ampholyte buffered pH are important factors in cIEF. cIEF methods that can produce a linear gradient over a wide pH range reduce the number of methods and internal standards an analyst must develop when working with multiple compounds.

The cIEF separations of a panel of synthetic peptide markers with pI points spanning the pH range of 4.1 to 10 (Table 3) were performed using the basic pH range method to assess linearity. This separation (Fig. 4) illustrates that the method has a wide functional range and is even capable of separating the acidic peptide markers when the mobilization time is extended.



BI (Inlet Buffer Tray) **BO (Outlet Buffer Tray)**

Figure 3: Buffer Trays Setup.

Incorporation of a slight modification can extend the method's range to include a pI 3.4 peptide marker. Analysis of pI versus detection time (Fig. 5) for the synthetic peptide markers shows that the gradient is linear between pH 10 and 4.1 with a correlation coefficient of 0.99.

Highly basic protein species not properly focused are sometimes mistaken as salt fronts. This is illustrated in Figure 4 as the cathodic pre-peaks. These peaks are actually a product of the bi-directional migration generated by the total capillary sample loading method employed by cIEF. These peaks are most prominent for compounds with basic pI points and can be quite useful as a diagnostic tool. Earlier detection times for cathodic peaks can be characteristic of a decrease in ampholyte concentration, increase of cathodic stabilizer volume, or increase in salt concentration in the sample solution. The opposite is true with increased detection times for cathodic peaks. Increased cathodic peak detection times may also be observed in older and/or expired capillaries, most likely due to the increase in the magnitude of electroosmotic flow (EOF).

pI Marker	Amino Acid Sequence	Mobilization Time Min
10.0	H-Trp-Tyr-Lys-Lys-OH	22.408
9.5	H-Trp-Tyr-Tyr-Lys-Lys-OH	23.462
8.4	H-Trp-Glu-Tyr-Tyr-Lys-Lys-OH	27.375
7.0	H-Trp-Glu-His-Arg-OH	29.429
6.7	H-Trp-Glu-His-His-OH	29.892
5.5	H-Trp-Glu-His-OH	32.808
4.1	H-Trp-Asp-Asp-Arg-OH	36.683

Table 3: Synthetic Peptide Markers Used to Assess Linearity.

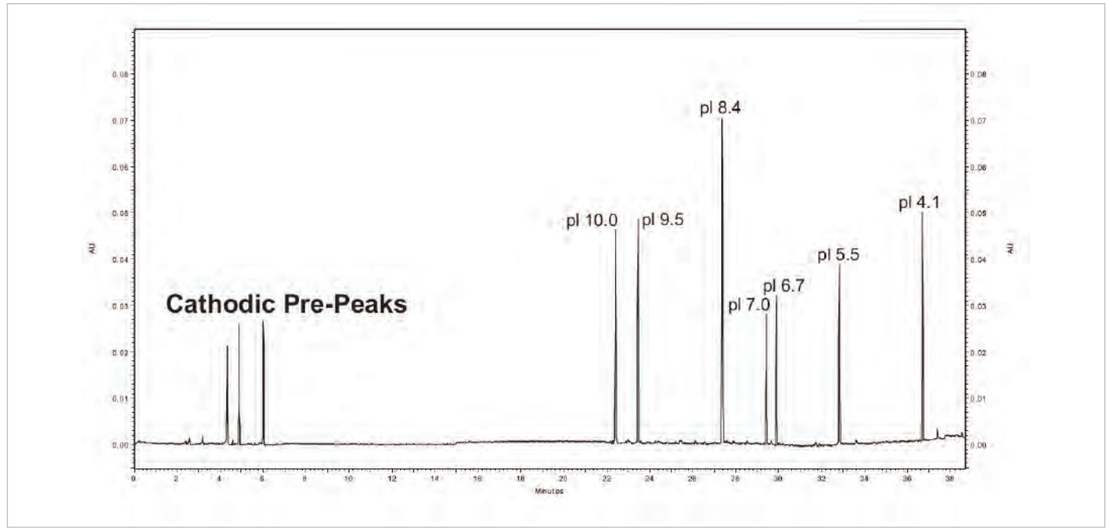


Figure 4: cIEF Separations of Synthetic Peptide pI Markers. Contains an electropherogram of seven synthetic peptide markers using the basic pH gradient cIEF separation method. The mobilization step was extended from 35 minutes to 40 minutes to allow for the detection of the pI 4.1 marker.

Separation of Three mAbs

mAbs tend to generate complex charge isoform profiles when separated by cIEF. These profiles in addition to actual pI values for the peaks generate a "mAb" fingerprint. It is for this reason that cIEF can be a powerful tool in identification and characterization of separate mAb preparations. The unique peak profile for different mAbs shows that a single method using a broad pH 3 to 10 ampholyte mixture is capable of resolving differences in pI up to a few hundredths of 1 pH unit across the basic range of the gradient (Figure 6).

Reproducibility of mAb Separation

In order to test intermediate precision for this method, a panel of three antibodies was separated in triplicate on two instruments using two different lots of neutral capillary and reagents on six separate days. All data was integrated using 32 Karat™ software. pI was determined through the qualitative analysis tool using the detection times of pI 10 and 7 synthetic peptide markers. The peaks were clustered into one of three isoform groups for each mAb, basic, main and acidic, and a percent composition was calculated for each isoform group. The pI point of seven signature peaks and the percent composition of three isoform groups for each of the three mAbs tested were recorded. These recorded values were then used to calculate percent coefficients of variation (%CV) which were used to gauge assay reproducibility (Figures 7, 8, and 9, and Tables 4, 5, and 6).

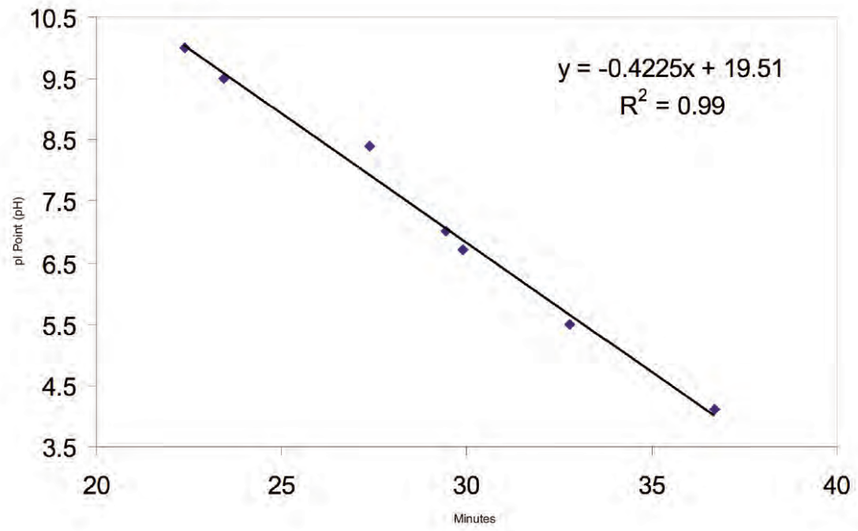


Figure 5: pI Marker Linear Model. A plot of pI values versus detection time observed for separation of synthetic peptides as summarized in Table 3. The mobilization of the pI markers fits a linear model with a correlation coefficient of 0.99 indicating that the mobilized pH gradient is highly linear.

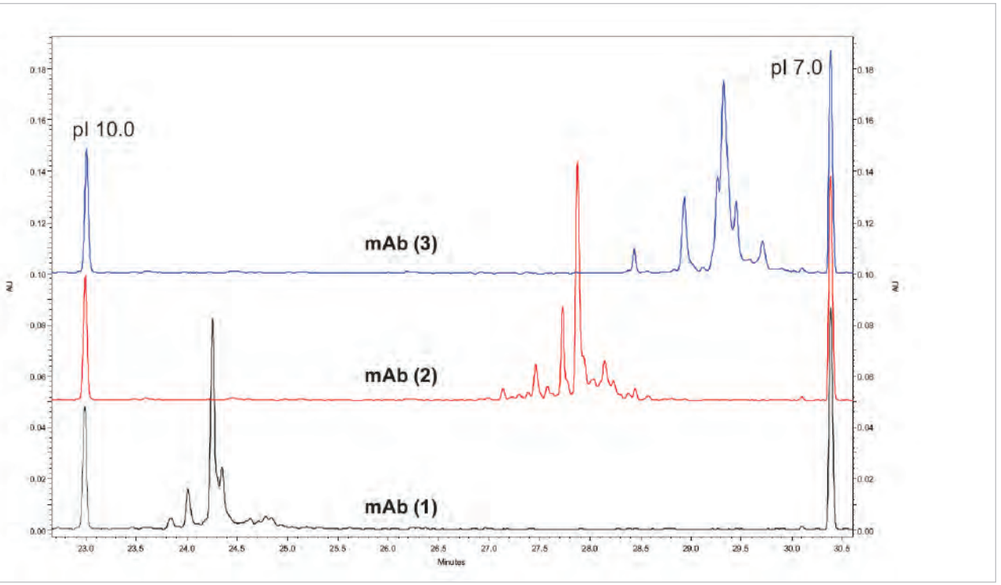


Figure 6: cIEF Identification and Characterization of Separate mAb Preparations.

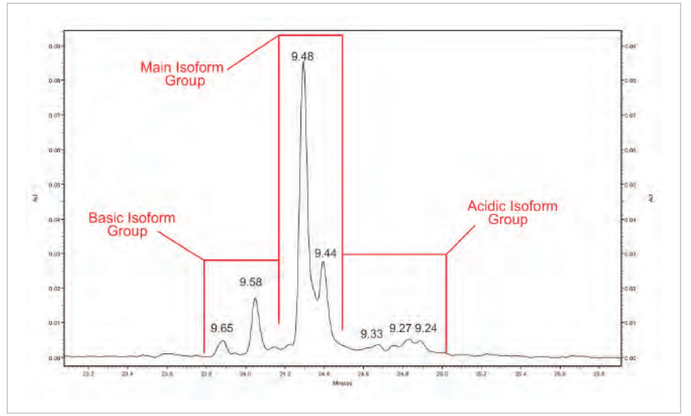


Figure 7: mAb (1) Peak Profile. A close up view of the mAb #1 cIEF separation.

n = 25

Calculated pI

Peaks	Average	Std Dev	CV
A	9.65	0.01	0.05%
B	9.58	0.01	0.06%
C	9.48	0.01	0.07%
D	9.44	0.01	0.09%
E	9.33	0.01	0.08%
F	9.27	0.01	0.07%
G	9.24	0.01	0.08%

Isoform Group Percent Composition

Group	Average	Std Dev	CV
Basic	13.94%	0.42%	3.04%
Main	71.97%	0.46%	0.64%
Acidic	14.09%	0.34%	2.38%

Table 4: Quantitative Analysis of mAb #1 cIEF Separation. Quantitative analysis was performed using the pI point of seven signature peaks and the percent composition of three isoform groups.

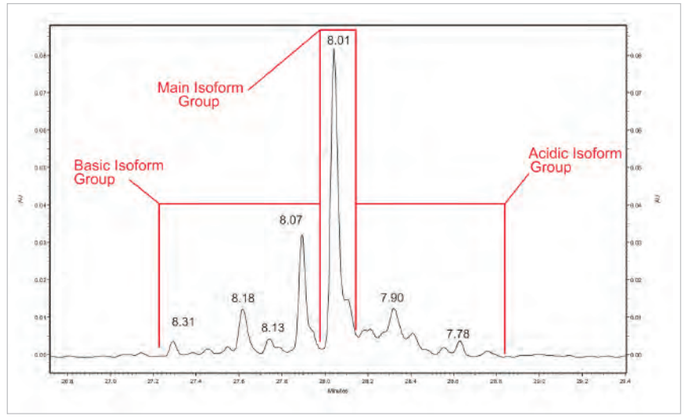


Figure 8: mAb #2 Peak Profile. A close up view of a mAb #2 cIEF separation.

n = 25

Calculated pI

Peaks	Average	Std Dev	CV
A	8.31	0.00	0.06%
B	8.18	0.01	0.07%
C	8.13	0.01	0.07%
D	8.07	0.01	0.07%
E	8.01	0.01	0.07%
F	7.90	0.01	0.07%
G	7.78	0.00	0.05%

Isoform Group Percent Composition

Group	Average	Std Dev	CV
Basic	30.97%	0.67%	2.17%
Main	45.01%	0.45%	0.99%
Acidic	24.02%	0.60%	2.50%

Table 5: Quantitative Analysis of mAb #2. Quantitative analysis was performed using the same method as mAb #1.

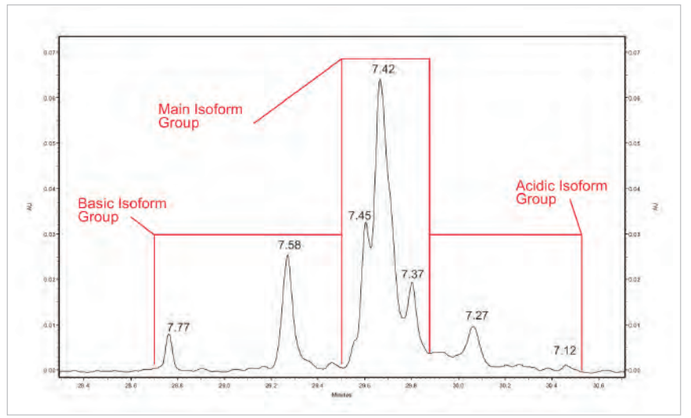


Figure 9: mAb #3 Peak Profile. A close up view of a mAb #3 cIEF separation.

n = 25

Calculated pI

Peaks	Average	Std Dev	CV
A	8.31	0.00	0.06%
B	8.18	0.01	0.07%
C	8.13	0.01	0.07%
D	8.07	0.01	0.07%
E	8.01	0.01	0.07%
F	7.90	0.01	0.07%
G	7.78	0.00	0.05%

Isoform Group Percent Composition

Group	Average	Std Dev	CV
Basic	30.97%	0.67%	2.17%
Main	45.01%	0.45%	0.99%
Acidic	24.02%	0.60%	2.50%

Table 6: Quantitative Analysis of mAb #3. Quantitative analysis was performed using the same method as mAb #1.

Conclusions

A single separation method can be employed to perform cIEF analysis of multiple mAbs across a broad pH range. The use of a single method allows for preparation of master separation mixes which can help reduce pipetting errors and inter-sample variability. Workflow is greatly simplified by the use of a platform method, reducing the chances for operator error and increasing overall efficiency in method development. The resulting reduction or elimination of method optimization also allows for easier scale up of cIEF analysis in routine use environments.

The analytical power of the cIEF separation technique is illustrated by the three very different peak profiles produced in each mAb separation. The reproducibility of the separation method was demonstrated by performing a series of separations on six separate days using multiple instruments with multiple lots of reagents. Statistical analysis of pI and isoform group percent composition confirms that highly reproducible cIEF separations of mAbs can be achieved even in the previously problematic basic region of the pH gradient.

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DEVELOPMENT AND QUALITY CONTROL

Separation of Recombinant Human Erythropoietin (rhEPO) using the European Pharmacopoeia Method on the SCIEX PA 800 Plus Pharmaceutical Analysis Systems

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Introduction

Erythropoietin (EPO) is a naturally occurring hormone that stimulates red blood cell production and release from bone marrow. EPO was one of the first therapeutic recombinant glycoproteins commercialized for the treatment of anemia. It is known to have complex N- and O-linked glycosylation patterns and can exist as numerous protein isoforms that play a critical role in the bioavailability, activity, potency and stability of EPO. Proper characterization of EPO is extremely important in order to ensure the comparability or efficacy of a biologic preparation.

Until 1999, gel-based isoelectric focusing (IEF) separation of EPO was the standard method adopted by the European Pharmacopeia (EuPh). However, this method lacked adequate quantitation and reproducibility. Because of this, a collaborative study initiated by the European Directorate for the Quality of Medicine (EDQM) was performed. The results of this study established Capillary Zone Electrophoresis (CZE) as the method of choice for the quantitation of EPO isoforms[1]. Additional collaborative studies by pharmaceutical companies, government agencies and academic institutions have refined this method and developed a suitability standard[2].

This article provides guidance for the preparation of an EPO suitability standard and separation buffer. Instrument configuration and set up of the EuPh method parameters for the analysis of EPO in 32 Karat software using the SCIEX PA 800 Plus Pharmaceutical Analysis System are also described.

Experimental

Sample Preparation

The EPO BRP3 – EuPh Reference Standard (LGC Promochem, Middlesex – UK) was used in this work. Two hundred and fifty milliliters of double distilled and deionized (ddi) water was added to reconstitute the lyophilized EPO standard. The resulting solution was desalted using a Millipore Microcon YM-3 (or equivalent) as described below.

Step 1 – YM-3 – Membrane Wash

- Assemble the YM-3 sample reservoir into the centrifuge tube provided according to instructions provided by Millipore.
- Dispense 250 µL of ddi water into the sample reservoir.
- Centrifuge at 13,000 g for 10 minutes. Discard retentate and eluent.

Step 2 – Sample Concentration

- Dispense 250 µL of reconstituted EPO to the washed sample reservoir.
- Centrifuge at 13,000 g for 10 minutes.

Step 3 – Sample Desalting (this step must be repeated 4 times)

- Dispense 250 µL of ddi water onto the sample reservoir.
- Centrifuge at 13,000 *g* for 10 minutes.
- Discard only the eluent

Final step – Desalted Sample RecoveryDispense 250 µL of ddi water onto the sample reservoir.

- Carefully place sample reservoir upside down into a new vial (provided by Millipore for this purpose).
- Centrifuge at 2,000 g for 2 minutes.
- The sample was stored at 4° C. The recovered volume was approximately 125 µL.

Determination of Sample Concentration

The protein content was measured spectrophotometrically at 280 nm as per the procedure also described in the EuPh of EPO using a DU 800 Spectrophotometer (Beckman Coulter, Inc.). The absorptivity of a 1% solution of EPO was 7.43. The concentration of desalted EPO used in this work was 0.17 mg/mL.

Preparation of CZE Buffer Concentrate

The buffer preparation was performed as described in the European Pharmacopoeia method. The following components were added to a 100 mL volumetric flask: 0.582 g of NaCl (S1249 - Spectrum), 1.793 g of Tricine (part no. T5816 – Sigma), 0.820 g of sodium acetate (part no. 3470-01 - J.T. Baker), ddi water was added up to 100 mL. This solution was filtered through a 0.2 µm membrane using a Nalgene filter unit (MF75) and stored at 4° C.

Preparation of 1M Putrescine Solution

A bottle of putrescine (part no. 3279 - Sigma) was placed in a water bath at 60° C and the contents were allowed to melt. This process took approximately 20-30 minutes. Using a disposable transfer pipette, 0.882 g of putrescine was weighed directly into a 10 mL volumetric flask, and ddi water was added to complete the volume to 10 mL. The solution was mixed and separated into 500 µL aliquots and stored at 4° C.

Preparation of CZE Running Buffer

The following components were added to a 50 mL polypropylene tube: 21 g of urea (part no. U1250 – Sigma), 5 mL of CZE Buffer Concentrate, 125 µL of 1 M Putrescine and 25 mL of ddi water. The pH was adjusted to 5.5 at 30° C with 2 N HAc (prepared from glacial acetic acid, part no. A6283 – Sigma). This solution was filtered through a 0.2 µm membrane using a Nalgene filter unit MF75 and stored at 4° C. This buffer is stable for one week.

Capillary

A bare fused silica capillary, 50 µm i.d., 110 cm total length with 100 cm effective length was used for this separation (Beckman Coulter part no. 338472).

Instrument Set-up Details

The instrument used to perform these separations was the PA 800 Plus Pharmaceutical Analysis System equipped with UV/vis detection configured with a 214 nm filter. System control was by 32 Karat v. 9.0 software. The cartridge and sample storage temperatures were set at 35° C and 4° C, respectively. The UV detector was set with a data rate of 0.5 Hz, the detector filter was set to normal and the filter peak width points were set to 16-25. These settings were used for all three methods in this work: equilibration, separation and shutdown which will be described later on this section.

Reagent Vial Volumes

For all methods used in this work, the reagent vials contained 1.5 mL of 0.1 N NaOH, ddi water and CZE running buffer. Both reagent and waste vials were incremented every 10 separations to avoid ionic depletion of run buffers and a decrease in volume of rinsing solutions.

Note: The waste vials must always contain 1.0 mL of ddi water to prevent crystallization and carryover of urea.

Capillary Equilibration Method

The capillary was rinsed with 0.1 N NaOH at 20 psi for 60 minutes followed by CZE running buffer at 20 psi for 60 minutes, and voltage equilibration performed at field strength of 181.8 V/cm for 720 minutes.

EPO CE Separation Method

The capillary was rinsed with ddi water at 20 psi for 10 minutes followed by 0.1 N NaOH at 20 psi for 5 minutes, CZE running buffer at 20 psi for 10 minutes and then sample injection at 0.7 psi for 20 s. Separation was performed at field strength of 143 V/cm. The total separation run time was 70 minutes. Auto-zero was performed 5 minutes after the start of the separation.

Important Note: For improved migration time, peak area and peak shape reproducibility, the use of 0.1 N NaOH solution which has been stored in borosilicate glass bottles may be needed. It was found that exposure of the 0.1 N NaOH rinse solution to borosilicate glass for a minimum of 24 hours improved the reproducibility of migration time for EPO. Studies indicated that using NaOH stored in a plastic container accounted for variation in EOF of 18.6 % (n=50) as compared to 3.4% (n=50) for NaOH stored in a borosilicate glass bottle for a period of 24 hours. For more information, see the poster , "Separation of Recombinant Human Erthyropoietin (rhEPO) using the European Pharmacopoeia Method on the PA 800 Plus", at sciex.com.

Shutdown Method

The capillary was rinsed with ddi water at 20 psi for 10 minutes followed by 0.1 N NaOH at 20 psi for 5 minutes, CZE running buffer at 20 psi for 10 minutes. At the end of this method, the lamp was turned off and the ends of the capillary were stored in water.

Typical electropherogram obtained of EPO BRP batch 3 (EuPh standard) is shown in Figure 1. The current throughout the separation is around 6+/- 0.5 µA.

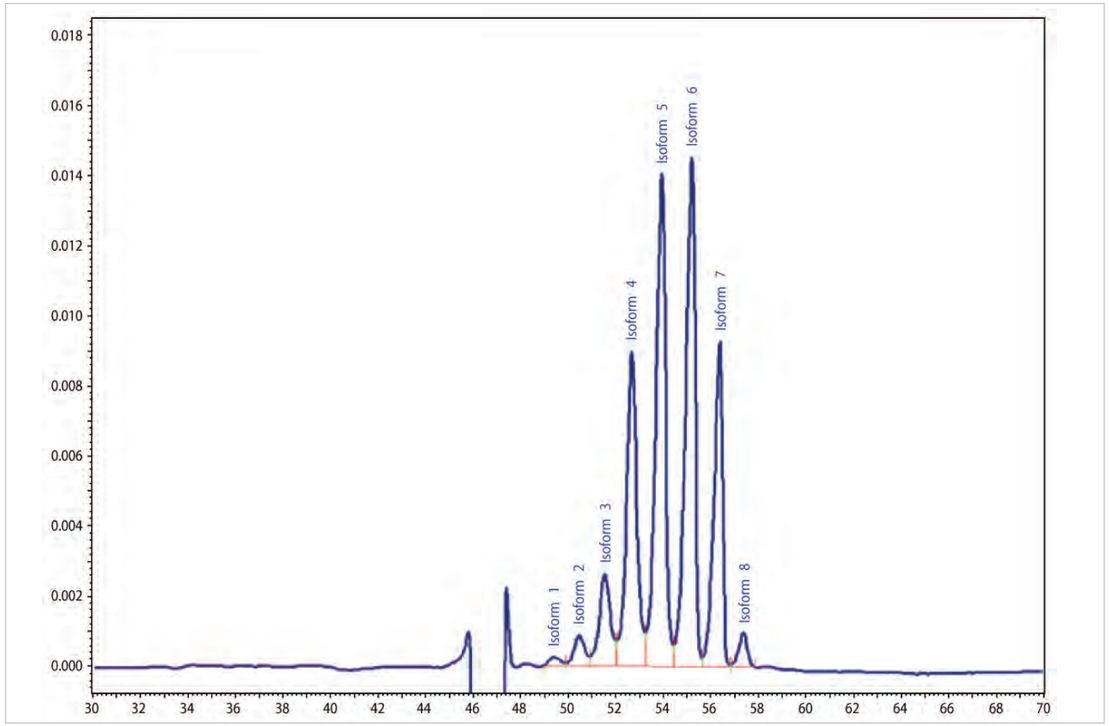

Figure 1: Typical electropherogram of EPO BRP batch 3.

Data Analysis Parameters and Peak ID Table Software Set Up

The software screen capture below (Figure 2) shows the parameters used to integrate the electropherogram in Figure 1. These parameters may change depending on the performance of the separation.

Following proper integration, a peak table can be created for proper peak identification and for system suitability reporting. Figure 3 shows the Peak ID table used to name the peaks on the electropherogram in Figure 1. The numbers displayed for the migration time and MT Window are populated by the software.

#		Event	Start Time	Stop Time	Value
1	☑	Width	0.000	0.000	0.5
2	☑	Threshold	0.000	0.000	75
3	☑	Shoulder Sensitivity	0.000	0.000	100000
4	☑	Minimum Cluster Distance	0.000	0.000	25
5	☑	Integration Off	0.000	48.000	0

Figure 2: Integration events table.

Named Peaks | Groups

#		Name	ID	Mig. Time	MT Window	Ref. ID #	ISTD. ID #
1	☑	Isoform 1	1	49.4	2.47	0	0
2	☑	Isoform 2	2	50.4333	2.52167	0	0
3	☑	Isoform 3	3	51.5333	2.57667	0	0
4	☑	Isoform 4	4	52.6667	2.63333	0	0
5	☑	Isoform 5	5	53.9333	2.69667	0	0
6	☑	Isoform 6	6	55.2	2.76	0	0
7	☑	Isoform 7	7	56.4	2.82	0	0
8	☑	Isoform 8	9	57.4	2.87	0	0

Figure 3: Peaks/Groups table.

System Suitability Parameters Set Up

According to EuPh the system suitability parameters are as follows.

Parameter System Suitability 1: The relative standard deviation of the migration time of the peak corresponding to isoform 2 is less than 2 %. In 32 Karat, the System Suitability screen should be set up as shown in Figure 4.

Compound:
Isoform 1
Isoform 2
Isoform 3
Isoform 4
Isoform 5
Isoform 6
Isoform 7
Isoform 8

#	Parameter	Min	Max	%RSD
1	Migration Time			2
2	Corrected Migration Time			2
3				

#	Test	Start	End	Value
1				

Figure 4: System suitability parameter for isoform 2.

Parameter System Suitability 2: The largest peak in the electropherogram is at least 50 times greater than baseline noise. On 32 Karat's system suitability table, parameters should be set up as described in Figure 5.

Compound:
Isoform 1
Isoform 2
Isoform 3
Isoform 4
Isoform 5
Isoform 6
Isoform 7
Isoform 8

#	Parameter	Min	Max	%RSD
1	S/N (ASTM)	50		
2				

#	Test	Start	End	Value
1				

Figure 5: System suitability parameter for S/N ratio.

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Parameter System Suitability 3: The resolution between isoforms 5 and 6 is not less than 1. Figure 6 shows software set up.

It is a requirement from the EuPh to perform 3 consecutive system suitability separations prior to each sequence table.

For more information on how to access these screens for software set up please consult 32 Karat online help.

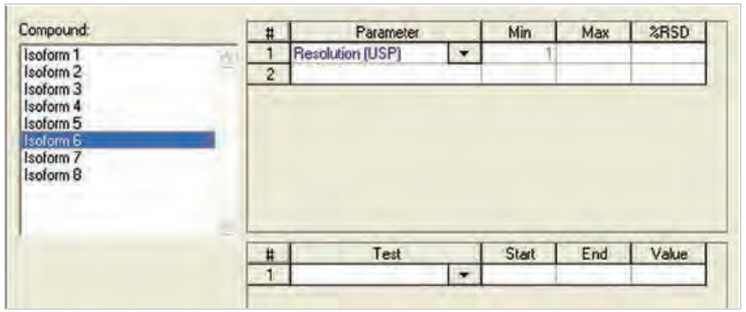

Figure 6: System suitability parameter for S/N ratio.

Limit of the Assay

The EuPh has also established the limits for isoform content based on the peak area percent composition. The peaks corresponding to isoforms 1-8 must have a percentage composition in peak area according the ranges below:

Peak Name	Content Allowed Range (Peak Area Percent)
Isoform 1	0-15
Isoform 2	0-15
Isoform 3	5-20
Isoform 4	10-35
Isoform 5	15-40
Isoform 6	10-35
Isoform 7	0-20
Isoform 8	0-15

Conclusion

This work demonstrates preparation, from reconstitution to desalting, of an EPO suitability sample. In addition, important parameters describing method and instrument set up, capillary conditioning and successful separation of the EPO isoforms by CZE including software parameters for data analysis and system suitability are described.

References

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Rapid Characterization of Biologics using a CESI 8000 – SCIEX TripleTOF® 5600+ System

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SCIEX, Brea, CA and Framingham, MA

Monoclonal antibodies (mAbs) make up an important class of biotherapeutics undergoing significant growth in the pharmaceutical industry today. Currently, more than 30 mAbs have been approved for use in treatment of a number of indications ranging from various forms of cancer to autoimmune and infectious diseases. Therapeutic pipelines for mAbs and mAb-like molecules like bi-specific antibodies, single chain variable fragments (scFv), and antibody drug conjugates (ADC) are expanding. Due to approaching innovator patent expirations, a growing number of mAb biosimilar and biobetter products are also in development. Consequently, there has been a shift towards more comprehensive characterization of both innovator mAbs as well as the alternatives since changes in primary amino acid sequences, quality attribute modifications, and/or such post translational modifications as glycosylation may impact therapeutic efficacy, bioavailability, and biosafety.

Capillary electrophoresis (CE) technology using absorbance- or fluorescence-based detection methods has long been applied in the biopharmaceutical industry for the characterization of mAb purity and heterogeneity in the forms of capillary zone electrophoresis (CZE), capillary isoelectric focusing (cIEF) and capillary SDS gel electrophoresis (CE-SDS). Studies exploring the transferability of this technique illustrate excellent robustness when performed in different laboratories in various geographical locations by different instrument operators[1,2]. The exceptionally high separation power of capillary electrophoresis (CE) in conjunction with the sample identification capability of mass spectrometry fulfills Level-3 characterization requirements necessary to reveal mAB modifications and degradations. Some important attributes including primary sequence, presence of degradation hotspots like oxidation, deamidation, isomerization, cyclization, and post-translational modifications like glycosylation are only detected, localized, and quantified by peptide analysis.

To facilitate this work, SCIEX has integrated capillary electrophoresis (CE) and electrospray ionization (ESI) into a single dynamic process, called CESI. This process was first described by Moini et al.[3], and further refined by Beckman Coulter to create the CESI 8000 High Performance Separation – ESI module[4]. In this article we describe the comprehensive characterization of a representative monoclonal antibody trastuzumab, illustrating the benefits of CESI coupled with high resolution mass spectrometry. This is a continuation of the work previously published by Gahoul et al. in the journal mAbs[5]. Small and large peptides in the range of 3 – 65 amino acids have been separated, identified with 100%

sequence coverage and quantified, including degradative hotspots such as asparagine-deamidation, methionine-oxidation, glutamic-acid-cyclization, and C-terminal lysine heterogeneity using a single separation of only 100 fmol of trypsin protease-digested sample. The low-flow rate of the system (~20 nL/min) maximized ionization efficiency and dramatically reduced ion suppression.

Benefits of CESI-MS technology

CESI provides a separation mechanism orthogonal to LC/MS-based approaches for separation, identification, quantification, and validation of sequence variants as well as post translational modifications (PTMs). The extremely low flow rate of CESI (~20 nL/min) is highly beneficial in maximizing ionization efficiency and minimizing ion suppression. An example of this is the strong ionization of such hydrophilic species as glycopeptides. The inherent separation efficiency of CE provides sharp peptide peaks for sensitive and reproducible identification, as well as relative quantitation. Using the open tubular format of CESI without any solid stationary phase, all peptides elute from the capillary (both hydrophobic and hydrophilic), supporting high mAb sequence coverage and essentially eliminating sample losses and carry-over. Based on the 100% protein sequence coverage routinely obtained by this approach, qualitative and quantitative analysis of mAb purity, stability, and glycoform heterogeneity is possible from a single CESI-MS run with the use of a single protease in the digestion reaction (trypsin). The migration times of the separating peptide components vary less than 30 sec (≤ 0.83% RSD) over the 60 minute separation and the relative abundance measurements of modified and unmodified peptides vary less than 2%.

Experimental Design

Sample preparation

100 µg of trastuzumab was solubilized using Rapigest followed by reduction with dithiothreitol (DTT) and alkylation by iodoacetamide (IAM). The resulting sample was digested overnight with trypsin at 37°C, dried, and resuspended in 300 µL of 133 mM ammonium acetate (pH 4), yielding a final concentration of 0.33 µg/µL of digested antibody. 50 µL was used for the sample injection.

CESI-MS and CESI-MS/MS

Analysis was performed on the SCIEX CESI 8000 High Performance Separation – ESI Module. Separations were performed in a 30 µm ID x 90 cm bare-fused-silica capillary housed in an OptiMS CESI cartridge, with recirculating liquid coolant set to 25°C, and coupled to the SCIEX TripleTOF® 5600+ mass spectrometer. 50 nL of sample (equivalent to 100 fmol of digested antibody) in 100 mM leading electrolyte was introduced into the bare-fused silica separation capillary and transient-isotachophoresis (t-ITP) was applied to focus the sample for the electrophoretic separation. The background electrolyte was 10% acetic acid and a voltage of 20 kV was applied for separation. Under the influence of the electric field, the analytes migrate within the separation capillary according to their charge to hydrodynamic volume ratio and upon reaching the porous sprayer tip of the capillary, are introduced into the mass spectrometer by ESI.

Information dependent acquisition (IDA) mode consisting of a high resolution TOF MS survey scan followed by several MS/MS scans was utilized to acquire the data. The IDA parameters were as follows: 100 msec TOF MS survey scan, 50 msec IDA on the top 30 ions which exceed 200 cps, rolling collision energy to induce fragmentation. The dynamic exclusion time was set to 10 sec. The total cycle time was equal to 1.8 sec and the IDA parameters were optimized so that the duty cycle of the MS readily supported the high speed CE separation. By enabling auto calibration during batches, the instrument was automatically calibrated once every 3 runs, limiting deviation of the mass measurement accuracy.

Data Analysis

Data analysis was performed using SCIEX BioPharmaView™, ProteinPilot™ and PeakView® Software. Some confirmation was manually performed as necessary.

Results and Discussion

Although a number of monoclonal antibodies have been approved by regulators for commercial use, few of these have been as comprehensively characterized as trastuzumab. This work utilized a bottom-up analysis approach in which trastuzumab was digested using only a single enzyme, trypsin. Development of a 'universal' enzyme approach for all biologics would greatly

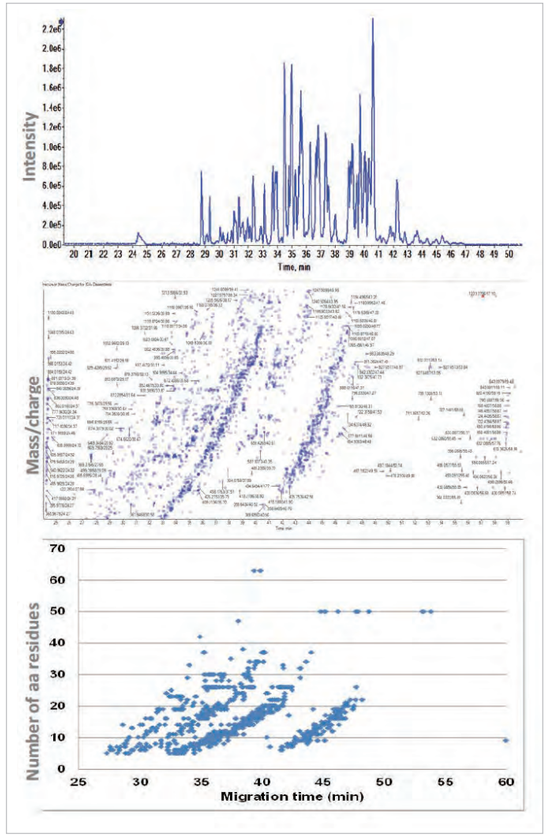

Figure 1: CESI-MS analysis of the tryptic digest of trastuzumab a) Base peak electropherogram; b) Distribution of precursor m/z values over time; c) Peptide elution times according to their number of amino acid residues.

simplify sample preparation and also help in overcoming matrix effects. From the viewpoint of workflow efficiency, the single enzyme approach can be beneficial under circumstances in which numerous molecules are analyzed in parallel and where it is unclear which choice of enzyme would provide the best results.

Figure 1 depicts the CESI-MS analysis of the tryptic digest of trastuzumab. The upper panel shows the base peak electropherogram of the experiment. In the middle panel of Figure 1, the selected precursor ions from the information dependent acquisition (IDA) are observed. The abundance of these ions over all the m/z value range increases the probability of obtaining more peptide identifications. These ions are analyzed by MS/MS, making it possible to identify tryptic peptides with a high confidence, including very small and very large species (up to 63 amino acids) as shown in the bottom panel of Figure 1. The open tubular arrangement of the CE capillary allows the detection of hydrophobic and hydrophilic peptides avoiding discrimination due of lack of interactions with the capillary surface.

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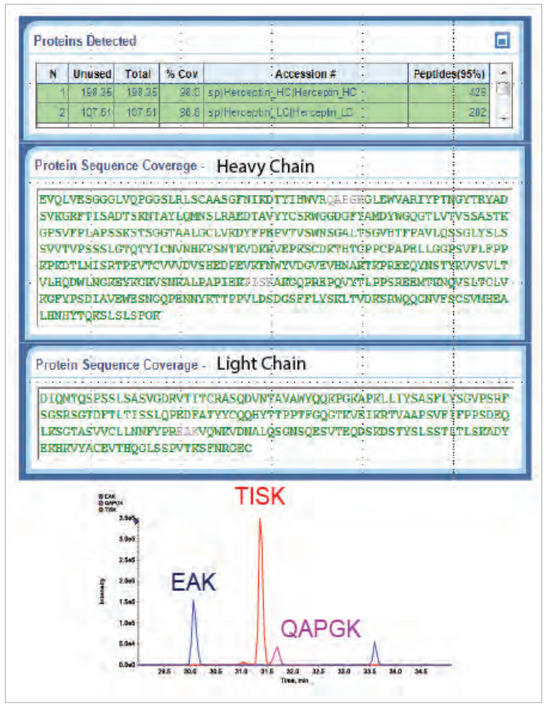

Figure 2: ProteinPilot™ Software Database Search Results of CESI 8000 – SCIEX TripleTOF 5600+ data and additional manual processing resulted in 100% sequence coverage of trastuzumab heavy and light chains.

Achieving 100% sequence coverage

Figure 2 depicts the protein sequence coverage using the ProteinPilot Software. The upper and middle panels depict the sequence information of the heavy and light chains, respectively. Manual determination was used for short peptide sequences (lower panel) that are commonly excluded from protein database search parameters (grey sequence sections). Using this combined approach, 100% amino acid sequence coverage was obtained from this single set of data for both the heavy (HC) and light (LC) chains of trastuzumab. Obtaining 100% sequence coverage and comprehensive characterization of a mAb from a single digestion step using only trypsin and a single 60 minute separation highlights the advantage of CE separation prior to MS analysis. The lower panel of Figure 2 further highlights the excellent separation capability of the CESI-MS setup for small peptides.

Detailed analysis of degradation hotspots

The characteristic highly resolved capillary electrophoresis peaks shown in Figure 1 were used for comparative peptide mass mapping, i.e., extracting peptide peak areas with the combined high separation power of CESI and high resolving power of MS in the mass dimension. To illustrate the capabilities of CESI-MS, identification and quantification of degradative hot spots on peptides within trastuzumab are delineated in Table 1 as pyroglutamate formation (N-terminal glutamate cyclization), methionine oxidation, and C-terminal lysine loss. Additional less abundant sites of the same degradative PTMs, among a few others, such as lysine glycation and/or tryptophan dioxidation were also identified but not described here due to their very low level. Extracted ion electropherograms (EIEs) were analyzed to determine the presence of degradation hotspots, which can commonly occur during processing and storage of mAbs. N-terminal modification can result in cyclization of glutamine to form pyroglutamate. Although not deemed critical, pyroglutamate formation has been proposed to increase the in vivo half-life of antibodies and is an indicator of manufacturing process control[6]. MS/MS spectra revealed both cyclized and unmodified forms of the N-terminal peptide, which differ by 18 amu. Extracted ion data suggested only a partial modification (Figure 3) of 3.26% pyroglutamate. Pyroglutamate cyclization leads to loss of a positive charge resulting in lower electrophoretic mobility of the modified peptide relative to the unmodified one. This is advantageous since the modified and unmodified forms can be well separated by CESI as shown in Figure 3 in addition to structural confirmation using MS/MS.

Methionine (Met) oxidation is another common modification also associated with mAb manufacturing and storage but linked to a decrease in mAb stability and biological activity[7]. Analyzing extracted ion data revealed oxidation at Met255 and Met83 on the heavy chain (Table 1) and indicated the presence of both oxidized and unmodified methionine at both sites in the trastuzumab sample. MS/MS diagnostic ions (Figure 4) confirmed the MetOx modification by a +16 amu molecular mass addition relative to unmodified Met in both sites. From EIEs, the relative abundances of MetOx255 and MetOx83 were determined to be 1.56% and 1.14%, respectively.

Extracted ion analysis of the data indicated quite a few incidences of Asp deamidation, specifically at Asn55, Asn387, Asn392, and Asn393 in the heavy chain and Asn30 and Asn152 in the light chain. MS/MS diagnostic ions for each of these identified the +1 amu difference confirming each instance of deamidation (Figure 5). With CESI, these two forms can be readily separated from each other, making their assignment and differentiation from ionization artifacts much easier. This is particularly important to emphasize since deamidated peptides are not always resolved by LC-MS analyses and may co-elute with unmodified peptides. Additionally, the isotopic envelopes of the peptides, only differing by 1 amu, can also overlap and be mis-assigned by automated identification softwares, even with high resolution MS and MS/MS data. Physical separation of these peptides by CESI-MS, can address both of these challenges with LC-MS.

The analysis of C-terminal lysine heterogeneity is depicted in Figure 6. Due to the loss of a positively charged lysine on the heavy chain, it can be confirmed by a large migration shift. From the extracted ion electropherograms (XIEs), greater than 99% of the heavy chain had C-terminal lysine loss.

Modification	Protein Localization	Sequence and associated mass shift	Monoisotopic mass [M+H]+	Migration time (min)	Relative Abundance (%)
Pyroglutamate formation	N-terminal	E(-18.010565)VQLVESGGGLVQPGGSLR	1863.9923	46.22	3.26
Methionine Oxidation	Met255	DTLM(15.994915)ISR	851.4291	35.79	1.56
Methionine Oxidation	Met83	NTAYLQM(15.994915)N	970.4299	14.62	1.14
Asparagine deamidation	Asn55	R.IYPTN(0.984016)GYTR.Y	1085.5262	36.65	92.47
	Asn387 Asn387 & Asn392 Asn387 & Asn393	K.GFYPSDIAVEWESN(0.984016)GQPENNY.K K.GFYPSDIAVEWESN(0.984016)GQPEN(0.984016)NY.K K.GFYPSDIAVEWESN(0.984016)GQPENN(0.984016)Y.K	2545.1154 2546.0994 2546.0994	40.82 4169 41.86	79.00*
	Asn30	R.ASQDVN(0.984016)TAVAWYQQ.P	1708.8289	39.07	48.06
	Asn152	K.VDNALQSG N(0.984016)SQESVTEQDSK.D	2136.9527	41.14	13.00
C-terminal lysine loss	C-terminal	K.SLSLSPGK(128.094963)	660.3563	40.5	>99

Table 1: Identification of degradation hotspots in trastuzumab. **Overall value*

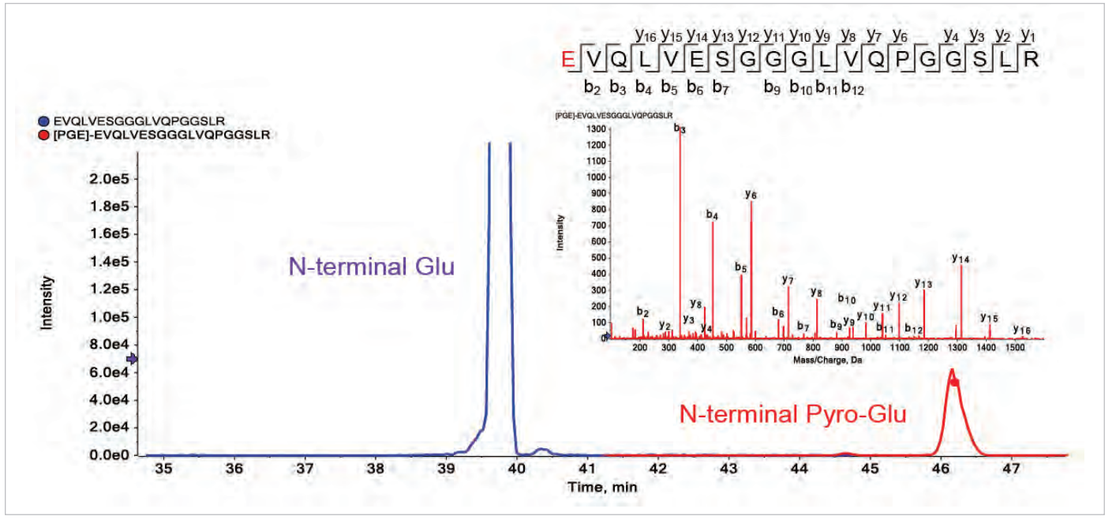

Figure 3: Extracted CESI-MS ion data revealed both cyclized (pyroGlu, red) and unmodified (Glu, blue) forms of the N-terminal peptide.

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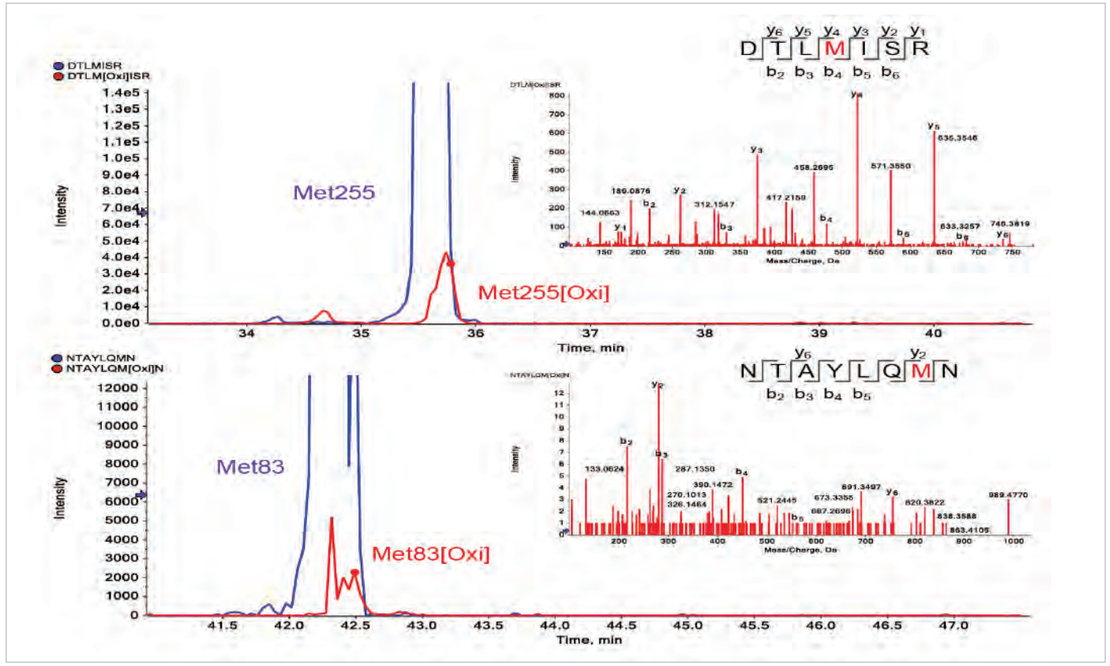

Figure 4: CESI-MS analysis of methionine oxidation (MetOx) at positions 255 and 83.

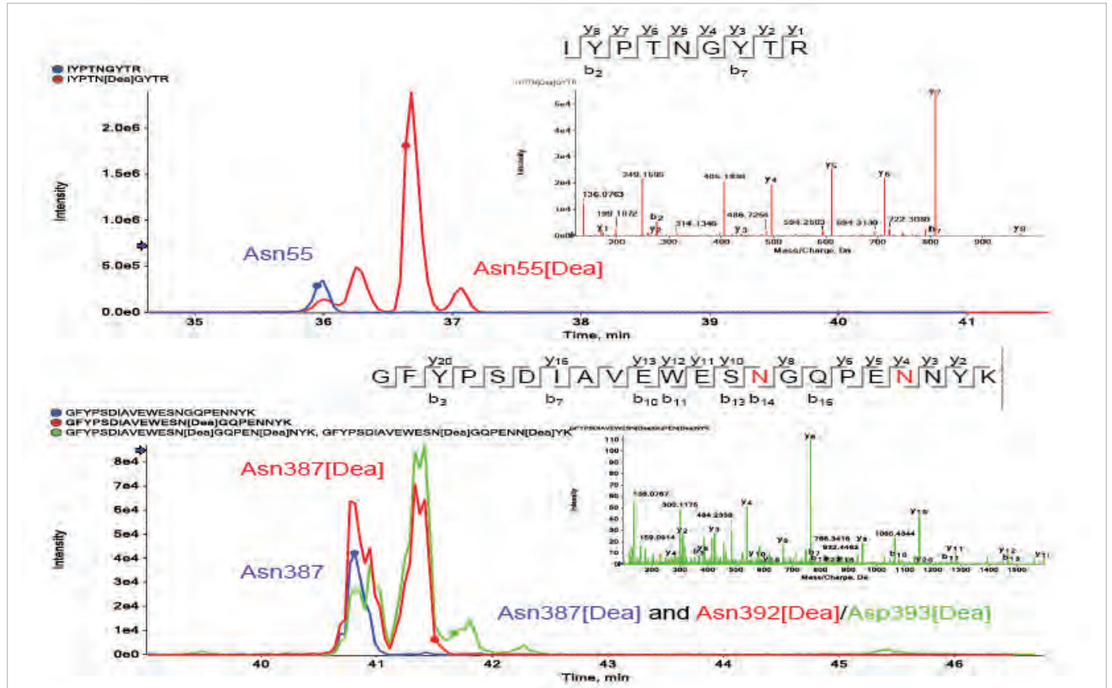

Figure 5: CESI-MS identification of asparagine deamidation sites. Peptide with unmodified residues are shown in blue, and deamidated forms are shown in red and green, with corresponding MS/MS.

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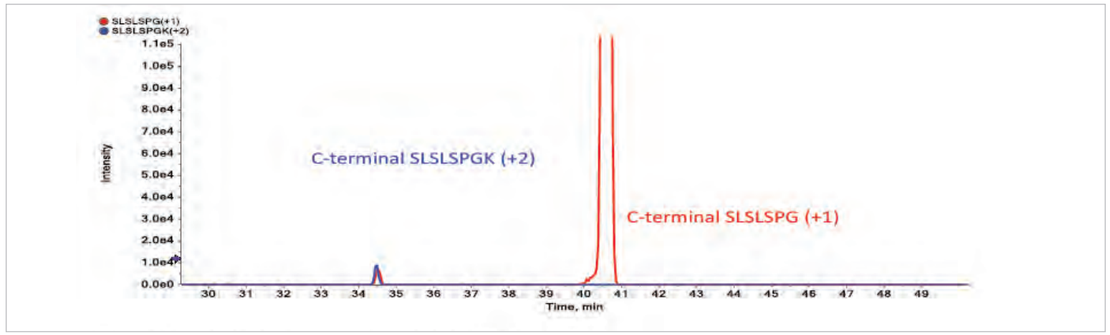

Figure 6: C-terminal lysine heterogeneity analysis by CESI-MS, with terminal lysine loss shown in red.

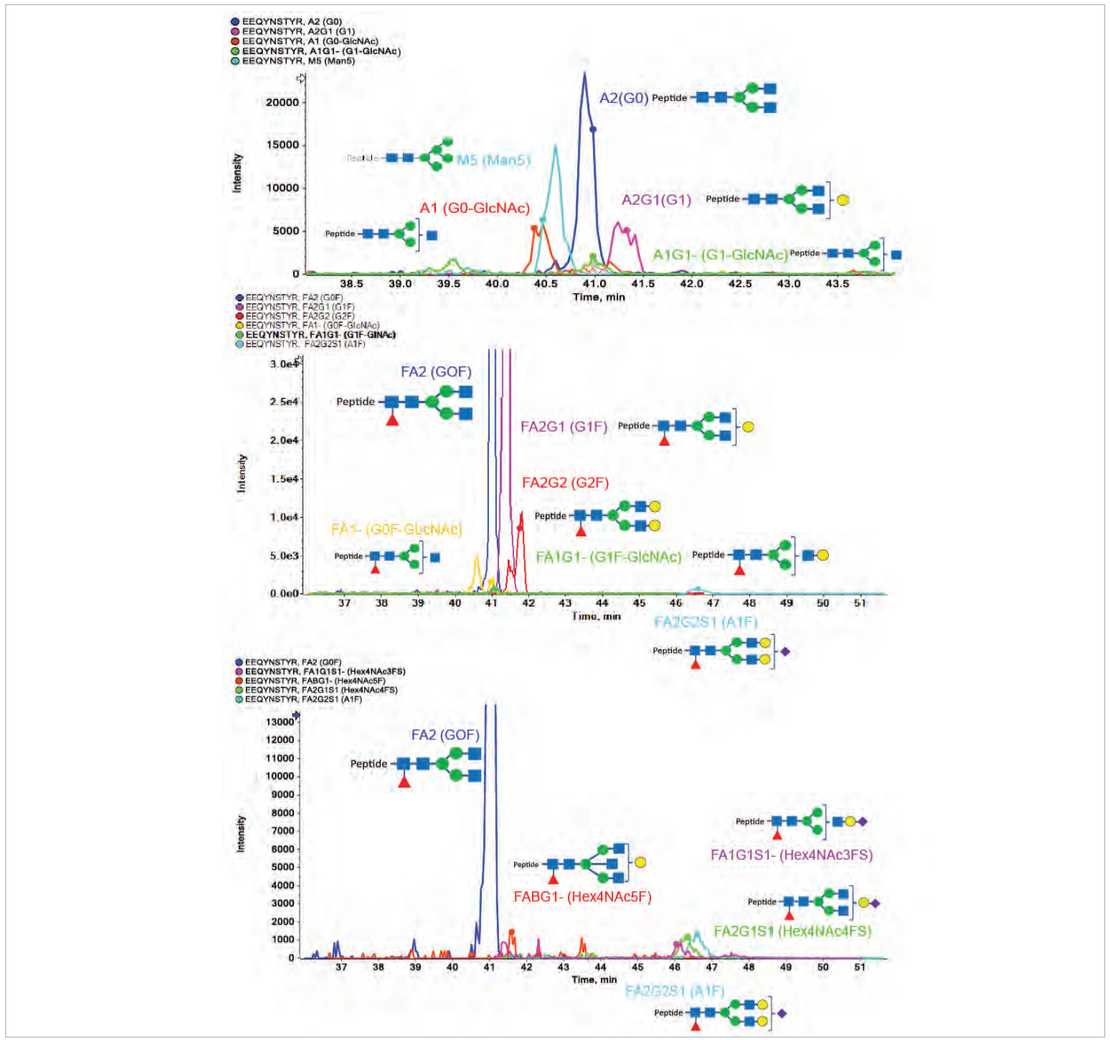

Figure 7A: Glycosylation structures on the EEQYNSTYR peptide identified by CESI-MS. Further glycopeptide characterization is shown in Table 2B. Glycan structure interpretation followed the CFG protocol.

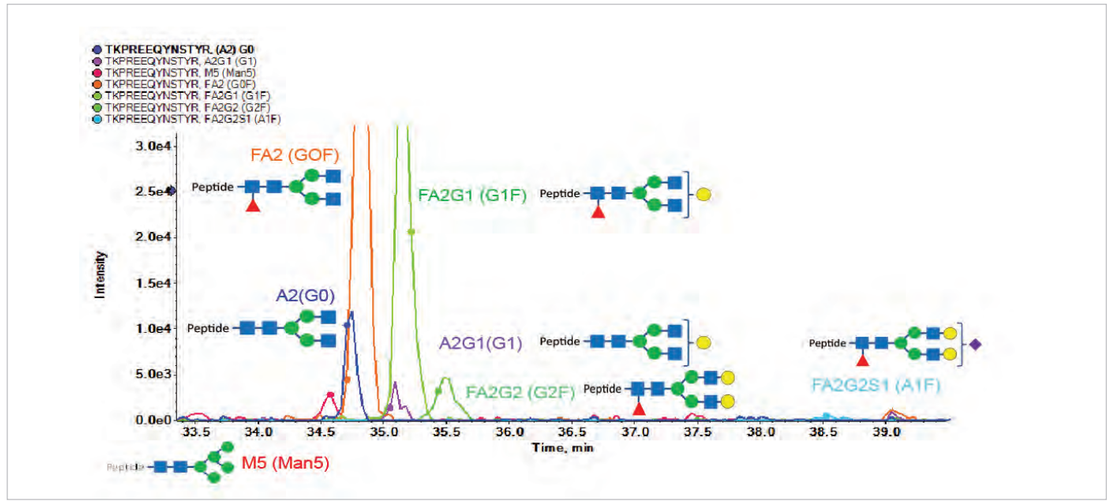

Figure 7B: Glycosylation structures on the TKPREEQYNSTYR peptide identified by CESI-MS. Further glycopeptide characterization is shown in Table 2A. Glycan structure interpretation followed the CFG protocol.

Glyan abbreviation (Structural name)	Glycopeptide identified as R.EEQYN (Glycan) STYR.V Asn300	Glycan mass (Da)	Monoisotopic mass [M+H]+	Migration time (min)	Relative Abundance (%)*
A1 (G0-GlcNAc)		1095.3966	2284.9086	40.42	1.39
M5 (Man5)		1216.4228	2405.9349	40.5	3.52
FA1 (G0F-GlcNAc)		1241.4545	2430.9665	41.02	2.41
A1G1 (G1-GlcNAc)		1257.4494	2446.9614	41.02	0.48
A2 (G0)		1298.4760	2487.9880	41.02	5.50
FA1G1 (G1F-GlcNAc)		1403.5073	2593.01931	41.08	0.33

** As a quantitative approximation, relative abundances were calculated using peak areas of glycosylated peptides of the same sequence and charge state detected (+4).*

Table 2A: Characterization of trastuzumab heavy chain glycosylation hot spot N300 on the TKPREEQYNSTYR peptide with structural identification and relative quantification. Glycan structure interpretation followed the CFG protocol.

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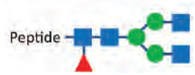
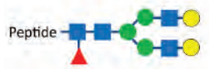
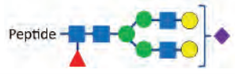
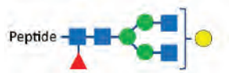
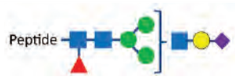
Glycan Abbreviation (Structural name)	Glycopeptide identified as R.EEQYN (Glycan) STYR.V Asn300	Glycan mass (Da)	Monoisotopic mass [M+H]+	Migration time (min)	Relative Abundance (%)*
M5 (Man5)		1216.4228	2888.2313	34.61	2.03
(A2) G0		1298.4760	2970.28452	34.73	7.49
FA2 (G0F)		1444.5339	3116.34242	34.74	47.37
A2G1 (G1)		1460.5288	3132.33732	35.07	2.25
FA2G1 (G1F)		1606.5867	3278.39522	35.24	35.38
FA2G2 (G2F)		1768.6395	3440.44802	35.47	4.94
FA2G2S1 (A1F)		2059.7349	3731.54342	38.56	0.54
FA2 (G0F)		1444.5339	2634.0459	41.00	39.06
A2G1 (G1)		1460.5288	2650.0408	41.35	1.86
FA2G1 (G1F)		1606.5867	2796.0987	41.36	36.68
FA1G1S1 (Hex4NAc3FS)		1694.6027	2884.1147	46.06	0.30
FA2G2 (G2F)		1768.6395	2958.1515	41.79	6.80
FA2BG1 (Hex4NAc5F)		1809.6661	2999.1781	41.62	0.29
FA2G1S1 (Hex4NAc4FS)		1897.6821	3087.1785	46.37	0.63
FA2G2S1 (A1F)		2059.7349	3249.2469	46.63	0.74

** As a quantitative approximation, relative abundances were calculated using peak areas of glycosylated peptides of the same sequence and charge state detected (+4).*

Table 2B: Characterization of trastuzumab heavy chain glycosylation hot spot Asn300 on the EEQYNSTYR glycopeptide with structural identification. Glycan structure interpretation followed the Consortium of Functional Glycomics (CFG) protocol.

Glycopeptide mass mapping

One of the most important effector function-related post translational modifications common to mAbs is glycosylation. The presence or absence of specific glycan residues can significantly alter the efficacy, stability, and immunogenicity of mAbs. Trastuzumab contains a consensus glycosylation site at Asn300 of the heavy chain on which various glycoforms can associate. Because mAbs are made up of two heavy chains, various combinations of glycoforms may be present, resulting in significant complexity. With CESI, very high ionization efficiency of the glycopeptides was achieved, generating strong signals and allowing for MS/MS identification of low abundance species. As the migration time of these glycans differs depending upon structure, the mobility of the peptide becomes a valuable aid in structural assignment. Since tryptic peptides separated by CESI-MS retain all associated glycosylation, specific amino acid linkage can be identified allowing confirmation that linkage occurs at Asn300. As Tables 2A and 2B depict, in this trastuzumab sample 14 different glycoforms were identified on the EEQYNSTYR peptide (Figure 7A) and 7 on the TKPREEQYNSTYR peptide (Figure 7B). The relative abundances of the glycans ranged from 47% to as low as 0.29%. Approximately half of these glycan species were present in low relative abundance but were still ionized and identified by CESI-MS. Since capillary electrophoresis has long been a robust technique for oligosaccharide analysis, application of this fundamental capability of CE to separate glycosylated species based on charge and hydrodynamic radius (i.e., differential electromigration) provides another dimension of analysis by which glycoform identity can be readily determined (Figure 7). Similarly to degradative PTM analysis, there are notable benefits from CESI-MS analysis of glycopeptides. First, with the low nL/min flow rates, glycopeptides are readily ionized among other peptides with better ionization efficiencies, illustrated by the comprehensiveness and dynamic range of glycopeptides identified. Additionally, since glycopeptides regularly co-elute with LC-MS analysis, CESI-MS separation of glycopeptide is advantageous. The migration-based separation of glycopeptides helps to confirm glycan structures. As expected, the identified glycans on peptides increase in size and negative charge as migration time increases. Any unwanted fragmentation of glycans prior to MS and MS/MS (i.e. in-source fragmentation) can be identified as electrophoretic peak shoulders and non-gaussian peak shapes. Thus the separation of glycopeptides by CESI-MS adds both sensitivity and confidence to glycan characterization.

Conclusions

In this article, we illustrated the use of CESI-MS for rapid characterization of the therapeutic antibody trastuzumab using the SCIEX CESI 8000 – TripleTOF 5600+ Platform. Starting with efficient sample preparation using a single enzyme, single digestion protocol followed by a single separation step, we were able to rapidly attain 100% primary sequence coverage for both the heavy and light chains of this important mAb therapeutic. In addition, identification of key amino acid modifications was accomplished, resulting in the elucidation of glutamine cyclization, methionine oxidation, asparagine deamidation, and C-terminal lysine heterogeneity. From the same CESI-MS separation, glycopeptide analysis was performed, resulting in the identification of glycan-amino acid linkage position for as many as 14 glycoforms, many in low abundance. Strong ionization signals for accurate mass measurement and subsequent MS/MS coupled with structure-based mobility separations greatly simplified the determination and assignment of the bound glycan structures. Because of its ultra-low-flow capability, CESI-MS provides remarkable sensitivity enhancements for poorly ionizing molecular species like glycopeptides, and when coupled to a fast accurate MS platform such as the TripleTOF 5600+, efficiently delivers broad mAb characterization from a single protease digestion and single CESI-MS run.

Acknowledgments

We thank Dr. Alain Beck at Pierre Fabre, France for providing trastuzumab.

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Studying Quality Attributes of Adalimumab by Mass Spectrometry

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Introduction

Peptide Mapping with mass spectrometric detection is now one of the default techniques for biotherapeutic analysis, whether in development or for release assays. SCIEX has innovated to develop state-of-the-art software that facilitates the data analysis from accurate mass instruments to speed up data review and reporting at any stage in the process of developing biotherapeutics.

This article will describe some of the elements that allow users to rapidly adapt the processing without any need for specialist mass spectrometry knowledge.

The Utility of Accurate Mass QTOF Analysis

Accurate mass QToF instruments are the dominant mass spectrometry platforms for characterization of biologics' critical quality attributes (CQAs), assisting in process development analyses, and in troubleshooting. QTof technology dominates this space, delivering high resolution (above 30k FWHM) at all mass ranges and fast acquisition rates. Additionally, there are no practical limitations for mass range, with TOF m/z ranges around 40kDa. Using SCIEX TripleTOF® platforms, therapeutic peptides and proteins of all sizes can be routinely analysed. BioPharmaView™ Software is used to automate data processing, and reduces analysis time from days to minutes compared to manual techniques. Although automation avoids human error, users often wish to review data themselves to ensure the appropriate processing parameters, or investigate batch failures. There are a number of ways in which this type of review can be facilitated in BioPharmaView Software.

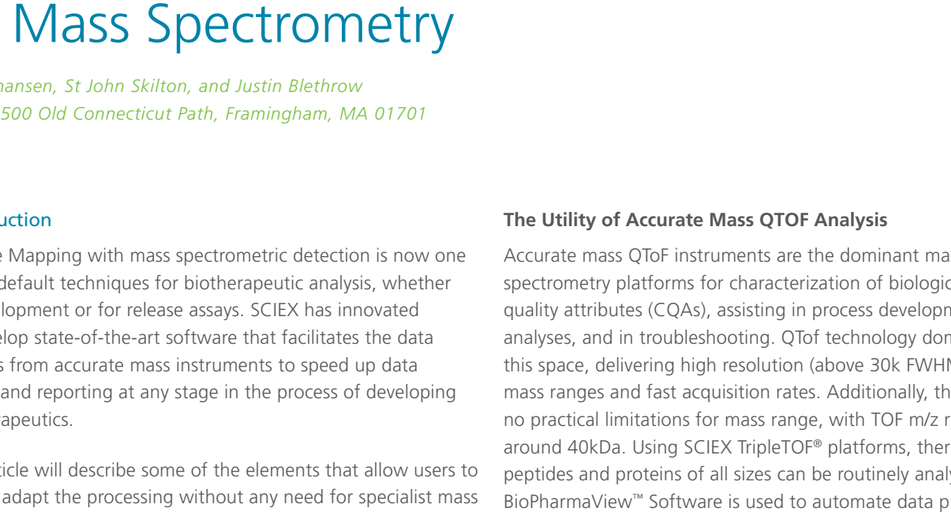

Figure 1: Screenshot of the filter function in BioPharmaView Software. On the left hand side is the pop out box with the options availbale to the reviewer, live-linked to the data in the review panes below. In this example the peptides are filtered for all Oxidized species with a m/z error less than 5ppm. The full list is not shown, which is in the middle panel. The two red 'filter' symbols show the columns where the multi-level filtering criteria have been applied.

Smart Filtering

A typical mAb peptide map might contain hundreds of peptides when considering all of the modifications, charge states and missed cleavages. Therefore, tools that allow smart filtering and multi-level filtering are always considered essential. The functionality displayed in Figure 1 shows how this is performed for a peptide map of the IgG mAb adalimumab, which is approximately 148 kDa and contains 1330 amino acids.[1]

BioPharmaView Software provides users with an exhaustive set of details regarding their biotherapeutics, but can simplify the information rapidly in the review stage. Figure 1 shows the panels of information available where the chromatographic trace is displayed above the tabular and spectral data in separate panels below. At the base are two panels depicting the spectrum (MS) and the fragment ions (MS/MS) for that peptide. The analyst only needs to glance at that data to know whether further investigation is needed.

When more refined information is needed, a user can further filter the data in a manner to align data for each peptide in a simple table, and verify that no assignments are false positives. In Figure 2, this is illustrated with the use of an additional filter level that is applied instantly. Figure 2 shows the filter applied to screen for all peptides that are deamidated using the prefix "De" in the filter panel.

The filter is also applied to show only those matches that fall within a 5ppm window of the target, and the peptide filter is set to exclude missed cleavages with a "NOT" Boolean logic operator applied for the "-" marker of missed cleavages in the peptide field.

Figure 3 shows the result when the logic operator is switched off again and the missed cleavages in that subset are once again displayed. A number of additional peptides are shown following a single mouse click. The filters change in real time and it is not necessary to re-process of re-sort the data.

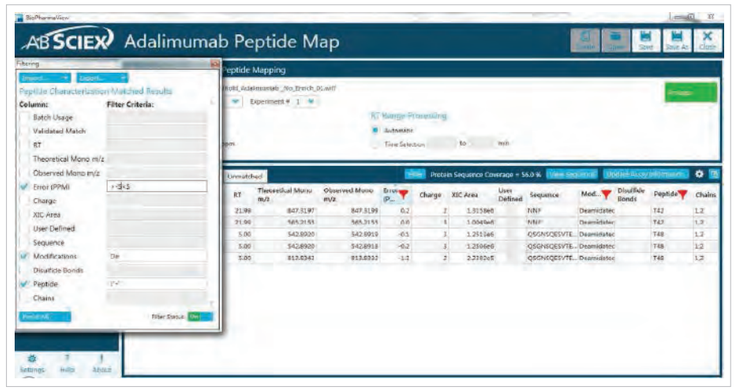

Figure 2: Smart filter applied to adalimumab peptide map with filtering for mass accuracy, deamidation, and no missed cleavages. This reduces the review time and allows a reviewer to home in on specific features that may be important quality attributes.

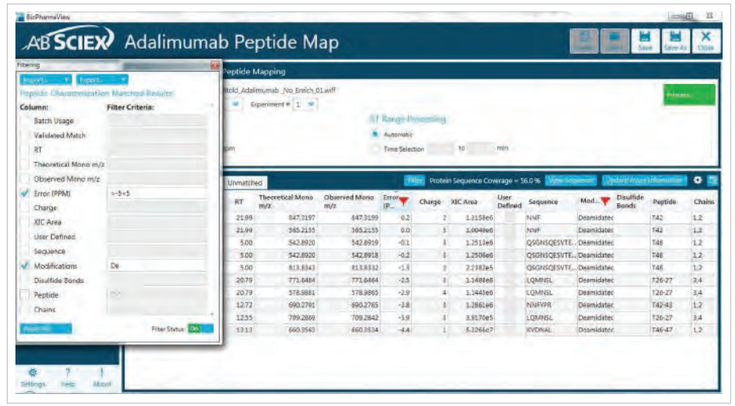

Figure 3: The same filter as applied in Figure 2 is shown here, but with the "Peptide" category unchecked. The result is the display of peptides with missed cleavages and the two-level filter based on mass accuracy and presence of a deamidation.

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The utility of the visual display is also apparent to signal when a filter is applied. The "on/ off" toggle can remove all filters or re-apply them with a simple click (Figure 4) allowing reviewers to see at a glance the effect of their filtering. Figure 5 is a zoom on the filter symbol which appears at the top of the relevant category (column) to show that a filter is being applied.

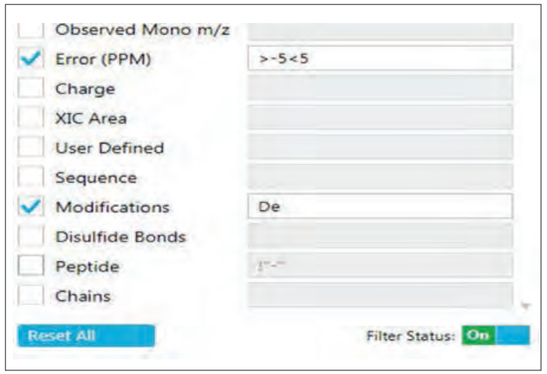

Figure 4: The "on/off" toggle switch for the filtering function in BioPharmaview Software.

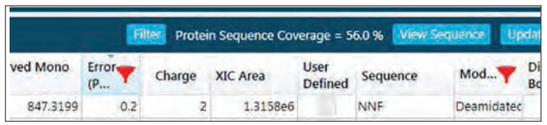

Figure 5: The "filter" symbol is shown in red at the top of each column category in BioPharmaview Software.

Conclusions

Efficient post-processing of peptide mapping data from TripleTOF® LC-MS systems is essential to providing SCIEX customers the rapid review capabilities they need. The flexibility of such a tool improves workflow efficiency by removing human bias, automating tedious procedures, and allowing in-depth analysis of a complex data set within seconds.

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Application of Data Independent Acquisition for Top-down Characterization of IgG Light Chain

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Introduction

Within the past few years, monoclonal human immunoglobulin gamma (IgG) antibodies have become popular biotherapeutic candidates. Mass spectrometry, a powerful technique, emerged to meet the need of high-throughput analysis for structural characterization of monoclonal antibodies (mAbs). Most commonly, the characterization of mAbs is performed by a "bottom-up" approach, in which the mAb is digested by protease and analyzed at the peptide level. However, due to some limitations with this approach, a "top-down" approach can be used to analyze the intact or the large domain of the mAbs. Here, we report a method using data-independent strategy with the TripleTOF® LC-MS/MS instrument to characterize light chain of IgG by top-down approach. Intact molecular weight of both light chain and heavy chain was determined in TOF MS and matched the value determined by primary sequence with known modifications. The high resolution MS/MS spectra were acquired by a data independent acquisition method, in which all charge species (+22 to +32) of light chain within mass range of m/z 755 to 1076 were isolated, one per isolation window, and fragmented by CID. The combined fragment information of the given precursors provided thorough information and improved sequence coverage for top-down protein sequencing. Data showed that such improvement was mainly effected by identification of precursor charge-dependent and precursor charge-favored fragment ions. This valuable information can be missed if using a traditional top-down method, in which only the most abundant precursors are selected for fragmentation.

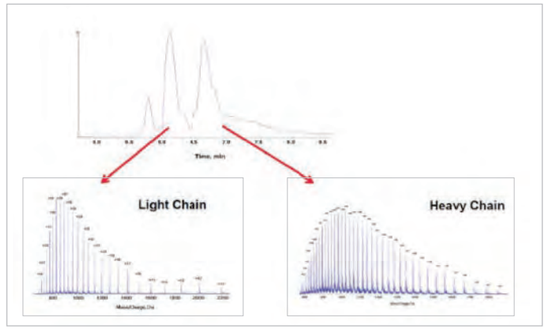

Figure 2: LC-MS chromatogram. Shown are the peaks from 1 µg reduced antibody sample. The light and heavy chains elutes at 6.2 min and 6.8 min respectively. Also shown below is the charge state envelope for each chain used for deconvoluting and confirming mass of the 2 chains.

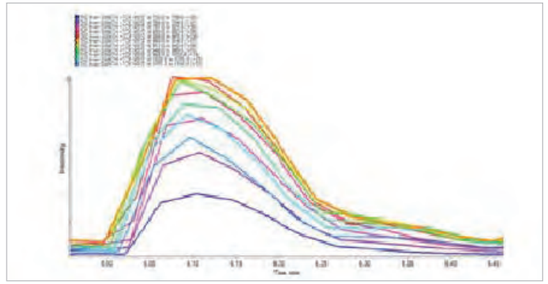

Figure 3: SWATH Acquisition of IgG light chain. Shown is the overlap of the TIC of SWATH Acquisition for the charge states ranging from +22 to +32. Variable mass window is chosen for each charge state to encompass the major peaks.

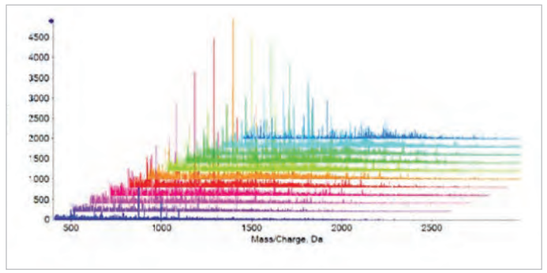

Figure 1: MS/MS spectrum from SWATH® Acquisition: In this SWATH Acquisition analysis, the instrument stepped through m/z of 755 to 1076 and generated a high resolution MS/MS spectrum (represented by each color) for this mass range with a cycle time of 2.4 sec. A variable Q1 window was applied to maintain fragmentation of one charge state per mass window.

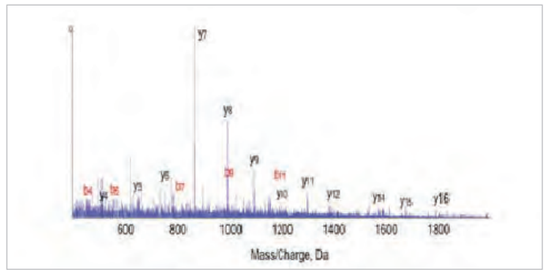

Figure 4: MS/MS analysis for +30 charge state. Shown are the MS/MS spectra of the m/z window of 791-821 which corresponds to the +30 charge state of the light chain. The abundant fragment ions observed are +1 charge of the N & C-term ion ladder. The peaks at lower signal, overlapping with noise, are highly multi-charged > +20.

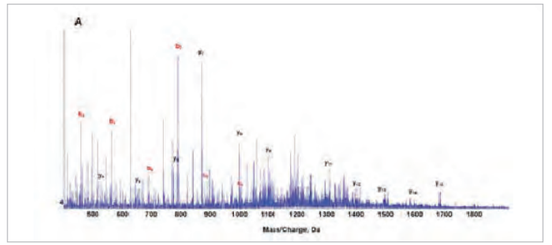

Figure 5A: MS/MS analysis for +22 charge state. The MS/MS spectra of the m/z window of 1075-1126 correspond to the +22 charge state. In addition to the terminal ion ladder, other fragment ions of higher charge states are also observed above the noise level. Figures 5B-D show the zoomed in regions from m/z 850-1500 to take a closer look at the fragment ions identified.

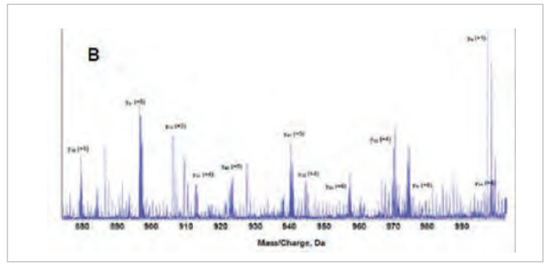

Figure 5B: shows the zoomed in MS/MS m/z region of 850-1000 with various multiply charged y ion MS/MS fragments.

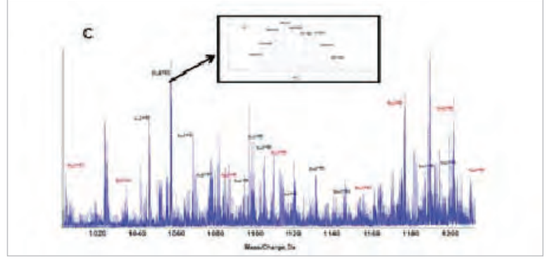

Figure 5C: shows the zoomed in MS/MS m/z region of 1000-1250 with the +9 charged MS/MS fragments from the b 82-99 and y 81-96 fragmentations. The inset shows the zoomed in area for the y82 ion confirming the +9 charge state.

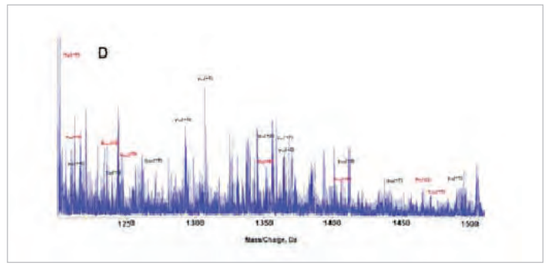

Figure 5D: shows the zoomed in MS/MS m/z region of 1200 -1500 identifying +9 charged MS/MS y97-114 and b99-122 fragments of the light chain.

DVLMTQTPLSLPVSLGDQASISCRSSQYIVHSNGNTYL
EWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDF
TLKISRVEAEDLGVYYCFQGSHVPLTFGAGTKLEIKRAD
AAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKW
KIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDE
YERHNSYTCEATHKTSTSPIVKSFNRNEC

Table 1: IgG1 light chain sequence coverage. Highlighted in blue and green are the regions of the light chain that were identified from the various charged b and y-ion fragments, respectively. As can be observed, a more complete coverage can be obtained via MS/MS spectra of the various charged state precursor masses of the IgG1 light chain.

Experimental

Sample Preparation

50 µg of Intact mAb (IgG1) was subjected to dithieothreitol (DTT) at 60°C for 45 min to reduce the antibody to the light and heavy chains.

Chromatography

Chromatographic separation of reduced light chain and heavy chain was performed on a high flow UHPLC system. Approximately 1 µg of reduced sample was loaded onto a Poroshell 300 SB-C8 column. Light and heavy chain were separated by a linear gradient of 20%-55% of B in 15 min, using 100% H2O, 0.1% FA (solvent A) and 100% ACN, 0.1% FA (solvent B).

SWATH Acquisition using TripleTOF 6600 LC-MS/MS system

The MS analysis was performed on a TripleTOF 6600 system using SWATH Acquisition. A continuous variable precursor isolation window was applied. The Q1 isolation window width was determined by the precursor charge state of light chain to maintain one charge species per window. High resolution MS/MS spectra were acquired by this SWATH Acquisition, which included all charge species (+22 to +32) of light chain within mass range of m/z 755 to 1076 and fragmented by CID. Data was processed using PeakView® Software.

Conclusions

- Shown in this study is a top down approach using data independent analysis to obtain better sequence coverage compared to the traditional data dependent approach.
- A variable Q1 mass window was used in SWATH Acquisition to maintain one charge species per mass window allowing for the selective generation of MS/MS for specific precursor charge states.
- MS/MS spectra from different charge states provide different fragment ion information. Cumulative fragment ion identification from the various charge states can provide a more complete sequence coverage.
- Further investigation can be performed using this top down SWATH Acquisition for sequencing of larger domains generated by enzymatically cleaved IgG heavy chain.

DEVELOPMENT AND QUALITY CONTROL

Unification of Charge Heterogeneity, Purity, and Molecular Weight Analyses of mAbs into a Single Analysis

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SCIEX Separations, [1]Brea, CA, [2]Framingham, MA

Key Benefits

- Simplified workflow with a single CESI-MS analysis for multiple mAbs characterization insights.
- Glycoforms, clipped species, impurities, and excipients all separated and identified by the same method with accurate mass detection.

Introduction

Immunoglobulin gammas (IgGs) are the most common molecules used for generation of therapeutic monoclonal antibodies (mAbs). The ability to characterize these molecules with high sensitivity and comprehensiveness is essential to their development and for regulation of their efficacy, bioavailability, and biosafety. Charge heterogeneity, purity, and molecular weight analyses are powerful CE- and MS-based methods for mAb characterization. Intact and reduced analysis of mAbs by CE are used in the mAb screening, analytical development, and quality control processes. Combining a similar CE separation with mass spectrometric (MS) detection of intact mAbs allows for unification of these three methods into one. Additionally, the high resolution and mass accuracy MS detection can facilitate identification of unknown CE peaks and may also provide more accurate and sensitive purity and molecular weight measurements than with optical detection alone.

The integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into one process (CESI) provides these possibilities while also lowering the sample mass analysis requirements. We present the analysis of intact and reduced IgGs using a single CESI-MS method which provides charge heterogeneity, purity, and molecular weight information similar to the multiple current industry-accepted CE-SDS, capillary isoelectric focusing (cIEF), and capillary zone electrophoresis (CZE) analysis methods combined.[1-3] Notably, the intact and reduced IgG analyses by CESI-MS demonstrate the capabilities for quickly screening and characterizing candidate or developed therapeutic IgG molecules which may have either charge and/or size heterogeneity, particularly due to stress or instability.

SCIEX CESI 8000 Plus High Performance Separation Module coupled to a TripleTOF® 6600 System via a Nanospray III source, CESI adapter, and neutral-coated OptiMS capillary cartridge.

The CESI-MS results are compared to existing industry-accepted, CE-based charge heterogeneity, purity, and molecular weight analyses. Charge heterogeneity separations by CESI-MS using a capillary zone electrophoresis (CZE)-based separation mechanism showed similar profiles to a cIEF-based method which has been previously demonstrated using optical detection methods.[3-6] With the MS-based detection, molecular changes that cause charge heterogeneity, such as glycoforms, were associated with CE migration shifts. Other peaks within the charge heterogeneity separation could also be primarily attributed to potential clipped IgG impurities within the samples. Collectively, the results demonstrate the advantages of using MS as the detector for a CZE-based charge heterogeneity analysis since it also provides molecular weight and purity information.

Materials and Methods

Sample Preparation: For CESI-MS experiments, IgG1, IgG2, and IgG4 molecules (20 mg/mL) were desalted and buffer exchanged into 50 mM ammonium acetate, pH 4 using Zeba spin columns (Thermo Fisher Scientific). For reduced analysis, IgG molecules were incubated for 45 min at 60° C in 10 mM DTT and 0.1% Rapigest SF Surfactant (Waters). Rapigest was cleaved (0.5% formic acid, 37° C, 10 min) and spun down with precipitants (14K x g). Concentrated (2 M) ammonium acetate, pH 4 was added for a 50 mM final concentration. For stand-alone CE experiments, IgG molecules were either diluted into a urea-gel-ampholyte solution (cIEF-based analysis) or SDS gel solution (CE-SDS analysis). Reductions in SDS gel solutions were performed at 60° C for 10 min in 10 mM DTT.

CESI 8000 Plus MS Mode Conditions: CESI experiments were carried out with a SCIEX CESI 8000 Plus system (P/N A98089) equipped with a temperature controlled auto sampler and a power supply with the ability to deliver up to 30 kV. An OptiMS Neutral Surface Cartridge (P/N B07368) with a porous tip was used. Solutions of 3% and 10% acetic acid were employed as background electrolytes (BGE) and conductive liquids, respectively. Pressure (5 psi) was applied for 10 sec to generate ~7.5 nL sample injections. Sample stacking after pressure injection was performed by transient isotachophoresis (tITP) due to the 50 mM ammonium acetate sample buffer. CESI separations were performed at 30 kV with 2 psi for 7 min (pre-separation), then 10 psi for 10 min (separation and ESI).

MS Conditions: A SCIEX TripleTOF 6600 system with a NanoSpray® III source and CESI adapter (P/N B07363) were used. Intact protein detection was performed with MS scans from 400 – 4500 m/z.

Data Analysis: High resolution MS spectra were analyzed using SCIEX PeakView® and BioPharmaView™ Software.

CESI 8000 Plus Stand-Alone CE Mode Conditions: CE-SDS and cIEF experiments were performed using SCIEX PA 800 Plus detectors (P/N B68372), kits (P/Ns A10663 & A80976), and protocols.

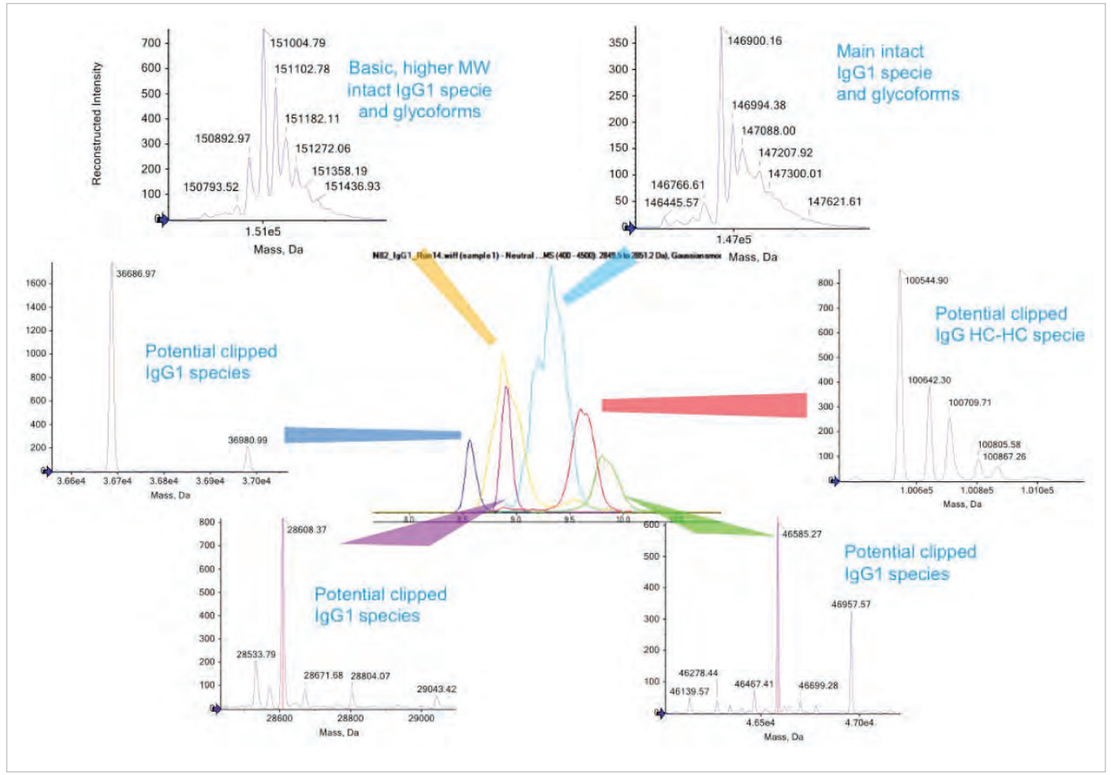

Figure 1: Charge heterogeneity separation of IgG1 with MS detection. Extraction and deconvolution of non-reduced IgG1 species MS spectra from the CESI separation. The extracted ion electropherograms (XIEs) from the most abundant charge states (~3 – 6) from the same charge state envelopes were summed. Spectra were integrated using 15 – 30 sec from each electrophoretic peak using their full width at half maximum height as the integration window.

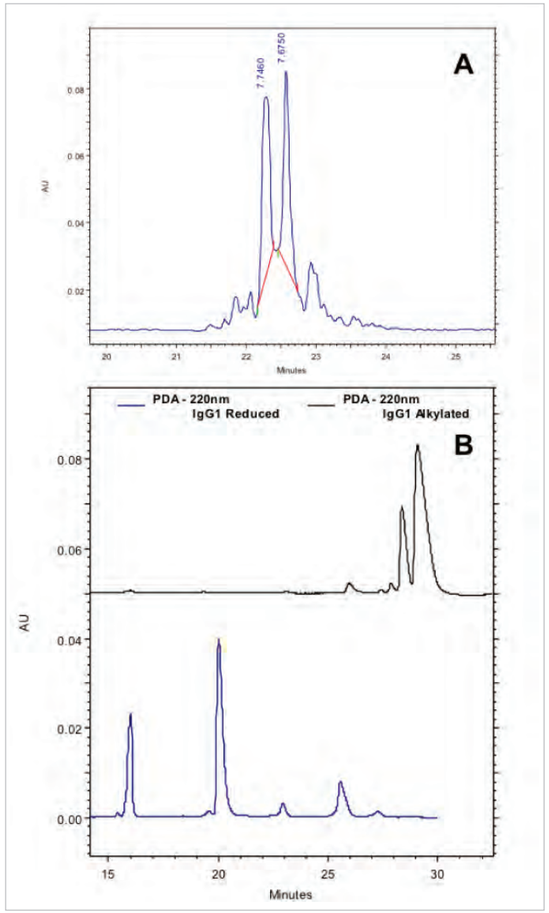

Figure 2: (A) cIEF analysis of non-reduced IgG1 molecules with UV detection. (B) CE-SDS analysis of non-reduced, alkylated (black trace) and reduced (blue trace) IgG1 molecules with PDA detection.

Results and Discussion

CESI-MS based analyses were performed at the non-reduced (intact) and reduced levels using the neutral-coated CESI OptiMS capillary. The CZE-based separation of non-reduced IgG1 (Figure 1) generated a charge heterogeneity separation and facilitated accurate molecular weight determination of IgG1 species and impurities through spectral deconvolution. Two intact IgG1 species were detected. The main charge variant had an average molecular weight of 146,900 Da while the basic IgG1 charge variant was 151,005 Da. In addition to average molecular weight differences, their glycoform profiles were different. Further data analysis using the high mass accuracy deconvoluted molecular weights would facilitate identification of candidate sequence and glycoform matching. While two main IgG1 variants were detected as electrophoretic peaks and intact MS spectra, their glycoform profiles and partially split electrophoretic peaks also imply that additional variants are also present in the sample.

Additional deconvolution analysis indicates a mixture of spectra from deamidations, disulfide breaks, and glycosylation variants (data not shown). Four other IgG1 species were also detected by MS which appeared to be impurities from potential in-solution clipping events. The IgG1 impurities included abundant molecular weights of 28,608, 36,687, and 46,585 Da. The highest molecular weight impurity was 100,545 Da which had a molecular weight and glycosylation profile consistent with a heavy chain dimer (HC-HC) impurity. Notably, the CESI-MS-based charge heterogeneity analysis provided results consistent with a cIEF (Figure 2A) and CE-SDS (Figure 2B) analysis of the same IgG1 sample. For example, a similar separation profile and number of species were detected between the cIEF and CESI-MS charge heterogeneity separations. Additionally, the number of molecular weight impurities from CE-SDS analysis is also consistent with the number of species detected with unique molecular weights by the CESI-MS based analysis. Thus, the CESI-MS analysis combines both aspects of the cIEF and CE-SDS analyses through a CZE-based charge heterogeneity separation and molecular weight determination by MS. Ultimately, the CESI-MS analysis allows for direct detection and candidate identification of potential IgG1 clipping events that might be otherwise misinterpreted as intact IgG1 charge isoforms or difficult to identify by UV detection alone. Representative raw MS data used for spectral deconvolution is shown for each electrophoretic peak in Figure 3.

The CZE-based method can also be used for reduced mAb analysis. Figure 4 shows the separation and detection of reduced IgG1 molecules. One IgG1 heavy chain was detected while two IgG1 light chain species were detect. This was also consistent with the three main reduced IgG1 species detected by the CE-SDS analysis. The molecular weight measurement of the two IgG1 light chain species contributes to the understanding of the molecular weight differences of the intact IgG1 forms. That is, the higher molecular weight IgG1 light chain is likely present in the acidic, higher molecular weight intact IgG1 charge variant. In combination with the high mass accuracy measurement by the MS, further data analyses could be used to confirm the different intact IgG1 forms using the reduced IgG1 analysis.

The same CESI-MS intact charge heterogeneity analyses were also performed on representative IgG2 and IgG4 molecules (Figures 5 and 6, respectively). Just as with the IgG1 analyses, multiple intact and clipped IgG2 and IgG4 species were detected in each analysis. Even though the intact IgG2 and IgG4 co-migrate with their respective forms, the spectral deconvolution process allows for identification of two different glycoforms of each in both cases. Similarly, potential clipped IgG2 and IgG4 species both migrate before their intact forms, allowing for sensitive identification of the impurities. In the case of the IgG2 analysis, a potential formulation excipient (e.g. polysorbate 80) was well resolved from IgG2 that might otherwise detrimentally affect MS spectral quality by nanoESI- or LC-MS-based analyses. Similar results with other non-ionic detergents would be expected since they will have the same electrophoretic mobilities and migration times.

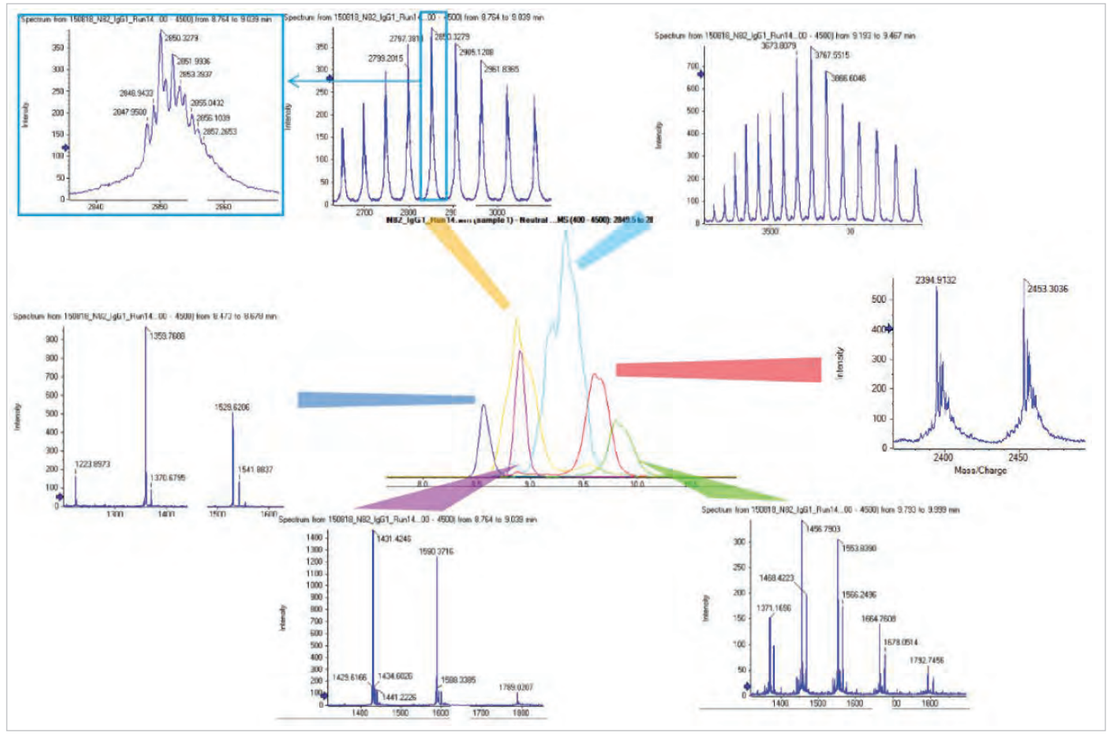

Figure 3: Extraction of representative raw MS spectra from non-reduced IgG1 species from the CESI separation used for generation of deconvoluted spectra in Figure 1. Spectra were integrated at 15 – 30 sec from each electrophoretic peak using their full width at half maximum height as the integration window.

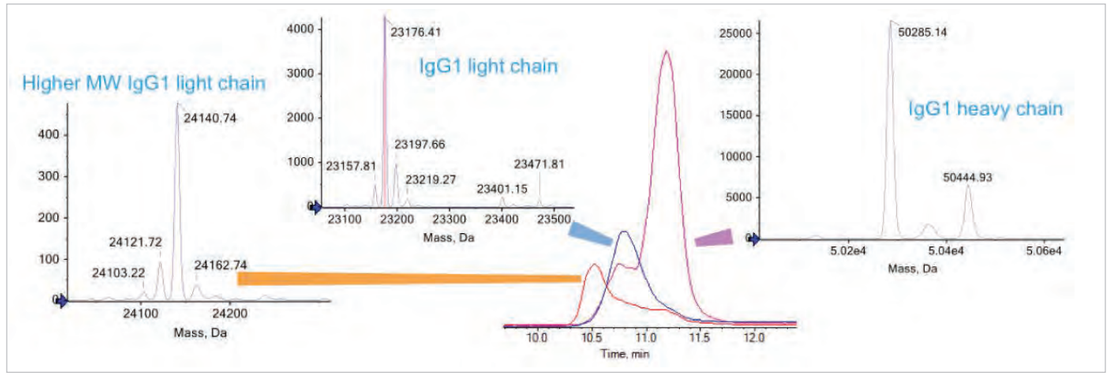

Figure 4: Reduced IgG1 analysis with MS detection. Extraction and deconvolution of reduced IgG1 species MS spectra from the CESI separation. The extracted ion electropherograms (XIEs) from the most abundant charge states (~3 – 6) from the same charge state envelopes were summed.

DEVELOPMENT AND QUALITY CONTROL

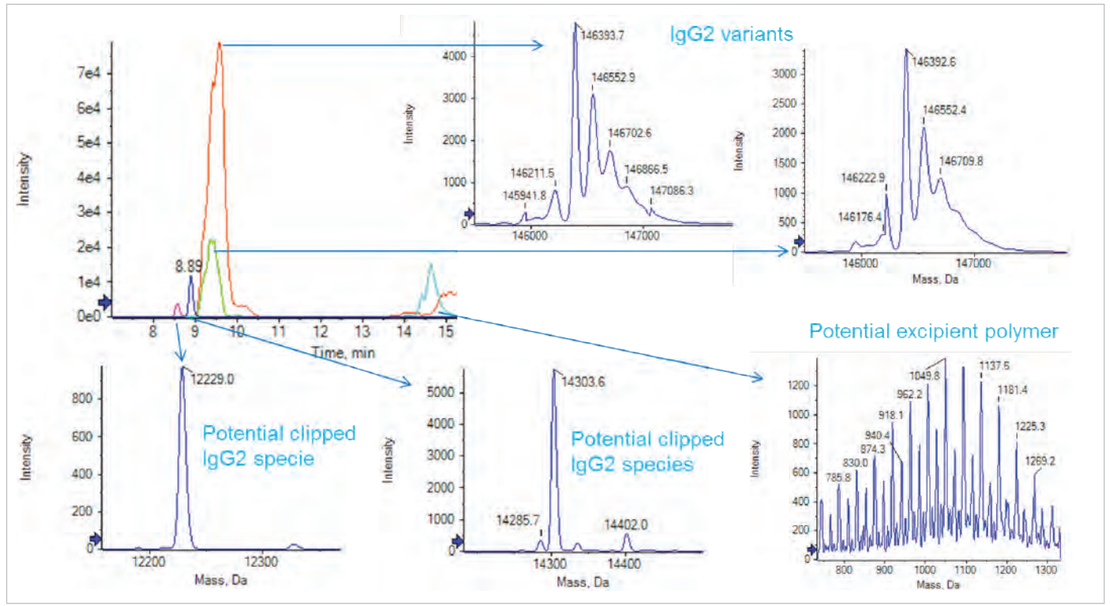

Figure 5: Charge heterogeneity separation of IgG2 with MS detection. Extraction and deconvolution of non-reduced IgG2 species MS spectra from the CESI separation. The extracted ion electropherograms (XIEs) from the most abundant charge states (~3 – 6) from the same charge state envelopes were summed.

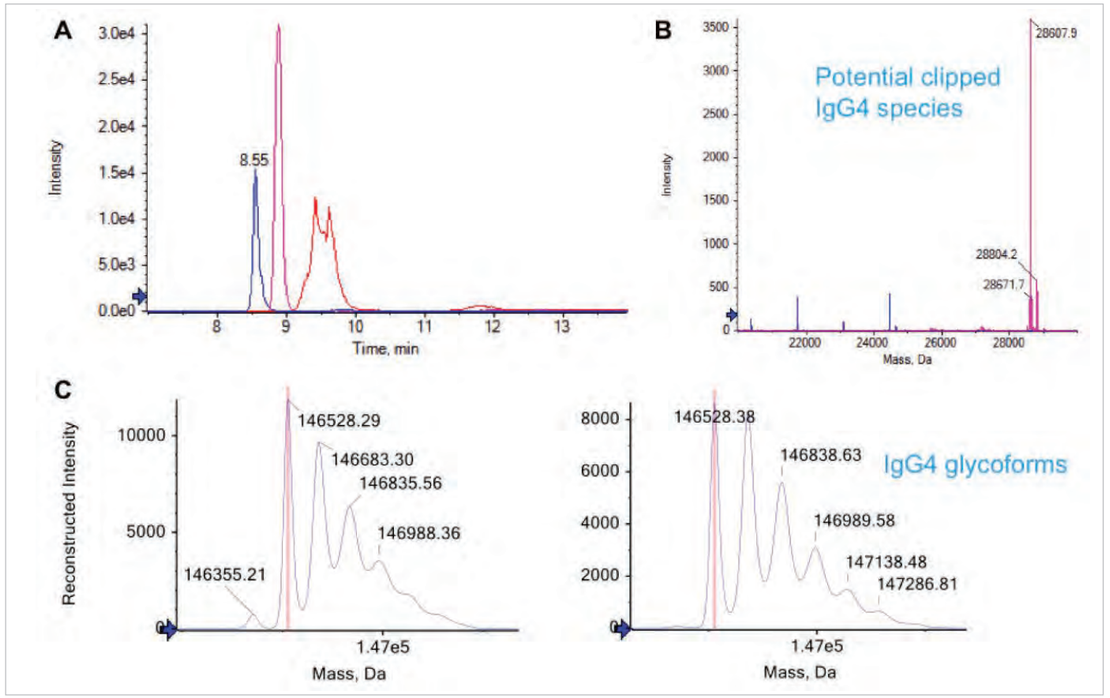

Figure 6: (A) Charge heterogeneity separation of IgG4 with MS detection. The extracted ion electropherograms (XIEs) from the most abundant charge states (~3 – 6) from the same charge state envelopes were summed. (B) Extraction and deconvolution of potential clipped IgG4 species MS spectra from the CESI separation. Deconvoluted MS spectra colors correspond to the XIE peaks of the same color. (C) Extraction and deconvolution of non-reduced IgG4 species MS spectra from the CESI separation. The left and right deconvoluted spectra correspond to spectral extraction of the left and right side of the orange, split XIE peak.

Conclusions

The combined CESI-MS and stand-alone CE functionality of the CESI 8000 Plus system provides powerful methods to characterize intact and reduced IgG forms. Both analyses deliver charge heterogeneity, purity, and molecular weight information. When coupled with the TripleTOF 6600 system, the CESI-based analysis using a neutral-coated capillary generates high resolution separations with high mass accuracy molecular weight information for characterization of mAb charge variants, glycoforms, and impurities. Additionally, the high sensitivity CESI-MS analyses are achieved from small sample amounts (~10 ng), particularly useful in the mass-limited development phase.

References

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DEVELOPMENT AND QUALITY CONTROL

Separation of Fucosylated, non-Fucosylated, and Complex Carbohydrates Associated with Monoclonal Antibodies using Capillary Electrophoresis

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Abstract

In order to gain a comprehensive understanding of therapeutic Monoclonal Antibody (MAb) function, it is necessary to critically characterize glycosylation associated with them. Carbohydrates are known to play an important role in the structure, function, and clearance of MAbs and have been shown to be responsible for invoking immune responses in humans. Changes in carbohydrate composition or concentration can significantly impact the overall efficacy of therapeutic MAbs and can also lead to side effects. Because of their link to Antibody Dependent Cellular Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC), accurate analysis of oligosaccharide fucosylation, sialylation, and antennary structure is critical for a complete understanding of MAb microheterogeneity. Capillary Electrophoresis (CE) technology has been successfully used to separate major IgG N-linked oligosaccharides G0, G1, and G2 structures from one another. The basis for this separation relies on electrophoresis of oligosaccharides labeled with 8-amino pyrene 1,3,6 trisulfonic acid (APTS). The complexity of glycans associated with many molecules calls for high resolution separation in order to assess heterogeneity among carbohydrate isomers and co-migrating carbohydrate species. Since CE is already an established technology for automated and quantitative analysis of N-linked oligosaccharides, we set out to develop methodology by which fucosylated, afucosylated, sialylated and complex antennary oligosaccharides can be differentiated from one another. Additional experimentation will focus on further development of these methods.

Introduction

Immunoglobulins or antibodies are soluble serum glycoproteins involved in passive immunity against foreign antigens. Monoclonal Antibodies (mAbs) have been developed as therapeutic reagents because of their specificity towards particular molecular targets associated with disease manifestation. There exists a high degree of structural and functional heterogeneity among antibodies, due in large part to the diversity of associated glycosylation.

Glycosylation on therapeutic monoclonal antibodies is a critical post-translational modification that has been associated with their bioactivity, structure, and pharmacokinetics.

A number of different carbohydrate moieties can potentially bind to mAbs, but it is generally thought that a core group of bi-antennary and high-mannose structures make up the most commonly associated species. MAb carbohydrate heterogeneity analysis and quantitation is essential as oligosaccharides linked to their Fc region play an important role in the regulation of Complement Dependent Cytotoxicity (CDC) and Antibody Dependent Cellular Cytotoxicity (ADCC).[1]

An increase in terminal galactose (Gal) on mAb N-linked oligosaccharides has been implicated in up-regulation of CDC.[2] Glycan species varying in terminal Gal content can be readily separated and analyzed using existing CE technology. Glycan sample preparation includes addition of both charge and fluorescence properties allowing oligosaccharides to be electrophoretically separated and then quantitated using laser-induced fluorescence (LIF) detection technology.

First, oligosaccharides are removed from the Asn[297] residue of the mAb backbone using the N-glycosidase F (PNGase F). This is followed by derivatization of the fluorophore 8-aminopyrene-1,3,6-tri-sulfonic acid (APTS) via reductive amination at the reducing end of the oligosaccharide (Figure 1).

Electrophoretic separation can be performed utilizing a polymeric separation matrix consisting of 0.4% polyethylene oxide (PEO). SCIEX has developed and commercialized technology to automate and simplify this process. It has been shown that the principle for this gel-based CE separation of oligosaccharides is based on both mobility and hydrodynamic volume.[3] This is illustrated in part by the fact that positional isomers, although identical in mass, can be resolved from one another (Figure 2).

Modifications on glycan structures including the presence of fucose, terminal sialic acid, or a bisecting N-Acetylglucosamine (GlcNAc) have been associated with changes in ADCC activity, thus have an impact on mAb efficacy.[4,5] High mannose structures also have been implicated in increased ADCC.[6] Because of the possibility of numerous different glycosylations, highly resolving separation of these species is necessary to accurately analyze glycan populations. Since the size difference between fucosylated and afucosylated glycans is as small as 16 daltons, and that they may have numerous positional isomers, separation has proven to be difficult for some of these species. Current methods have been incapable of resolving a large number of the major co-migrating glycan species from one another. Previously, CE conditions capable of separating fucosylated and afucosylated N-linked oligosaccharides were presented.[7] That work, in addition to the success CE technology has in resolving differences in terminal galactosylation, suggests that it should also be capable of separating glycans that are fucosylated, sialylated, or bisected from each other as well as high mannose species.

Methods and Materials

All separations were performed using the PA 800 Plus Pharmaceutical Analysis System configured with a 488 nm solid state laser and LIF detection with an emission band-pass filter of 520 nm ± 10 nm. N-CHO capillaries were used for separation of oligosaccharides. All other assay conditions were as described in the standard operating procedure for the Carbohydrate Labeling and Analysis Assay Kit (SCIEX p/n 477600) with the exception that carbohydrate separation buffer was substituted with a new separation buffer formulation where indicated. Final concentration for oligosaccharide samples was 1.25 µM. Glycan standards for fucosylated and afucosylated species of G0, G1, G1', and G2 were purchased from Glyko ProZyme, Inc. (Hayward, CA). The therapeutic mAb was obtained from Genentech, Inc. (S. San Francisco, CA). See Table 1 for additional information.

Experimental details for this work were as follow (unless otherwise indicated):

- Carbohydrate separation gels used:
  - Carbohydrate assay gel (contains polyethylene oxide (PEO)) buffer or,
  - New separation gel buffer was 1:1 mixture of:
    - Carbohydrate separation gel buffer (PEO) – BEC p/n 477623
    - dsDNA1000 separation gel buffer (LPA) – BEC p/n 477628
- Capillary length: total length = 60.2 cm, length to detector = 50 cm
- Capillary diameter: 50 µm I.D.
- Injection conditions: 0.5 psi for 10 sec unless otherwise stated
- Separation Voltage: 30 kv
- Field Strength: 500 volts/cm
- Capillary cartridge temperature: 20° C
- Sample storage temperature: 10° C

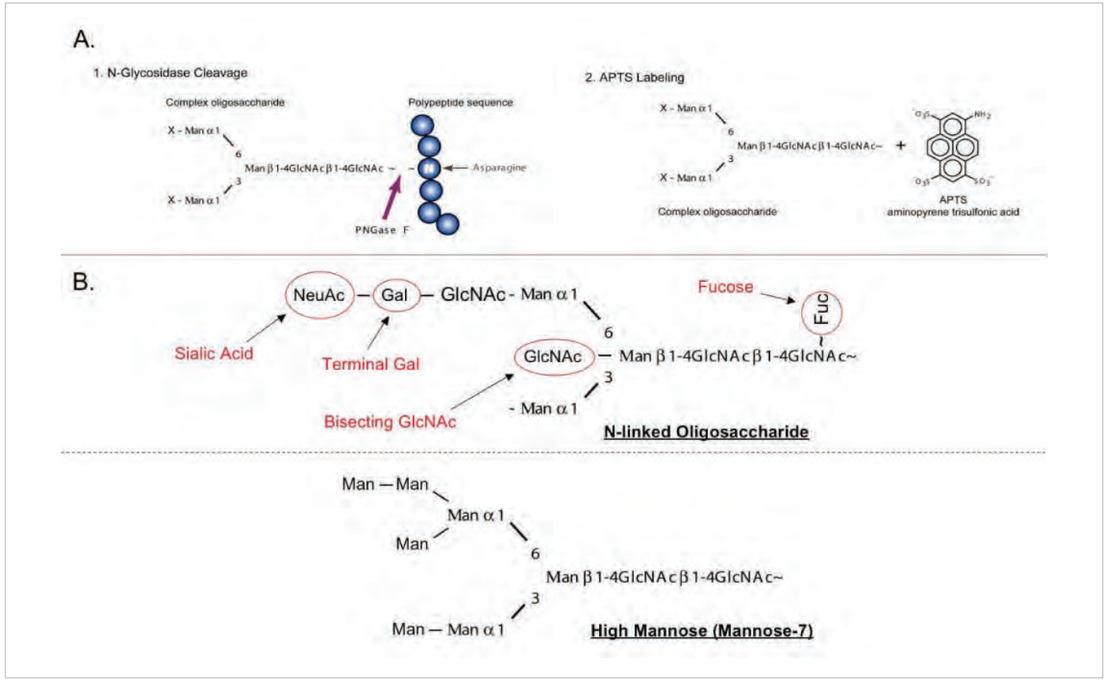

Figure 1: Schematics of glycan analysis sample preparation and various carbohydrate structures. A. Glycan cleavage and APTS derivatization strategy for analysis of N-linked oligosaccharides. B. Examples of 2 glycan species: N-linked oligosaccharide illustrating putative important modifications (left) and high mannose structure (right).

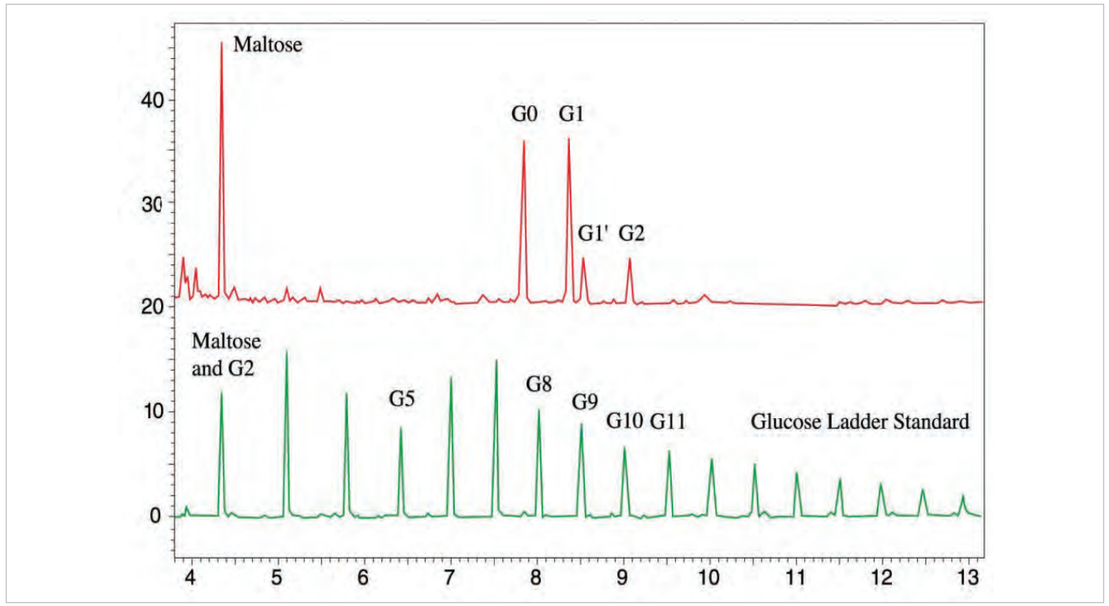

Figure 2: Separation of G0, G1, and G2 glycan species. Representative data (top trace) shows separation of N-linked oligosaccharides G0, G1, G1', and G2 using the Carbohydrate Labeling & Analysis Assay Kit (SCIEX p/n 477600). G1 positional isomers are resolved from one another illustrating the mobility-based and hydrodynamic volume-based separation. The bottom trace shows separation of a glucose ladder standard. The 'G' designation for the glucose ladder standards refers to the number of glucose subunits making up that standard.

Abbreviation	Description	Compound Name	MW (Da)
-G	Trimannosyl core	M3N2	911
-GF	Trimannosyl core, substituted with fucose	M3N2F	1057
Man-5	Oligomannose 5	Man-5	1235
G0	Asialo, agalacto, biantennary complex	NGA2	1317
Man-6	Oligomannose 6	Man-6	1398
G0F	Asialo, agalacto, biantennary complex, core substituted with fucose	NGA2F	1463
G1 / G1'	Asialo, monogalactosylated, biantennary complex	NA2G1	1480
Man-7	Oligomannose 7	Man-7	1560
G1F / G1'F	Asialo, mono-galactosylated, biantennary complex, core substituted with fucose	NA2G1F	1626
G2	Asialo, galactosylated, biantennary complex	NA2	1641
G0FB	Asialo-, agalacto-, biantennary, core-substituted with fucose and bisecting N-acetylglucosamine (GlcNAc)	NGA2FB	1667
Man-8	Oligomannose 8	Man-8	1722
G2F	Asialo, galactosylated, biantennary complex, core-substituted with fucose	NA2F	1787
Man-9	Oligomannose 9	Man-9	1884
G2S1	Mono-sialylate, galactosylated, biantennary complex	A1	1933
G2S1F	Mono-sialylate, galactosylated, biantennary complex, core-substituted with fucose	A1F	2079

Table 1: Glycan abbreviations and descriptions. In the course of this work, separation of standards was utilized to help identify various glycan peak positions. This table indicates the compound names, abbreviations used in the data, as well as descriptions and molecular weight of each glycan species.

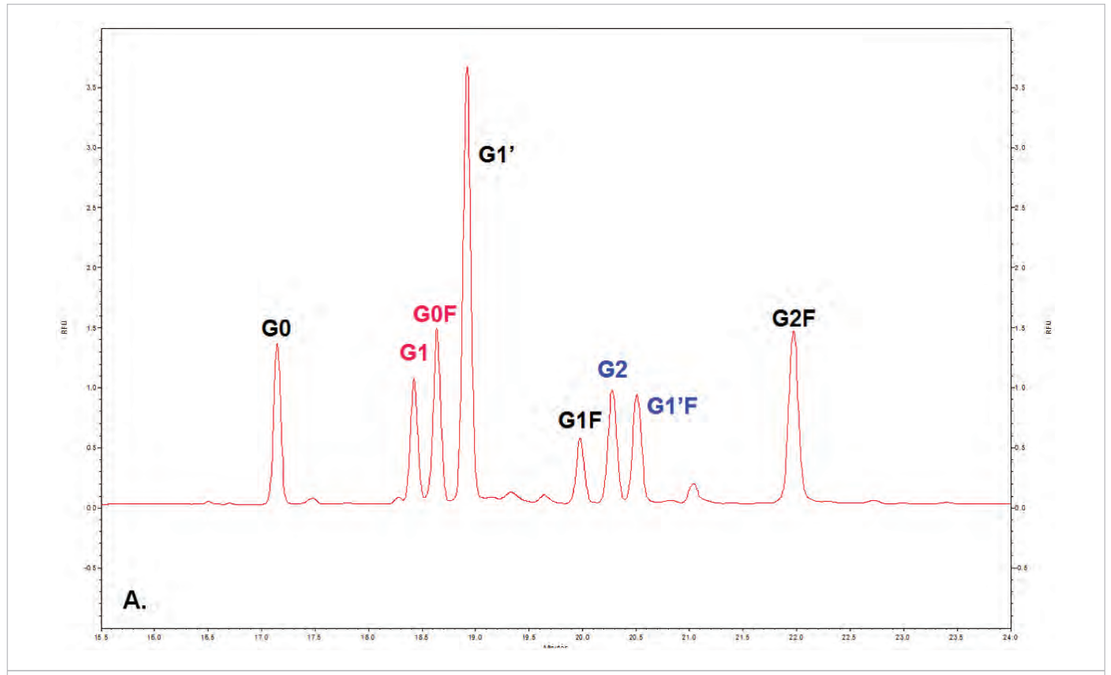

Figure 3: Optimization of the carbohydrate separation buffer allows for resolution between closely-migrating oligosaccharide pairs. Using standard sample preparation protocols, oligosaccharide standards were APTS labeled and separated by CE. Resolution of co-migrating was facilitated by combining existing SCIEX separation buffers. Separation buffer consisted of a 1:1 mixture of Carbohydrate Separation Buffer (SCIEX p/n 477623) containing 0.4% PEO and dsDNA 1000 Gel Buffer (SCIEX p/n 477628) containing a low percentage of linear polyacrylamide (LPA). Separations were performed on an N-CHO capillary (p/n 477600) with an effective length of 50cm. Separation conditions were 20kV following 0.5psi injection for 5 seconds. Field strength was 333V/cm. (A) Closely migrating fucosylated and afucosylated N-linked oligosaccharide standards G0F and G1 (red labels) as well as G1'F and G2 (blue labels) were separated from one another. (B) Closely migrating high mannose oligosaccharide standards were separated from one another. This separation resolved Man-5 from G0, Man-6 from G0F, and Man-7 from both G1F and G2.

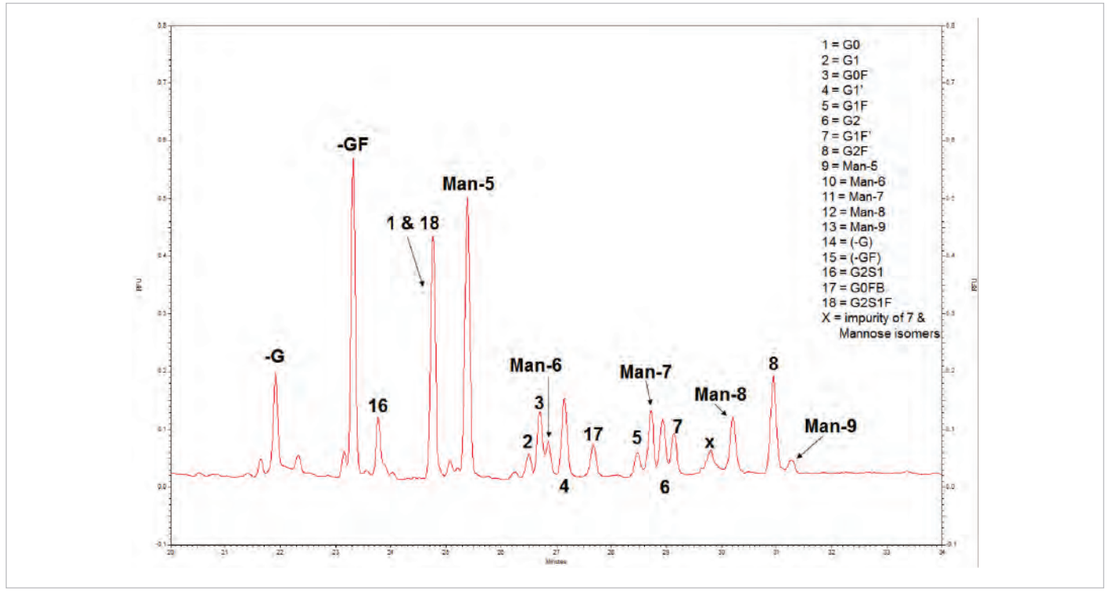

Figure 4: Optimization of the carbohydrate separation buffer allows for resolution of a number of mAb-associated oligosaccharides. Using standard sample preparation protocols, oligosaccharide standards were APTS labeled and separated by CE. Resolution of N-linked oligosaccharide standards was facilitated by combining existing SCIEX separation buffers. Separation buffer consisted of a 1:1 mixture of Carbohydrate Separation Buffer (SCIEX p/n 477623) containing 0.4% PEO and dsDNA 1000 Gel Buffer (SCIEX p/n 477628) containing a low percentage of linear polyacrylamide (LPA). To date, we have been able to separate and identify important glycans differing in galactosylation, fucosylation, sialylation, as well as high mannose structures. Separation conditions were 20kV following 0.5psi injection for 5 seconds. Field strength was 333V/cm.

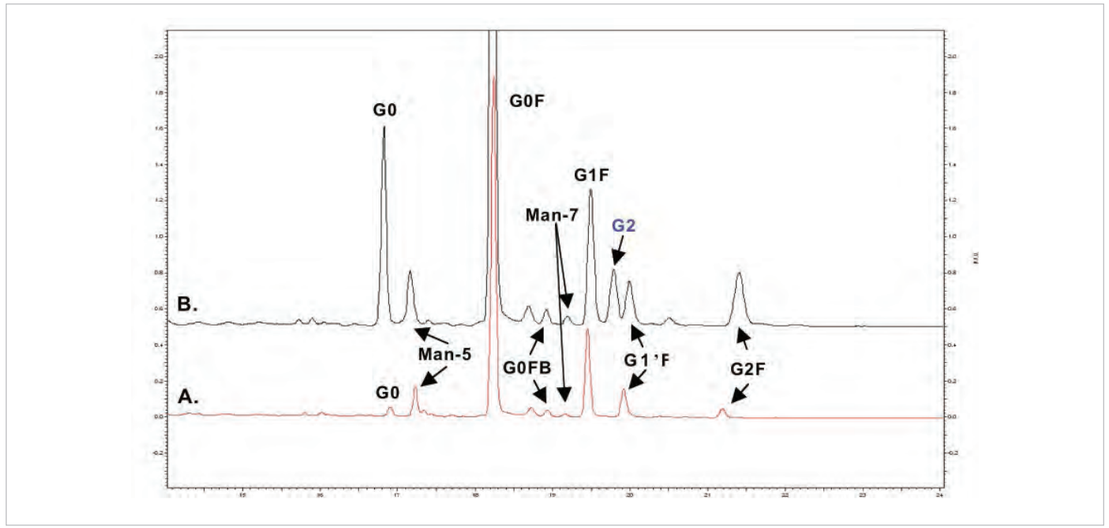

Figure 5: Separation of oligosaccharides associated with a recombinant therapeutic mAb. Oligosaccharides were cleaved from a therapeutic mAb, APTS labeled, and separated by CE using the new buffer formulation. A number of oligosaccharide species associated with this mAb were resolved from one another (A). In order to identify and help illustrate resolution between co-migrating glycan species, the MAb sample was spiked with standards (B). Relative to the oligosaccharide standards, we were able to quantifiably identify G0, Man-5, G0F, G0FB, Man-7, G1F, G1'F, and G2F. G2 standard (in blue) was also spiked into the mixture to indicate the location in the separation at which this oligosaccharide species would reside. Conditions for both separations were the same except that injection for the MAb alone was 0.5 psi and that for the mAb + glycan standards was 1.5 psi.

Results and Discussion

The goal of this study was to achieve separation of major complex glycan species associated with monoclonal antibodies. This glycan population includes oligosaccharides with and without core fucose moieties, terminal galactose subunits, terminal sialic acids, and bisecting GlcNAc residues in addition to numerous positional isomers. We set out to characterize the separation limitations for existing separation chemistry, the SCIEX Carbohydrate Labeling & Analysis Assay Kit. Employing standard protocols (included with the Carbohydrate Labeling & Analysis Assay) for instrument configuration, sample preparation, and separation conditions, we easily attained baseline resolution between G0, G1 positional isomers (G1 and G1'), and G2 oligosaccharide species (Figure 2). A systematic approach was devised in which standards were spiked into samples to help identify additional peaks in this separation and also to better define co-migration of glycans that may be occurring. The G0, G1 and G2 species are shown in Figure 3.

Spiking experiments using oligosaccharide standards illustrated that individual separated peaks may contain multiple glycan species. This was previously demonstrated by co-migration of G0+fucose (GoF) with G1, and co-migration of G1'+fucose (G1'F) with G27. Modification of separation parameters such as capillary length, separation voltage, and temperature did not offer improved resolution (data not shown). By developing a new separation buffer formulation, we were able to better resolve these co-migrating species (Figure 3). Additional spiking experiments illustrated the power of the CE separation developed (Figure 4).

In order to test this separation method on an antibody, we obtained a therapeutic mAb and analyzed its associated glycans (Figure 5).

Spiking with oligosaccharide standards to help identify glycan species, we showed good resolution between many of the major oligosaccharides which were previously difficult to separate by CE.

Conclusion

High resolution CE separations based on mobility and hydrodynamic volume have been developed for quantitative analysis of glycans. Using published protocols and commercially available reagents, we have shown this technology to be capable of separating oligosaccharides differing in terminal galactose. We also showed that by combining standard PEO separation gel buffer with a LPA gel buffer, we were able to separate fucosylated from afucosylated N-linked oligosaccharides, high mannose structures, and numerous other glycan moieties. This work suggests that CE can be used to successfully separate and quantify a wide array of N-linked oligosaccharides associated with mAbs.

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Rapid Sample Preparation and Analysis of Monoclonal Antibody N-glycans by Magnetic Bead Technology and CE-LIF

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1SCIEX Separations, Brea, CA, 2NIBRT, Dublin, IR

One of the fastest growing segments in the modern pharmaceutical industry is the development of biotherapeutics[1]. With the recent expiration of quite a few innovator biopharmaceutical patents, an increasing number of biosimilar and biobetter products, mostly monoclonal antibodies (mAb) are under development[2]. This emerging global biopharmaceuticals market requires sensitive and high resolution bioanalytical techniques for comprehensive product characterization, including the analysis of important post translational modifications (PTMS), such as glycosylation[3]. In fact, biotherapeutics require rapid characterization methods in all aspects of the product development chain, with glycosylation being a critical quality attribute. Therefore, the carbohydrate moieties on therapeutic antibodies should be closely monitored during all steps of the manufacturing process.

The variability of mAb glycosylation is mostly dependent on the host cell line used and the expression conditions. Structural diversities include the presence or absence of core fucosylation, differences in galactosylation and sialylation as well as the existence of a bisecting N-acetylglucosamine[4]. The glycosylation structures at the conserved Fc site in the CH2 domain (Figure 1) influence biological activity, physicochemical properties and most importantly antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) functions.

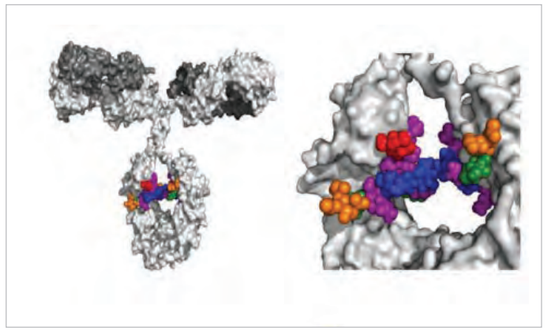

Figure 1: Structural interpretation of IgG1 (left panel) showing the more detailed glycosylation structure at the conserved Asn297 site (right panel) at the CH2 domain of the Fc region[5].

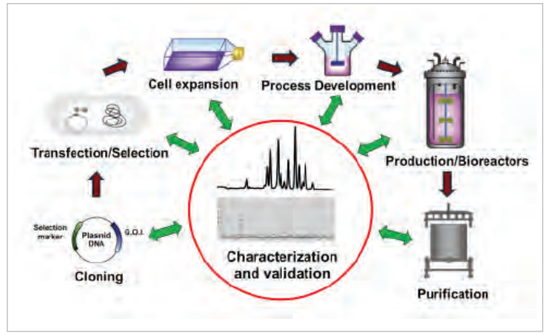

Figure 2: Main steps of the manufacturing process of recombinant therapeutic antibodies emphasizing their glycoanalytical aspects.

As sugars are involved in all key aspects of bioprocessing, sugar to function relationships should be considered as part of the quality by design process (QBD). For example, core fucosylation is critical in influencing ADCC function in a counterproductive manner (lower core fucosylation results in enhanced ADCC function). The presence of a bisecting GlcNAc also increases ADCC function. Terminal sialylation influences anti-inflammatory properties, while galactosylation enhances CDC function. Trace analysis of certain immunogenic sugar residues like alpha 1-3 galactosylation and the presence of N-glycolylneuraminic acid (Neu5Gc) in antibody therapeutic products is also of high importance[6]. All of these glycosylation features should be thoroughly analyzed in each step of the manufacturing process from clone selection, through transfection, cell expansion, process development, production and purification as delineated in Figure 2. Among the many factors that may contribute to alterations in glycan processing on recombinant glycoproteins are expression levels of the processing enzymes in host cell lines, monosaccharide nucleotide donor levels, cell signaling pathways like cytokines/hormones, media components, loss of cellular organelle organization due to e.g., pH changes, gene mutations, silencing and overexpression, as well as the bioprocessing environment (temperature, oxygen level, etc.). Therefore the use of proper analytical toolsets to ensure that the product has the required glycosylation pattern for optimal activity is crucial.

There are various methods available for the characterization of N-glycans on therapeutic proteins, such as capillary electrophoresis (CE), liquid chromatography (LC), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR) to mention the more frequently used ones.

Capillary electrophoresis offers rapid separation times, high resolving power and excellent detection sensitivity. CE technology utilizing UV/visible absorbance- or fluorescence-based detection methods has long been applied in the biopharmaceutical industry to characterize mAb purity and heterogeneity in the forms of capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF) for charge distribution studies, capillary SDS gel electrophoresis (CE-SDS) for molecular mass assessment, as well as capillary gel electrophoresis (CGE) for glycosylation analysis. Studies exploring the transferability of these techniques illustrated excellent robustness when performed in different laboratories in by different instrument operators.[7-10]

In this article, an optimized, rapid magnetic bead based sample preparation approach is introduced for N-glycosylation analysis of therapeutic antibodies. Please note that all sample preparation steps can be readily automated using an automated liquid handler without the need for centrifugation or overnight incubations. In its entirety, the sample preparation process takes less than 4 hours.

Experimental Design

Chemicals: IgG, acetic acid and sodium-cyanoborohydride (1 M in THF) were purchased from Sigma Aldrich (St. Louis, MO). The 8-aminopyrene-1,3,6-trisulfonate (APTS), N-CHO Carbohydrate Separation Gel Buffer, maltooligosaccharide ladder and Agencourt CleanSEQ magnetic beads were from Beckman Coulter (Brea, CA, USA). The PNGase F enzyme was from ProZyme (Hayward, CA) and the digestion reaction mixtures were prepared following the manufacturer's protocol.

Sample Preparation: Accelerated glycan release was accomplished at 50°C for 1 hour incubation using the PNGase F reaction protocol supplied by the vendor. This was followed by magnetic bead based partitioning of the released glycans from the remaining polypeptide chains and the endoglycosidase using 200µL Agencourt CleanSEQ magnetic bead suspension in 87.5% final acetonitrile concentration. Following thorough mixing, the tube containing the glycan - magnetic bead mixture was placed on a strong magnet for fast partitioning. The supernatant was discarded and the captured glycans were eluted from the beads into the same tube by the addition of 21 µL of 40 mM APTS in 20% acetic acid.

The elution step was immediately followed by initiating reductive amination with the addition of 7 µL sodium cyanoborohydrate (1 M in THF). The reaction mixture was incubated at 37°C for 2 hours, followed by removal of the excess dye using the same Agencourt CleanSEQ magnetic beads from the digestion reaction, again in 87.5% final acetonitrile concentration. The supernatant was discarded and the captured APTS-labeled glycans were eluted from the magnetic beads using 25 µL of water and partitioning by placing the tube on a strong magnet. The supernatants containing the APTS-labeled released glycans were then analyzed by CE-LIF.

Capillary Electrophoresis: All capillary electrophoresis separations were performed in a SCIEX PA 800 Plus system, equipped with a solid state laser induced fluorescence detection system (excitation: 488 nm, emission: 520 nm). The effective length of the N-CHO separation capillary was 50 cm (60 cm total length, 50 µm i.d.). The applied electric field strength was 500 V/cm in reversed polarity mode (cathode at the injection side). Samples were pressure injected by applying 1 psi (6.89 kPa) for 5 sec. SCIEX 32 Karat™ version 9.1 software was used for data acquisition and analysis.

Results and Discussion

Comprehensive glycosylation analysis of monoclonal antibodies is a challenging task as carbohydrates usually feature very complex, highly diversified structures. In addition, they do not possess any chromophore / fluorophore groups that would accommodate routinely used detection systems (UV/vis or FL) of liquid phase separation devices. In addition, most of the glycan structures do not carry any charge, which would otherwise be necessary to drive their electric field mediated migration process. Historically, methods such as NMR, mass spectrometry, high performance liquid chromatography and capillary electrophoresis or their combinations (LC-MS, CE-MS) have been used for glycosylation analysis. Among them, CE with laser induced fluorescent detection can readily address the above listed issues offering a robust, highly efficient and reproducible glycan analysis method. Capillary electrophoresis is also capable of accommodating high throughput separation modes with rapid analysis. Similar to LC and most MS based methods, CE requires pre-separation labeling. Reductive amination with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) is one of the most frequently used sugar tagging methods for capillary electrophoresis[11], providing strong fluorescent signal and the necessary charge for rapid electromigration. This simple, one step reaction results in a stable APTS - glycan conjugate where the fluorophore is always at the reductive end assuring good quantification (one dye per glycan structure). The fluorescent characteristics of the labeling agent readily support the blue solid state laser of the PA 800 Plus system with 488 nm excitation wavelength.

Glycan Release and Fluorophore Labeling: Glycan analysis of therapeutic monoclonal antibodies starts with the removal of the sugar moieties from the polypeptide backbone with an appropriate endoglycosydase enzyme, PNGase F in this instance. This process was optimized with respect to incubation time and temperature to support the rapid sample preparation requirement. It was found that the release of the sugar structures was complete within 1 hour of incubation at 50°C[8].

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Similarly, the APTS labeling reaction process was also optimized for rapid and efficient tagging of the released sugar structures, paying particular attention to avoid possible losses of labile sugar residues, such as terminal sialic acids[8]. For this step, the reaction time of 2 hours at 37°C was found to be optimal, ensuring efficient labeling without any significant sialic acid loss (< 2%). Additional labeling parameters such as the concentrations of acetic acid (catalyst) and APTS were also optimized in the reaction mixture (20% and 40 mM, respectively). The APTS concentration was chosen considering reaction volumes of larger than 15 µL to accommodate automated liquid handling based automated procedures for large scale / high throughput applications, like clone selection.

Magnetic Bead based Sample Preparation Process: Purification after the glycan release and labeling steps were necessary to remove the remaining polypeptide chain of the glycoprotein, the endoglyosidase and the excess labeling reagent, respectively. Conventionally, these steps required ethanol precipitation (after PNGase F release) and Sephadex or normal phase chromatography (excess APTS removal) requiring centrifugation and pre-concentration steps using vacuum centrifugation. We have eliminated these steps by introducing a magnetic bead based approach using Beckman Coulter's Agencourt CleanSEQ beads, which are primarily used for DNA purification. Bearing in mind the fact that the backbone of deoxyribonucleic acid molecules is made up of sugars (deoxyriboses), we considered the use of these magnetic beads as appropriate agents for both of the purification processes, i.e., after glycan release to partition the liberated carbohydrates, and after APTS labeling, to pull down the labeled sugars from the reaction mixture.

The complete sample preparation process utilizing magnetic bead based partitioning to avoid the necessity of any centrifugation or vacuum centrifugation steps for easy automation for high throughput processing is illustrated in Figure 3. The first step is PNGase F digestion for one hour at 50°C (Step A, Figure 3). The reaction is then stopped by the addition of acetonitrile (final concentration 87.5%). At this stage the released glycans are bound to the magnetic beads via hydrophilic interaction and pulled down to the bottom of the reaction vial by a strong magnet (Step B, Figure 3).

After removal of the supernatant, the partitioned glycans are eluted from the beads by the addition of the APTS reaction mixture (40 mM APTS in 20% acetic acid), eliminating in this way the vacuum centrifugation based pre-concentration step normally required in conventional protocols (Step C, Figure 3). After the addition of the reducing agent (1 M sodium-cyanoborohydrate in tetrahydrofuran) the labeling reaction is incubated for 2 hours at 37°C and the reaction is stopped by the addition of appropriate volume of acetonitrile to reach 87.5% final concentration again. Capture of the APTS labeled glycans is accomplished with the same magnetic beads used in the earlier step. After thorough mixing with the beads, a strong magnet is used to pull down the captured APTS labeled glycan bound beads (Step D, Figure 3). After discarding the supernatant, the labeled sugars are eluted with water from the capturing beads by breaking the hydrophilic interaction bonds (Step E, Figure 3). The beads are then pulled down with a strong magnet making the APTS labeled sugar solution (supernatant) available for CE-LIF analysis (Step E, Figure 3).

Figure 4 demonstrates the efficiency of the magnetic bead based sample preparation protocol. Traces (a) and (b) depict the electropherograms of the APTS labeled IgG N-glycans before (trace a) and after (trace b) magnetic bead based sample preparation. It is immediately apparent from the electropherograms that almost all the excess derivatization reagent was effectively removed with magnetic bead based purification (migration regime of 5-8 min) not overlapping with any of the glycans in the sample. Table 1 depicts the structures of the main IgG glycans of interest (Peaks 1-6).

It is important to note that peak distribution remained the same after the excess dye removal (Table 2), demonstrating that the magnetic based partitioning process did not cause any binding/ removal bias for these most frequently analyzed glycan structures. In addition, the magnetic bead based sample preparation process provided clean electropherograms by CE-LIF analysis.

The entire sample preparation process from glycan release to data interpretation of the electropherograms took less than four hours. Table 3 depicts the timing of the individual steps of the workflow below.

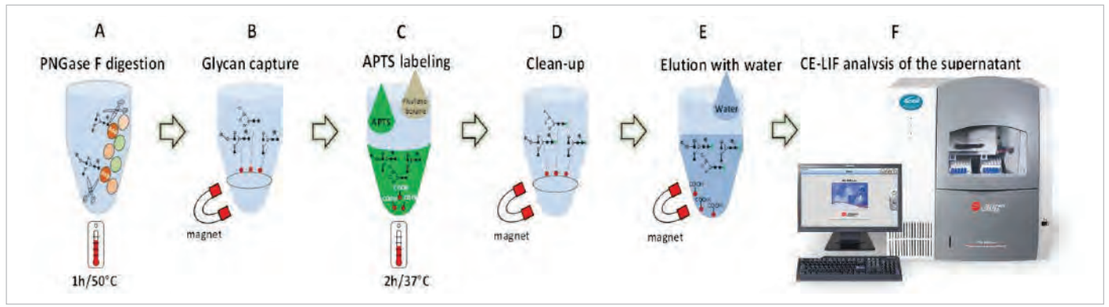

Figure 3: Magnetic bead based sample preparation flowchart for N-glycosylation analysis of therapeutic antibodies by CE-LIF.[12]

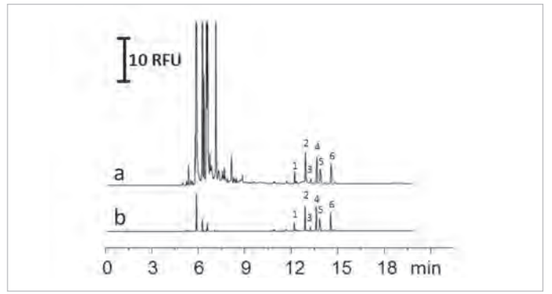

Figure 4: CE-LIF analysis demonstrating the effectivity of magnetic bead based APTS clean-up. a) unpurified IgG glycans; b) magnetic bead purified IgG glycans. Conditions: N-CHO capillary (50 cm effective and 60 cm total length, 50 µm i.d.), N-CHO separation buffer. LIF Detection: excitation / emission: 488 nm / 520 nm. Applied electric field: 500 V/cm. Injection: 1 psi / 5 sec.of the Fc region.[5]

Peak	Abbreviated Structure	Structure
1	FA2G2S1	
2	FA2	
3	FA2B	
4	FA2(6)G1	
5	FA2(3)G1	
6	FA2G2	

Table 1: Structures corresponding to the main IgG glycans of interest.

Conclusions

In this article, we demonstrate the utility of magnetic bead based technology for rapid and efficient sample preparation enabling CE-LIF based N-glycosylation analysis of therapeutic antibodies. Using the novel sample preparation protocol described here, the full workflow of glycoprotein sample preparation took less than 4 hours, without the need for any centrifugation and/or vacuum centrifugation steps, suggesting compatibility with fully automated liquid handling systems (as described in the next article). Another benefit for this approach includes unbiased glycan labeling and electrokinetic sample injection allowing unambiguous detection for highly sialylated carbohydrates which otherwise may co-migrate with excess APTS dye.

Peak	Abbreviated Structure	Magnetic bead protocol (area %)	Overnight protocol (area %)	Difference (area %)
1	FA2G2S1	7.48	7.48	0.03
2	FA2	22.23	22.23	0.11
3	FA2B	3.97	3.97	0.00
4	FA2(6)G1	23.01	23.01	0.08
5	FA2(3)G1	11.57	11.57	0.02
6	FA2G2	18.3	18.3	0.17

Table 2: Peak distribution comparison of the two labeling methods.

PNGase F Digestion	60 min
Glycan capture	10 min
APTS labeling	120 min
Cleanup	10 min
Elution	10 min
CE-LIF	20 min
Total analysis time	230 min

Table 3: Magnetic bead method timeline.

Acknowledgments

CsV appreciates the help of Dr. Jeff Chapman for providing funds for his internship to accomplish this work.

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DEVELOPMENT AND QUALITY CONTROL

Fully Automated Sample Preparation with Ultrafast N-Glycosylation Analysis of Therapeutic Antibodies

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There is a growing demand in the biopharmaceutical industry for high throughput, large scale N-glycosylation profiling of therapeutic antibodies in all phases of product development, but especially during clone selection where hundreds of samples must be analyzed in a short period of time. SCIEX has recently developed a magnetic bead based protocol for N-glycosylation analysis of glycoproteins to replace centrifugation and vacuum-centrifugation steps the currently used. Glycan release, fluorophore labeling and clean-up were all optimized resulting in a <4 hours magnetic bead mediated process with excellent yield and high reproducibility. This article demonstrates an extension of this work by fully automating all steps of the optimized magnetic bead based protocol. Optimization includes endoglycosidase digestion through rapid fluorophore labeling and clean-up using high throughput 96-well plate sample processing in an automated liquid handling. Capillary Electrophoresis – Laser Induced Fluorescence (CE-LIF) analysis of the fluorophore labeled glycans was also optimized to enable rapid (<3 min) separations in order to accommodate high throughput of the automated sample preparation process.

Liquid phase-based glycoanalytical methods generally require labor intensive and time consuming sample preparation and derivatization steps including glycan release, purification, fluorophore labeling and pre-concentration prior to analysis. In addition, current protocols include numerous centrifugation and vacuum-centrifugation steps that make full automation of the process by liquid handling robots difficult and expensive. Utilizing a magnetic bead mediated sample preparation protocol, a large number of samples can be processed in less than 4 hours in a 96 well plate format without the requirement centrifugation or vacuum-centrifugation steps. This protocol has been tested using a Beckman Coulter Biomek FX^P Laboratory Automation Workstation for preparation of immunoglobulin G samples. Ultrafast analysis of the resulting fluorophore labeled glycans was accomplished in less than 3 minutes per sample using the SCIEX PA 800 Plus capillary electrophoresis system configured with laser induced fluorescent detection (Figure 1).

Figure 1: The SCIEX PA 800 Plus Pharmaceutical Analysis System with laser induced fluorescence detection (CE-LIF).

Experimental Setup

Automated sample preparation was performed on a Biomek FX^P Laboratory Automation Workstation (Figure 2) which was set up with 96 well plate holders, a magnetic stand, 1000 µl and 25 µl pipette tips, a quarter reservoir, along with sample and reagent vials. The quarter reservoir contained acetonitrile (Sigma Aldrich, MO) and the Agencourt CleanSeq magnetic beads (Beckman Coulter). The reagent vials contained reagents for the PNGase F digestion (Prozyme, Hayward, CA), 8-aminopyrene-1,3,6-trisulfonate (APTS) (SCIEX) in 20% acetic acid and 1 M sodium-cyanoborohydrate (in THF) (SCIEX). To reduce evaporation induced volume loss, a pipette box lid was used to cover the quarter reservoirs. The glycoprotein samples were incubated in a Biomek vortex heater block. For adequate re-suspension, an extra empty sample plate was applied under the actual sample plate, in which case the magnets were positioned under the sample tubes, rather that of at the side. In this configuration the magnet could pulls down the magnetic beads to the bottom of the vials and with fast aspiration/dispensing the beads could be easily re-suspended.

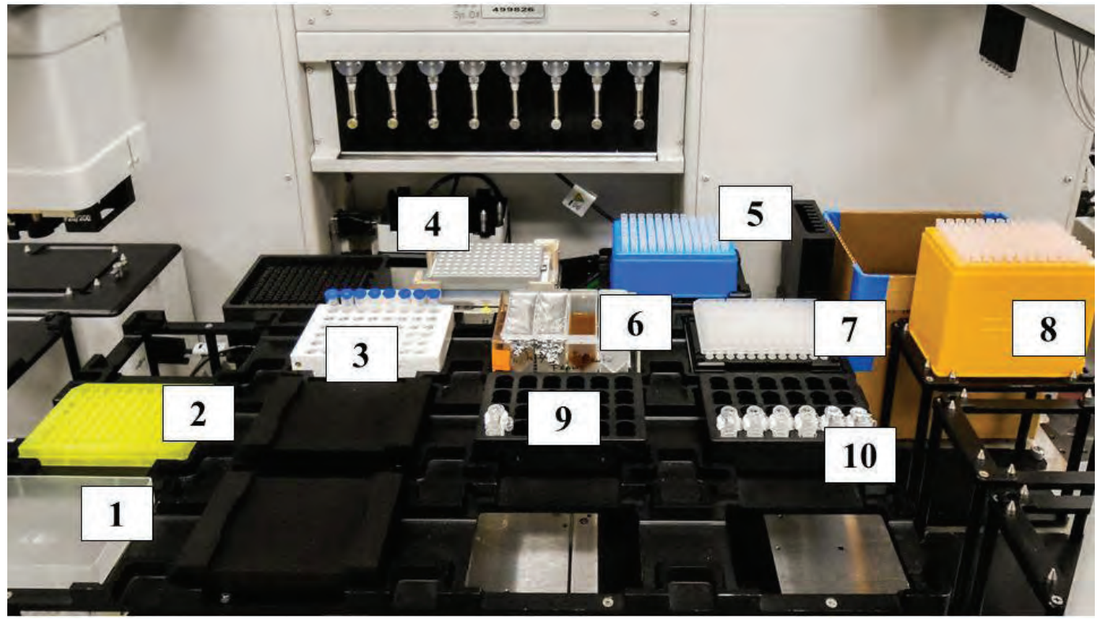

Figure 2: Experimental setup of the Biomek FX^P Laboratory Automation Workstation

No.	Labware	Function
1	Pipette tip box lid	Reducing the evaporation of acetonitrile
2	96 well plate (Nunc U96 0.5 ml)	Stacking plates for resuspension
3	Result sample tray	Beckman PA 800 Plus vials
4	Shaker Peltier 96	For incubation, mixing and resuspension
5	Pipette tip box (P20)	Small reagent amounts
6	Quarter reservoir	Acetonitrile (100% and 87.5%) and Agencourt CleanSeq magnetic beads
7	Sample plate (Thermowell 96 well PCR plate) on magnetic stand	Sample preparation and capturing magnetic beads on side of the plate vials
8	Pipette box (P1000)	Large reagent amounts
9	Sample tray (24 well tray)	Protein sample
10	Reagent tray (24 well tray)	For PNGase F digestion and labeling reagents

Methods

The individual steps of the manual approach of the magnetic bead mediated sample preparation protocol was published by Varadi et al.[1] and described in the previous article. The entire workflow is shown in Figure 3. Enzymatic digestion using PNGase F was performed at 50°C for 1 hour followed by glycan capture on the magnetic beads in 87.5% acetonitrile medium[2]. APTS labeling of the bound carbohydrates was initiated *in situ* on the beads by the addition of sodium cyanoborohydride followed by incubation at 37°C for 2 hours. The fluorophore labeled glycans were eluted from the beads by the addition of 25 µl of HPLC grade water and were analyzed by CE-LIF analysis using a PA 800 Plus equipped with LIF detection (488 nm excitation, 520 nm emission) (SCIEX).

For the separation, 20 cm effective length NCHO capillaries SCIEX were used (30 cm total length, 50 µm ID) with 25 mM lithium acetate (pH 4.75) background electrolyte containing 1 % PEO (900,000, Sigma-Aldrich). The applied voltage was 30 kV and the separation temperature was 20oC. The samples were pressure injected by 3 psi for 6 seconds. The entire liquid handling protocol was programmed using Biomek Software version 4.0 (Figure 4). The CE-LIF data were acquired and analyzed by the SCIEX 32 Karat software package (SCIEX).

DEVELOPMENT AND QUALITY CONTROL

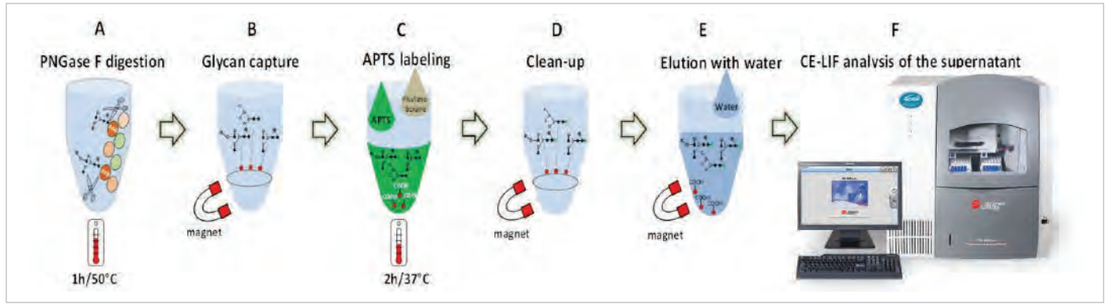

Figure 3: Magnetic bead mediated sample preparation flowchart for N-glycosylation analysis of therapeutic antibodies by CE-LIF.

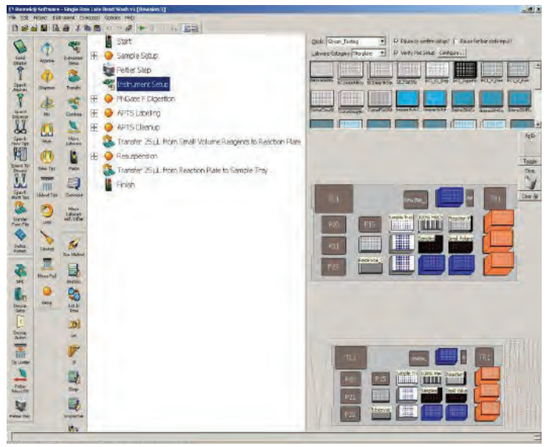

Figure 4: The liquid handling protocol, programmed by the Biomek Software ver 4.

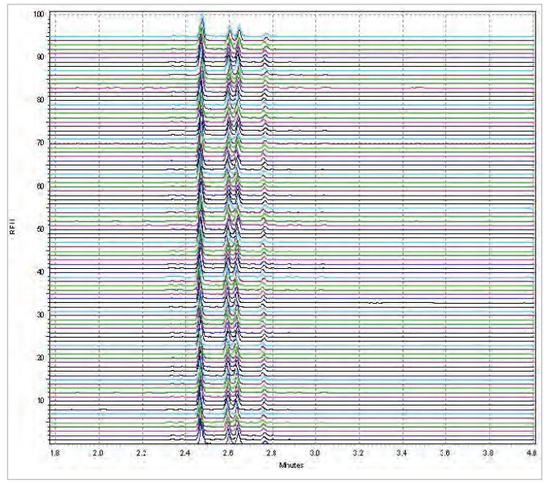

Figure 5: Ultrafast CE-LIF analysis of APTS labeled IgG glycans prepared by a liquid handling robot in a 96 well plate format utilizing the magnetic bead based protocol.

Results and Discussion

In this work, our earlier published magnetic bead mediated glycan release, APTS-labeling and sample clean-up protocol[1] was applied to a 96 well plate format utilizing the Biomek FX[P] Laboratory Automation Workstation (Figure 2). Results illustrated that by applying full automation, the process could be shortened to < 3.5 hours of processing time with excellent yield and high reproducibility. Programming of the liquid handling platform was simple and the system was flexible and robust, capable of handling a large number of samples. The Laboratory Automation Workstation offered a fast sample preparation option, reduced flow-induced shear strain on native biological sample matrices and minimized contamination risks.

The large amount of deck space available in the liquid handling system enabled buffer preparation for CE-LIF analysis to be performed using the Biomek FX as well, to automate the solubilization step of the separation polymer matrix as defined in the Methods section.

The resulting fluorophore labeled glycans were analyzed using CE-LIF optimized for rapid separation to accommodate the high throughput of the fully automated sample preparation process. APTS labeled IgG glycan were injected consecutively from the 96-well plate taken directly from the liquid handler (Figure 5). Please note that baseline separation of the major IgG glycans was obtained in less than 3 minutes. This separation method can be readily applied to large scale processes like clone selection where rapid analysis of hundreds of samples is crucial.

In summary, the PA 800 Plus capillary electrophoresis system in combination with automated liquid handling used in these work was capable of precise and robust high throughput sample preparation for rapid N-glycosylation analysis of IgG molecules.

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Acknowledgement

The generous support of Don Arnold and Navaline Quach as well as the help of Mike Kowalski and Bee Na Lee is greatly appreciated. The authors also acknowledge the support of the MTA-PE Translation Glycomics project (#97101).

DEVELOPMENT AND QUALITY CONTROL

Differential Mobility Spectrometry Analysis of Glycans and Glycopeptides

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Introduction

Glycans and glycosylated biomolecules perform numerous roles through their interactions in cellular environments, with more than half of human proteins glycosylated[1]. Characterization of glycosylation in biopharmaceuticals is essential for determination of their function and efficacy[2]. Thus, glycan analysis is gaining great interest. However, carbohydrate sidechains are highly variable in structure owing to differences in their anomeric configurations, monomer stereochemistry and inter-residue linkage positions. The separation of carbohydrates has been a great challenge for current analytical techniques[3-4]. Differential mobility separation (DMS) is a technology that can separate gas-phase ions prior to analysis by mass spectrometry (MS)[5]. It has been used in the separation of various isomeric species, including stereoisomers[6], structural isomers[7], and tautomers[8].
In this study, we employed DMS-MS for the analysis of glycans and glycopeptides.

The separation of the isomeric glycan and glycopeptide species studied here was accomplished because of differences in the DMS behavior between each isomer pair[9]. The ions bear subtle structural differences that allow the DMS to separate them based on their different mobilities during the high- and low-field portions of the asymmetric waveform applied across the DMS cell. As such, each isomer required different DC compensation voltages (CVs) to bring their trajectories on-axis for successful sampling by the MS. Besides the differences between the isomers' structures, differences in how these species bind to added volatile chemical modifiers in the DMS cell made their DMS behavior differences more prominent, yielding increased selectivity and peak capacity for the DMS experiments.

Experimental

Sample Preparation: Glycans were purchased from Sigma-Aldrich (Oakville ON, Canada) and from Dextra Laboratories (Reading, UK). For work in positive ionization mode, glycans were diluted to 1 µg/mL in methanol and water (50/50, v/v) containing 50 µM sodium chloride. For negative mode work, glycans were diluted to 1-5 µg/mL with acetonitrile and water (20/80, v/v) containing 10 mM ammonium bicarbonate. Two glycopeptides, supplied by Anaspec (Fremont, CA), were diluted to 10 µg/mL with acetonitrile and water (50/50, v/v) containing 0.1% formic acid.

DMS-MS Conditions: The SCIEX SelexION® differential mobility separation device was mounted in the atmospheric region between the SCIEX QTRAP® 5500 or 6500 system's sampling orifice and a Turbo V™ source (Figure 1). The temperature of the DMS cell was maintained at 150 °C, and the nitrogen curtain gas was operated at 30 psi. Chemical modifiers (water, methanol, or acetonitrile) were added into the nitrogen curtain gas flow at 1.5% (v/v). The fundamentals of the DMS device have been described elsewhere[5]. In this study, both separation voltage (SV) and compensation voltage (CoV) were scanned. As the SV was stepped from 0 to 4000 V, CoV was scanned from -40 V to +20 V. These data were plotted as dispersion plots, with SV as the x-axis, and the optimal CoV for ion transmission as the y-axis.

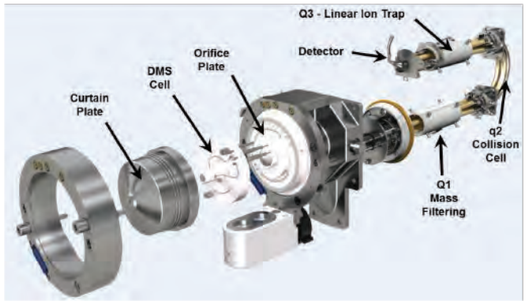

Figure 1: The instrument setup of the SelexION Differential Mobility Spectrometry device.

Results

Disaccharides and Trisaccharides:

DMS-MS was employed to analyze sodiated or deprotonated molecular ions of disaccharides and trisaccharides. When these carbohydrates were analyzed without chemical modifiers, molecular ions could not be fully separated (Figure 2). The controlled addition of water or methanol vapor in the DMS cell induced different shifts in optimal CoVs, enhancing the separation of isomeric carbohydrates. (Figures 2 to 4).

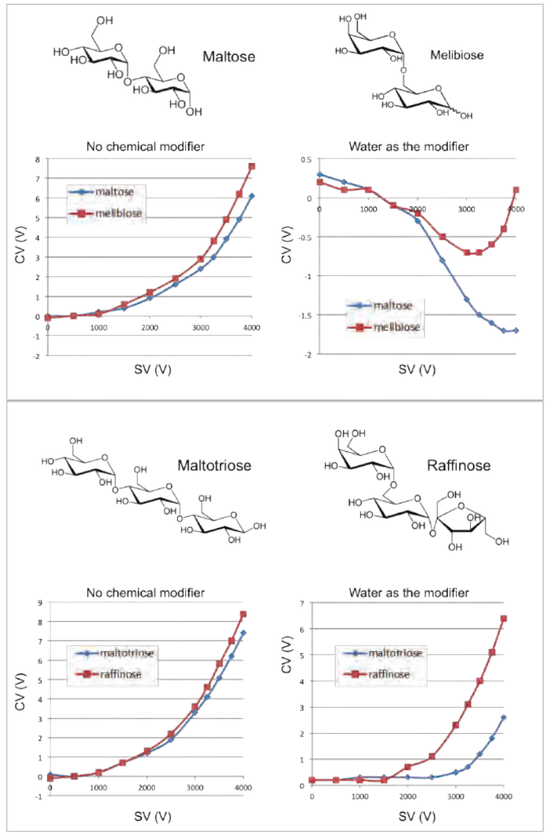

Figure 2: Isomeric separation of sodiatedglycans with DMS-MS

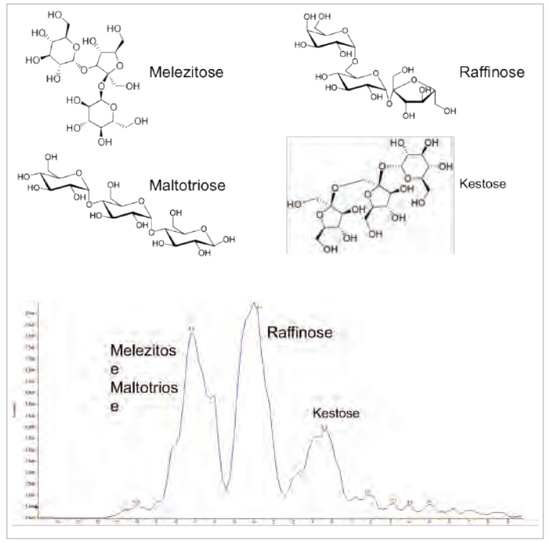

Figure 3: Ionograms of four sodiatedtrisaccharides. Separation voltage was set to 4000 V, and methanol was used as the chemical modifier.

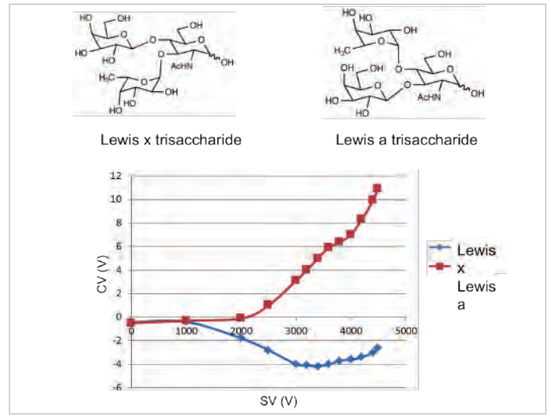

Figure 4: Isomeric separation of deprotonated Lewis a and Lewis xtrisaccharides with DMS-MS, using methanol as the modifier.

Differentiation of 2-3 and 2-6 linked Sialic acid:

Differentiation of 2-3 and 2-6 linked sialic acid was achieved using methanol as a modifier. Two examples are shown in Figures 5 and 6.

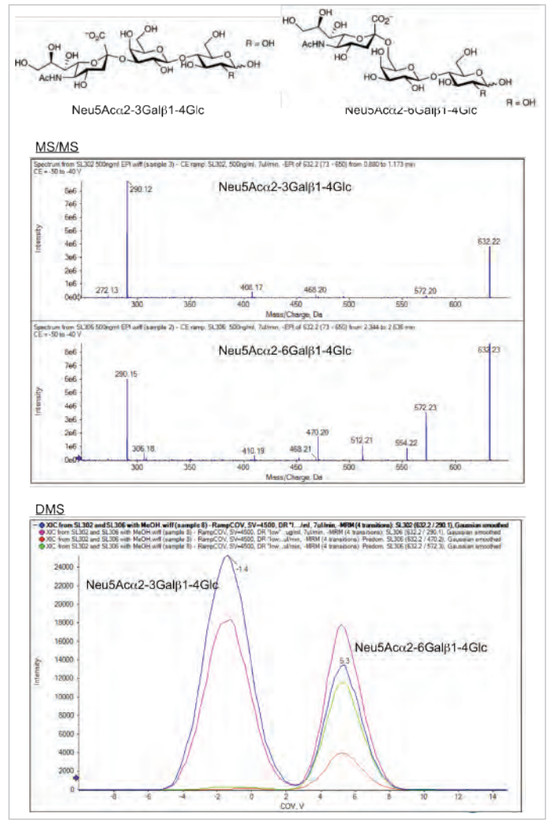

Figure 5: Structure, MS/MS and DMS analysis of a pair of deprotonated isomers (Neu5Acα2-3Galβ1-4Glc and Neu5Acα2-6Galβ1-4Glc). Methanol was used as the chemical modifier for the DMS analysis; separation voltage was set to 4500V.

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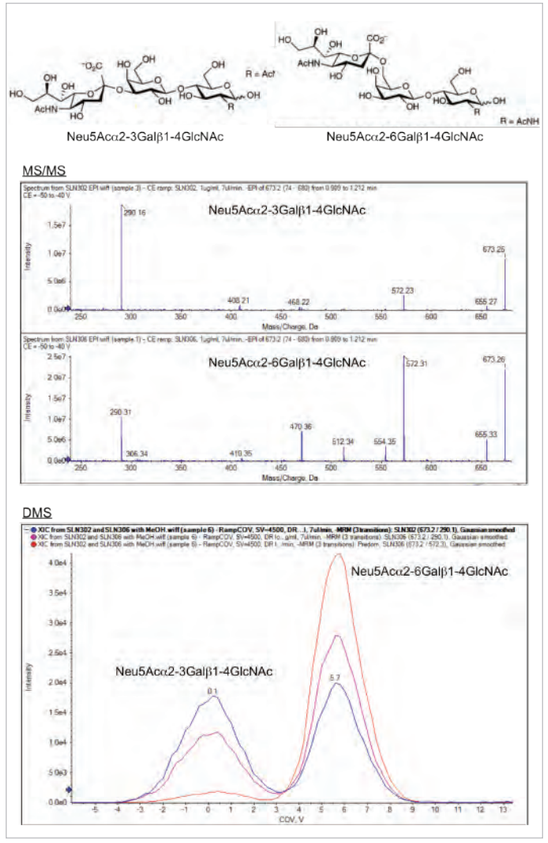

Figure 6: Structure, MS/MS and DMS analysis of a pair of deprotonated isomers (Neu5Acα2-3Galβ1-4GlcNAc and Neu5Acα2-6Galβ1-4GlcNAC). Methanol was used as the chemical modifier for the DMS analysis; separation voltage was set at 4500V.

O-Linked Glycopeptides MUC5AC-3 and MUC5AC-13:

We also used DMS to separate isomeric glycopeptides MUC5AC-3 and MUC5AC-13 (3 and 13 indicate site of glycosylation). Using acetonitrile vapor as a chemical modifier, we separated these isomers and clearly identified characteristic b- and y-ions for each isomer that retain the O-linked GalNAc modification, as shown in Figure 7.

Conclusions

In this study, differential mobility spectrometry was used to analyze biologically relevant glycan and glycopeptide isomers. With the addition of gas-phase chemical modifiers to the DMS, various isomeric species were successfully separated including sodiated and deprotonated glycans, deprotonated glycans containing 2-3 or 2-6 linked sialic acid, and protonated glycopeptides. In addition, fragments containing the GalNAc modification were identified for O-linked glycopeptides with CID fragmentation mechanism. This method can be used as a fast and convenient approach for the glycan/glycopeptide analyses.

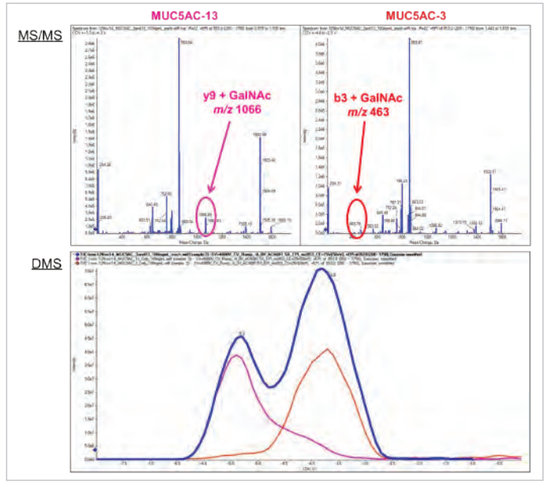

Figure 7: MS/MS and DMS analysis of a pair of glycopeptide isomers (MUC5AC-3, GT**T**PSPVPTTSTTSAP, and MUC5AC-13, GTTPSPVPTTST**T**SAP). **T** represents the threonine residue modified with GalNAc. Acetonitrile was used as the chemical modifier for the DMS analysis.

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DEVELOPMENT AND QUALITY CONTROL

Profiling the Distribution of N-Glycosylation in Therapeutic Antibodies Using the QTRAP® 6500 System

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[1]SCIEX, USA, [2]PREMIER Biosoft, USA

Immunoglobulin G (IgG) molecules have become attractive as targeted therapeutic proteins, due to their high specificity and long circulation time. Glycosylation patterns determine the stability and bio-disposition of these recombinant protein drugs in vivo, as well as the efficacy, folding, binding affinity, specificity and pharmacokinetic properties. Therefore, a complete characterization of the biotherapeutic IgG glycosylation is desirable.

In this study, we demonstrated how a comprehensive MS/MS analysis of the glycopeptides can be achieved by targeting the known nature of the glycosylation structures. We used trastuzumab, a humanized mAb, and Sigma-Aldrich SilumAb standard protein in this proof of concept study. Monoclonal IgGs have a known glycosylation site and set of glycan isotypes (complex, high mannose and hybrid). We applied a robust MRM triggered MS/MS workflow to profile and confirm glycopeptides and their glycan isoforms. Overlaying several specific MRMs for each glycopeptide group and searching each full scan MS/MS spectrum with SimGlycan software provides added confidence to the identification of each glycopeptide isoform (Figure 1).

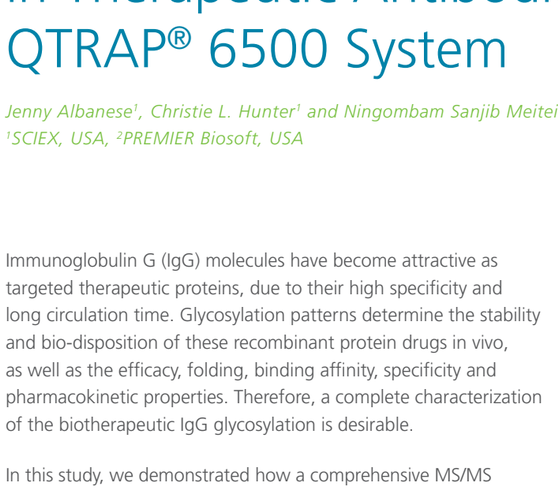
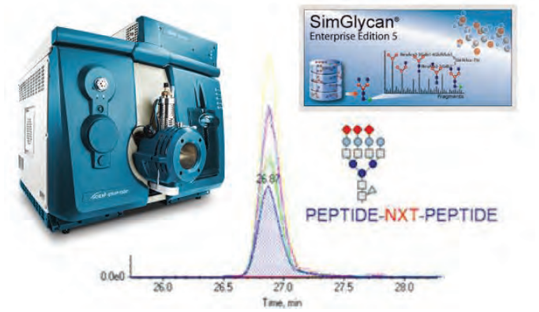


Figure 1: Targeted Detection and Characterization of Glycopeptides using Unique QTRAP® System Functionality. MRM transitions specific to a set of IgG1 glycopeptides (inset) were used to trigger full scan MS/MS. The example shown for EEQYNSTYR-GO-F highlights the multiple levels of information used to successfully identify the glycopeptide (matched MS/MS spectra, multiple overlaid MRMs to structural fragments and a SimGlycan MS/MS search score).



Key Advantages of Using the QTRAP 6500 System for Quantitative Glycopeptide Profiling

- The QTRAP 6500 system with 2000 m/z upper mass limit can sufficiently detect complex, hybrid and high mannose type containing glycopeptides.
- High sensitivity of full scan MS/MS (EPI) acquired with low energy CID provides both carbohydrate fragment ions (mainly B/Y) and peptide fragment ions (b/y)
- High information content MS/MS for identification of N-glycopeptides using SimGlycan software
- Expanded functionality of *Scheduled* MRM™ Pro Algorithm enables:
  - More glycopeptides to be screened - Group triggered MRM improves dwell and cycle time by only acquiring secondary transitions when primary transitions to the glycopeptides are above the set threshold.
  - More specific acquisition of MS/MS - Group triggered MIDAS™ workflow triggers MS/MS only when multiple primary transitions for a glycopeptide are detected.
  - Increased efficiency of targeted detection for glycopeptides without enrichment strategies.

Materials and Methods

Sample Preparation: Monoclonal antibodies (mAb human) were supplied by Bristol-Myers Squibb (Sunnyvale, USA). The mAb solution (30-60 µg in 30 µL) was first denatured 8M guanidine HCl (60 µL) and reduced with 800 mM DTT (5 µL), incubated at 37°C for 1 hour. 10 µL of 800 mM iodoacetamide was added and incubated at RT for 45 min. A Zeba column (PN PI89883, VWR) was used to remove guanidine and any impurities before digestion (use according to manufacturers' guidelines). Recovery of 65% was observed. A 1 µg aliquot of trypsin was added and solution was incubated at 37°C for 2 hours. A second aliquot of 1 µg of trypsin was added and incubate at 37°C for 2 more hours. The digests were diluted to a final concentration of 3.3 pmol/µL prior to LC-MS analysis.

LC Analysis: Microflow LC was performed using the SCIEX NanoLC™ 425 system using a C18 RP column (CXP CL18-120 0.5x150mm, SCIEX) running at 15 µL/min. The autosampler was equipped with a 10 µL sample loop. Samples were directly loaded onto a heated column at 30°C using a 30 min gradient to 2-35% acetonitrile, 0.1% formic acid). Injections of 3.3 to 6 pmol of protein digest on column per run were performed.

Mass Spectrometry: Microflow LC was performed using the SCIEX NanoLC 425 system using a C18 RP column (CXP CL18-120 0.5x150mm, SCIEX) running at 15 µL/min. The autosampler was equipped with a 10 µL sample loop. Samples were directly loaded onto a heated column at 30°C using a 30 min gradient to 2-35% acetonitrile, 0.1% formic acid). Injections of 3.3 to 6 pmol of protein digest on column per run were performed).

Data Analysis: Full scan MS/MS spectra acquired on the targeted glycopeptides were then analyzed using SimGlycan Enterprise Software 5.4. A search template containing the known sequence of the glycopeptide is first created then searched against a database containing all possible N-glycan structures. Spectra were annotated using the symbolic peak representation. Results are reported in Excel along with glycan scores and structures for further downstream data analysis. MRM peak integration was performed using MultiQuant™ Software 3.0 and exports of RT and area were collected to set up time-scheduled MRM methods containing specific marker ions. PeakView® Software 2.2 was used to overlay multiple MRM transitions for the same species allowing quick assessment of peak shape to scan for possible peak interferences.

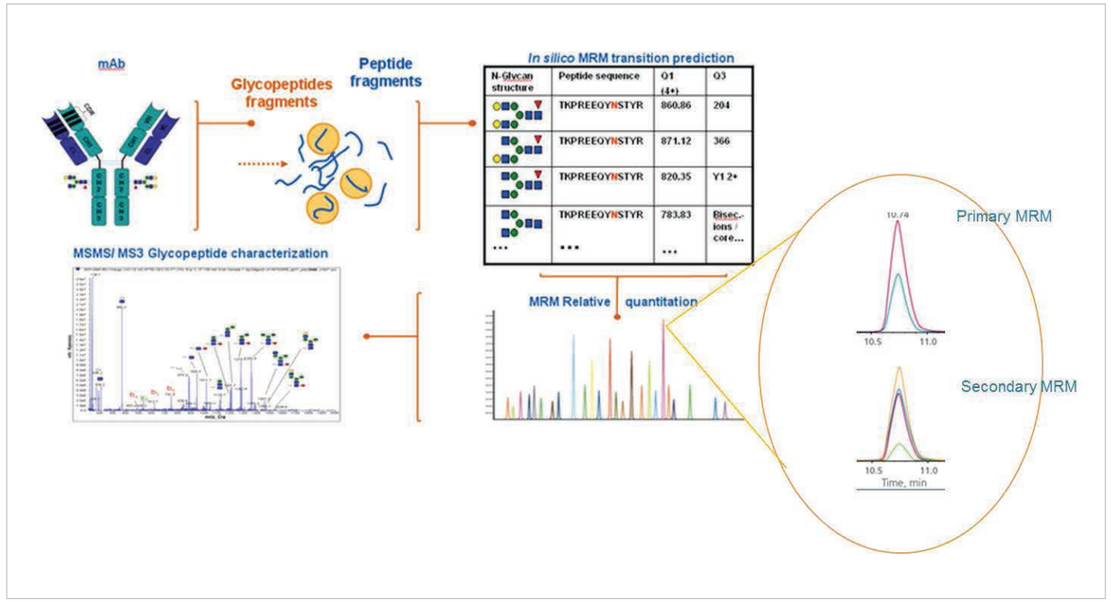


Figure 2: Group MIDAS Workflow Uses *In Silico* MRM Prediction for Detection and Ion Trap MS/MS for Confirmation. As the glycans on IgG proteins are constructed in a known way on known peptide sites, the glycopeptides formed during digestion can be easily predicted. This enables the *in silico* prediction of MRM transitions to these potential glycopeptides. Multiple transitions per glycopeptide (both primary and secondary MRM transition) are used to increase the specificity of detection with group triggered MRM workflow in the *Scheduled* MRM Pro Algorithm. Both additional MRMs and full scan MS/MS spectra can be collected in a targeted manner to characterize and quantitatively profile glycopeptides from IgG.

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In Silico Prediction of MRM Assays for IgG Glycopeptides

Because of the controlled manner in which glycosylation is constructed on an IgG and the known glycosylation site in the Fc domain, the tryptic glycopeptides can be easily predicted. The production of IgG molecules in cell culture results in varied glycoprotein structure due to incomplete processing of the galactose and fucose residues from the biantennary glycans, adding to the complexity. Targeted MRM assays can be easily predicted for all possible IgG glycan structures, using the known structures and peptide sequence for parent ion computation and the common known fragment ions that are produced during MS/MS, including diagnostic marker ions (Table 1). An Excel spreadsheet was constructed that enabled the computation of specific MRM transitions for all possible IgG glycopeptides. Also included were some unusual IgG glycan forms that had been reported in the literature.

Group Triggered MRM for Specific Detection

In the *Scheduled* MRM Pro Algorithm, there are a number of additional features that can be used to improve the efficiency and specificity of targeted detection workflows. First is the ability to define sets of transitions as a group for a specific glycopeptides. Within the group, MRMs expected to be most intense can be set as primary transitions, and additional confirmatory MRMs can be defined as secondary transitions (Figure 2).

MRM type	Q3 ion	Sugar	Symbol	Description
P	204.1	HexNAc		Confirms a glycan is attached to peptide
P	366.1	HexHexNAc		
S	512	HexHexNAcFuc		B-ion
S	982.4^{2+}	Pep1–HexNac3 Hex3		Confirms bisecting glycan portion
S	1042.4^{2+}	Pep1–HexNac2 Hex3		Confirms non-bisecting glycan portion
S	274	NeuAc-H2O		Confirms sialic acid
S	657	NeuAcHexHexNAc		
S	803	NeuAcHexHexNAcFuc		
S	147	Fuc		

Table 1: Significant Fragment Ions for IgG Glycopeptides. The fragment ions produced by MS/MS of different glycoforms can act as diagnostic marker ions and can be predicted and monitored by MRM for specific detection and quantitation. An Excel spreadsheet was constructed to automatically compute MRM transitions for the known peptides (peptide EEQYNSTYR in this case) using these expected fragment ions.

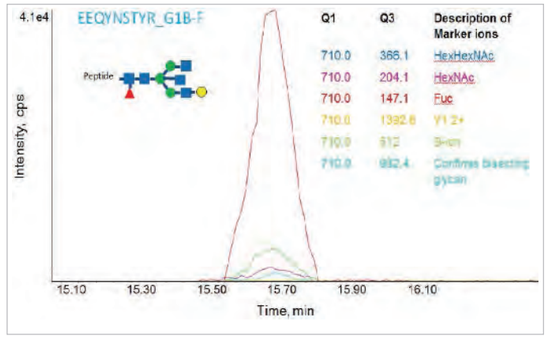

Figure 3: Detection of a Specific Tryptic Glycopeptide using Sets of Specific MRM Transitions. For the targeted detection of the EEQYNSTYR-G1FB, primary MRM transitions from the predicted parent mass to the diagnostic sugar ions, (m/z 204.1 and 366.1) were used to trigger the secondary MRMs when they exceeded the threshold of 200 cps. For this glycopeptide, secondary MRM transitions were used that confirmed the presence of fucose (m/z 147.1), the peptide core (m/z 1392.6^{2+}), the B-ion (m/z 512) and the bisecting core (m/z 982.4^{2+}).

Historically, a precursor ion scan was done on specific sugar ions, mainly 204 (GlucNAc) or 366 (HexHexNAc), to find out which peptides are glycosylated[1]. As these are typically of good intensity, these were used as primary MRM transitions for the first round of glycopeptide detection, triggering additional structurally unique secondary MRMs to further characterize the sugar structure (Figure 3, 4). Using group-triggered MIDAS Workflow, improved dwell and cycle times are achieved by only acquiring secondary transitions when a glycopeptide is eluting as detected by primary transitions. In order to explore the feasibility, sensitivity and specificity of a group-triggered MIDAS Workflow, we used a tryptic digest of two IgG1 antibodies containing glycopeptide 1 (EEQYNSTYR) and peptide 2 (TKPREEQYNSTYR).

This initial MRM method served two purposes: first it provided initial evidence regarding the presence of the specific glycopeptide form, and second it provided a retention time for further optimization of a time-scheduled MRM assay.

We conducted several runs for peptide 1 (Figure 3) and peptide 2 (Figure 4) of IgG1 isoform. In a second run, the retention times from the first run were used to schedule the MRM transitions, which enabled the use of more specific marker ion such as Fucose, bisecting-ions, core-ions, Y1^{2+} ion, B-ion (HexHexNAcFuc+H+)$^{+}$ and sialic acid-ions. In summary, we used 204 and 366 ion as primary MRM transition and all other ions as secondary transition for all non-sialic acid glycoforms.

By overlaying the results of these additional MRM transitions in a group, specificity was achieved for each glycopeptide isoform. Detection of a bisecting ion confirms a G1B-F glycoform on peptide 1, as demonstrated in Figure 3 by the presence of signal for the bisecting core ion (m/z 982.4^{2+}).

Increased Glycopeptide Detection using Both Peptide Forms

Previous work[1] has shown that a large glycan structure can mask the tryptic cleavage site and therefore neglecting this longer peptide would result in an incomplete glycan profile. Therefore, Peptide 2 was also included in the study (TKPREEQYNSTYR contains 1 missed tryptic cleavage). The same diagnostic marker ions were monitored for peptide 2 (Table 1). However, the fragment ion masses for the core and the bisecting glycoforms were modified accordingly.

Here, the overlay of the additional secondary MRMs highlights a high mannose type glycoform, mannose-7 (Figure 4). The specific core ion (m/z 1283.9^{2+}) confirms the high mannose type structure. It was found that by monitoring peptide 2, additional high mannose glycoforms could be detected (such as mannose-7 and 9) and a tri-antennary A3 isoform as shown in Table 3.

Chromatographic Profiling of Glycopeptides

When studying glycopeptides, there are multiple challenges due to broad dynamic range that must be overcome. The targeted profiling of glycopeptides using MRMs to the m/z 204 marker ion shows that specific glycoforms can be up to 10 times more abundant than other glycopeptides (Figure 5). In addition, the abundance of the non-glycosylated form of the peptide can again be as much as 10x more abundant than the glycosylated form (VVSVLTVLHQDWLNGK - data not shown).

In order to detect lower abundant glycopeptides, a significant amount of digest was loaded (pmoles of IgG digest on column).

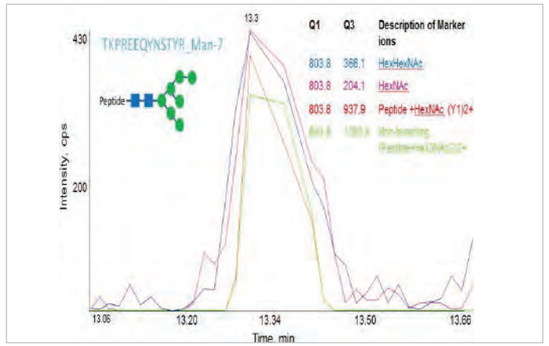

Figure 4: Detection of a Specific Non-Tryptic Glycopeptide using Sets of Specific MRM Transitions. For the targeted detection of the TKPREEQYNSTYR-Mannose7, primary MRM transitions from the predicted parent mass to the diagnostic sugar ions, (m/z 204.1 and 366.1) were used to trigger the secondary MRMs when they exceeded the threshold of 200 cps. For this glycopeptide, secondary MRM transitions that confirmed the correct branching of a high mannose type were used such as m/z 937.9^{2+} for the Y1^{2+} ion (Peptide 2 + HexNAc)$^{2+}$ and 1283.9^{2+} for the core (Peptide 2 + HexNac2 Hex3)$^{2+}$.

The glycopeptide chromatographic profile (Figure 5) highlights the variation in abundance observed as well as the co-elution that is often observed when using a C18 column. However, good quality MS/MS on many glycopeptide isoforms is still obtained because of the specificity gained by targeted detection (Figure 5, inset). Group-triggered MIDAS Workflow was key to obtain improved peak sampling and more MS/MS acquisition when many targeted glycopeptides co-elute. These can then be used for glycoform identification because of the quality of the spectra (highlighted in Figure 1).

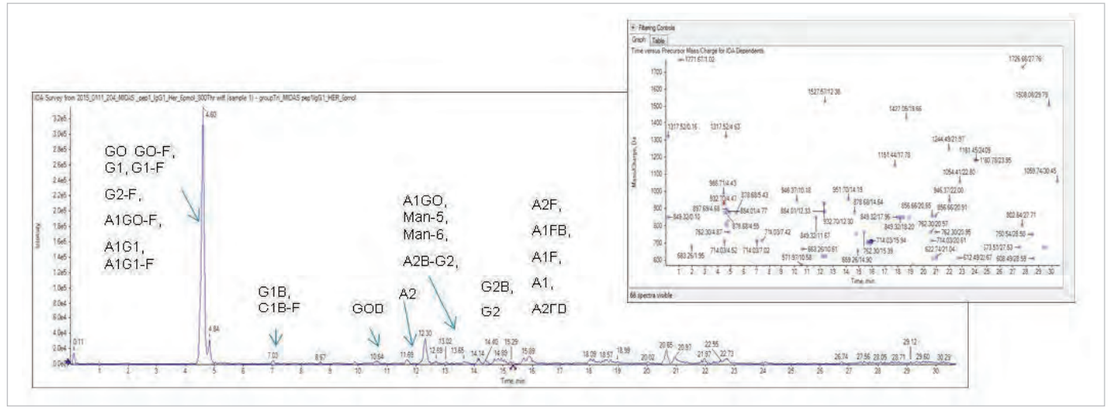

Figure 5: Targeted Quantitative Profiling of Glycopeptides using the MIDAS Workflow. The chromatogram shows the MRM transitions for the IgG1 glycopeptides on peptide 1 (EEQYNSTYR) to the 204 marker ions. When signal is detected in the MRM transition, a full scan MS/MS spectrum is collected, to confirm the glycopeptide structure (inset demonstrates the m/z and retention where each selected glycopeptide was sent for MS/MS).

DEVELOPMENT AND QUALITY CONTROL

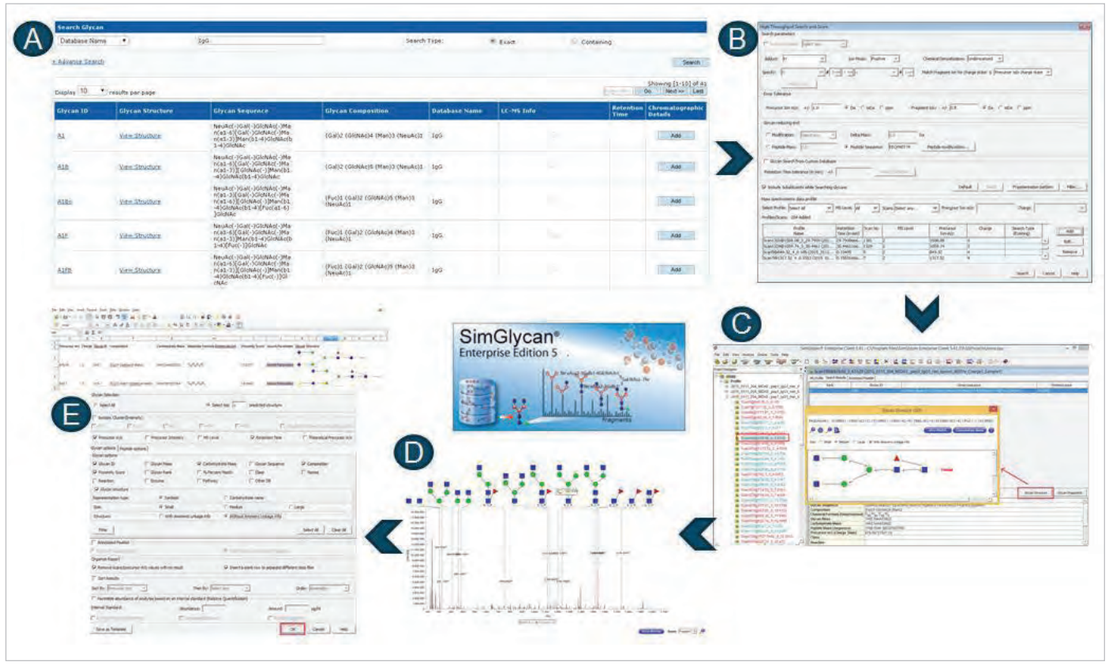

Figure 6: Glycopeptide Identification Workflow in SimGlycan Enterprise Edition software. A) A specific database is created containing the known glycoforms for IgGs. B) MIDAS workflow files are then submitted for batch searching. C) Search results are then returned and all matched glycoforms are shown in green. Selecting a result will show the glycan structure. D) Each full scan MS/MS can be annotated using the search results and the known glycopeptide structure. E) A report is then generated using the report creation window.

Semi-Automated Identification of Glycopeptides using SimGlycan Software

SimGlycan® software predicts the structural information of many glycans and glycopeptides and stores the information in its large relational database, containing 22,456 glycans, 22814 glycoproteins, 11,438 glycans with known biological sources, 11,918 glycans with known classes. When performing targeted detection experiments as described here, the known peptide sequences can be used as input in SimGlycan software. Data generated from all SCIEX instruments can be processed using version 5.4, including the new support for the MIDAS Workflow (Figure 6).

Both the MIDAS Workflow data using MRMs to m/z 204 and the Group triggered MIDAS data targeted the additional structural ions were submitted for search. Using the high throughput functionality for glycopeptides, each LC-MS run was analyzed in batch mode. Results from the searches are summarized in Table 2 and 3, including the SimGlycan software proximity scores to highlight successfully confirmed MS/MS spectra.

For each MS/MS scan, SimGlycan software creates an initial list of candidate glycopeptides based on precursor m/z (and retention times were possible). The program compares the *in silico* fragment ions of the candidates against the observed MS/MS spectra. Mostly higher scores (proximity score >70) are assigned to candidate glycopeptides with the carbohydrate fragments matched to high intensity peaks in the MS/MS data. In addition, the retention time of the SimGlycan software search result matched the precursor ion which was also used for group triggered MRM transitions.

		Trastuzumab 6 pmol			SiluMab 6pmol		
Symbolic glycan structure	Glycopeptide & glycan name	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)
	EEQYNSTYR_Man-7	-	-	-	63.65#	14.46	3150
	EEQYNSTYR_Man-9	-	-	256	73.56	18.93	3814
	EEQYNSTYR_G2-F	73.80	4.5	14877	71.92	5.63	38668
	EEQYNSTYR_A1-G1-F	-	4.59	15412	72.65	4.51	9692
	EEQYNSTYR_GO_F	73.02	4.60	58687	72.52	5.7	743131
	EEQYNSTYR_A1-GO-F	76.09	4.63	113938	68.57#	4.54	18426
	EEQYNSTYR_A1-G1	-	4.82	3002	-	4.55	23346
	EEQYNSTYR_G1-F	72.65	5.12	343124	72.72	5.62	25609
	EEQYNSTYR_G1	73.55	5.51	656	-	-	-
	EEQYNSTYR_G1B-F	74.36	7.01	362	-	-	-
	EEQYNSTYR_G1B	77.59	7.02	88578	78.71	14.63*	1125
	EEQYNSTYR_GOB	77.47	10.57	2190	71.42	10.57	1011
	EEQYNSTYR_A2	72.76	12.17	564	-	-	433
	EEQYNSTYR_A1-GO	72.25	12.19	12795	-	10.52	23346
	EEQYNSTYR_A2B-G2	36.42#	12.23	1098	-	-	-
	EEQYNSTYR_GO	74.23	12.28	164989	74.96	4.64*	242
	EEQYNSTYR_Man-6	76.98	12.30	3757	73.08	14.42	2521
	EEQYNSTYR_Man-5	76.04	12.57	5562	74.52	13.6	1497
	EEQYNSTYR_G2	-	14.16	2182	71.43	18.60*	645
	EEQYNSTYR_G2B	75.09	14.8	7192	-	-	-
	EEQYNSTYR_A1	-	15.85	1229	75.27	28.54	980
	EEQYNSTYR_A1FB	65.90#	15.86	2176	-	-	1358
	EEQYNSTYR_GOB-F	55.78#	15.88	22379	73.33	15.8	25609
	EEQYNSTYR_A1F	-	15.89	573	-	-	340
	EEQYNSTYR_A2F	75.04	15.90	705	-	-	996
	EEQYNSTYR_A2FB	-	15.92	550	-	-	-
	EEQYNSTYR_G2B-F	72.71	22.8	211	71.03	9.41*	282

*# * – See Table 3 for explanation.*

Table 2: Summary of Glycopeptides Found on EEQYNSTYR Peptide for trastuzumab and SiluMab. A glycopeptide was considered detected when both the MRM area of the m/z 204 peak was ~200 cps or greater, multiple MRMs per glycopeptide were overlaid, and when the SimGlycan score on the triggered MS/MS spectrum exceeded 70.

Symbolic glycan structure	Glycopeptide & glycan name	Trastuzumab 6 pmol			SiluMab 6pmol		
		SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)
	TKPREEQYNSTYR_GO-F	65.62#	4.73	4750	-	4.6	1774
	TKPREEQYNSTYR_G1S1	76.93	12.99	1405	-	-	-
	TKPREEQYNSTYR_Man-8	-	13.34	3018	-	12.17	2148
	TKPREEQYNSTYR_G2-F	76.91	13.35	2053	-	13.32	836
	TKPREEQYNSTYR_Man-5	76.47	13.35	2027	-	12.12	1034
	TKPREEQYNSTYR_Man-6	74.52	13.35	1307	-	12.4	303
	TKPREEQYNSTYR_Man-7	76.55	13.35	3626	-	12.43	308
	TKPREEQYNSTYR_G2	76.86	13.7	1540	-	13.7	199
	TKPREEQYNSTYR_A1	76.90	15.8	802	-	16.91	895
	TKPREEQYNSTYR_A2FB	-	16.9	1126	-	17.95	1373
	TKPREEQYNSTYR_A3	74.18	19.46	1366	-	-	-
	TKPREEQYNSTYR_Man-9	74.29	25	3110	-	24.4	414

** Retention times marked with an asterisk in Table 2 and 3 are derived from a different file, the unscheduled MIDAS workflow file for the 204 m/z fragment. In these cases, the identity was confirmed by SimGlycan search results and the observation that multiple charge state MRMs overlapped.*

 # For SimGlycan scores below 70, the full scan MS/MS was manually evaluated and included if significant marker ions confirming structure were present.

Table 3: Summary of Glycopeptides Found on TKPREEQYNSTYR Peptide for trastuzumab and SiluMab. A glycopeptide was considered detected when both the MRM area of the m/z 204 peak was ~200 cps or greater, multiple MRMs per glycopeptide were overlaid, and when the SimGlycan score on the triggered MS/MS spectrum exceeded 70.

Quantitative Profiling of Therapeutic Antibodies

Starting with efficient sample preparation using single tryptic digestion protocol followed by a single LC separation step, we were able to identify and quantify total 23 glycopeptide isoforms for trastuzumab and 14 for SiluMab (Table 2 and 3). Only glycopeptide isoforms who showed multiple MRM transitions as well as significant SimGlycan scores on the MS/MS were considered confidently identified.

For trastuzumab, MRM signal was detected for 23 different glycopeptides on peptide 1, however only 17 of these were further confirmed by MS/MS. When monitoring peptide 2, MRM signal confirmed 11 glycoforms, and 10 of these were further confirmed by MS/MS, providing a total coverage of 23 unique glycopeptide isoforms.

Comparing these data to an untargeted CESI glycan profiling approach of the same mAb (trastuzumab)[2, 3], 13 glycoforms were found in common between these two strategies, confirming this approach. However additional high mannose type isoforms and bisecting glycan isoforms were found with this targeted method.

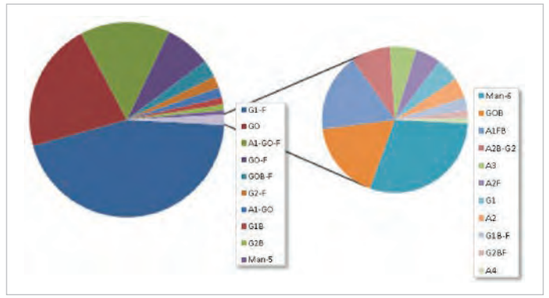

Figure 7: High Abundance Variation of the Glycopeptide isoforms on Trastuzumab. MRM peak areas for m/z 204 for each of the glycopeptide 1 isoforms are compared. Inset shows the isoforms that make up the lowest 1.5% of glycopeptide MRM signal.

To test the robustness of the strategy, an additional IgG1 isotype was analyzed. For SiluMab, 16 glycoforms on peptide 1 were confirmed by MRM and MS/MS. Interestingly; no glycoforms on peptide 2 were confirmed by MS/MS with a high enough MS/MS score, probably because of the much lower abundance of this peptide form.

Conclusions

In this article, the feasibility of the Group triggered MIDAS workflow was demonstrated for the characterization and profiling of glycopeptides derived from therapeutically relevant antibodies (trastuzumab and SiluMab) using the QTRAP 6500 System.

The resulting specific MRM data for sugar marker ions (HexNAc, HexHexNac, Fucose, Hexose and Sialic acid) as well as differentiating core and bisecting glycopeptide ions on the digested IgG sample provided more specificity because of multiple co-eluting transitions. In addition, MRM triggered MS/MS further confirmed the identity of glycopeptide structures. SimGlycan Enterprise Edition software help to confirm these MS/MS glycopeptides and also confirmed the retention time of the correct precursor ion found in the MRM survey scans. The wide abundance variation in the detected glycopeptides highlights the benefit of using targeted detection on a high sensitivity QTRAP system.

The Group MIDAS workflow has been well optimized; however there is an opportunity to further optimize the LC separation. The three major co-eluting LC regions on the RP C18 column could be better separated and therefore more MRM and MS/MS spectra could be collected to look for lower abundant glycopeptide isoforms.

Future work will test the approach with a broader range of separation techniques (e.g. column types or in combination with CESI-MS). Further investigation of possible in-source fragmentation of the fucose will be enabled with improved separation workflows. Combining the optimized separation with this Group MIDAS workflow will provide a powerful strategy for the confirmation and quantitation of glycopeptides.

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[3] Rapid Level 3 Characterization of Biologics using a CESI 8000, Bryan Fonslow and Andras Guttman, Technical Note 10400614-01, SCIEX.

DEVELOPMENT AND QUALITY CONTROL

Developing a Routine LC-MS/MS Platform for Quantitative Identification of Host Cell Proteins (HCPs)

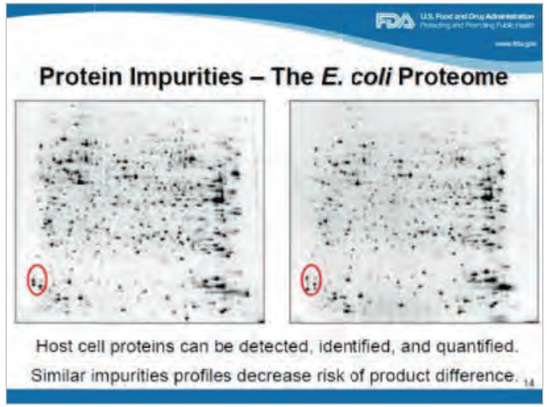
Introduction

Unknown HCPs are a threat to income

Regulators classify residual host cell proteins (HCPs) from recombinant production as process-related impurities. It is known that HCPs may elicit an inappropriate response in patients and must be monitored as part of guidelines. EMA (European Union) and FDA guidelines have been in effect since the 1990s and reflect ICH documents that the removal of HCPs should be proven:

"6.2 Validation of the purification procedure – …The ability of the purification process to remove other specific contaminants such as host-cell proteins…should also be demonstrated"

The repercussions of unwanted HCPs can be extremely costly, such as needing to change formulations, or repeating clinical trials. In 2006-2008 a product about to launch was the subject of scrutiny when 100% of the patients enrolled in two clinical studies developed anti-HCP antibodies[2] forcing the organization to repeat trials and incur unexpeted costs and delays in getting to market. The cause was excess host cell protein levels, later resolved by process change in purification[1]. When the therapeutic was marketed in the US, the USFDA was forced to respond to objections from other innovator companies, who highlighted the potential immunogenicity identified with the early version of the product[3], potentially forcing delay in launch in a new geography.



Slide from an FDA Product Advisory Committee meeting in August 2012 indicating how HCP profiling can demonstrate process control. *[http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/advisorycommitteeforpharmaceuticalscienceandclinicalpharmacology/ucm315764.pdf][10]*

Even worse, unwanted, undetected contaminants may lead to severe loss of revenue, as a biotech in the US discovered when it had to halt production of what was then the only product of its kind on the market[4]. Because of supply shortages, patient groups brought litigation against the company[5]. Additionally, other companies were able to leverage US legislation which allows early market entry, partly to try and prevent shortages, potentially opening markets to competitors prior to patent expiry and threatening income from what had been a unique product[6,7,9]. Therefore the appropriate identification and monitoring of contaminants has the potential to save significant amount of time and money for an organization marketing a biotherapeutic.

Today many analytical methods are used for HCP analysis, such as ELISA, SDS gels, Western Blots, and HPLC with optical detection. Some can be expensive to develop: ELISAs, for example, may take up to eight months and require the ethical housing of test animals. Additionally, antibodies raised in animals such as rabbits may not adequately reflect the situation in humans, leaving open the question of whether such animal models are appropriate. Techniques such as gels and blots may be subjective, or may require pre-knowledge about the contaminant proteins. Many of these techniques have been established as the biotechnology industry developed, reflecting the analytical techniques of their time. Some years ago, forward thinking scientists were aware of the possibilities of the use of SCIEX QTOF MS for HCP analysis. In 2008 the Laboratory of the Government Chemist (LGC) in the UK applied SCIEX iTRAQ® labeling techniques for the quantification of host cell protein impurities for Somatropin Growth hormone[8]. With next generation instruments, and the use of SWATH® Acquisition, SCIEX continues to advance this field.

Analytical Challenges for HCP Analysis

The following challenges are associated with the analysis of HCPs:

- **Complexity:** The HCP component of a biotherapeutic is extremely complex, and could be comprised potentially of any protein that could be expressed by the host organism. The HCP composition may change with different production and purification procedures. Therefore an analytical technique must not make any assumptions and needs to cope with the associated complexity.
- **Dynamic Range:** Most HCPs are present at extremely low levels. It has been suggested that monitoring should occur at least in the ppm range (1-100 ppm). Therefore an appropriate analytical technique requires good dynamic range that can also cope with a dominant proportion of the therapeutic protein.
- **Identification:** Early techniques relied on prior knowledge of the contaminant proteins (e.g., ELISA), but an unbiased analytical technique has the potential to discover proteins that may not have been targeted, such as viral contaminants.
- **Time/Cost:** Method development can be expensive and time consuming – therefore a generic methodology that can be applied at all stages can significantly reduce the cost, time and expertise required to profiling the HCPs.

Earlier LC-MS work focused on complex strategies, or on workflows developed for proteomics, some of which include two-dimensional LC. While these procedures allowed many proteins to be found, they can take weeks to complete and may require careful oversight of the systems by expert users. In this article, we demonstrate an analysis that can be used for default techniques such as peptide mapping and intact mass analysis, as well as the analysis of HCPs. The timescale of this analysis means that the system can be used when needed, and does not need weeks of dedication to the task. Simplification of the workflow results in a robust system, minimizing the need for troubleshooting and reducing the need for expert supervision.

Results

In this analysis we use a TripleTOF® 5600 system with an Eksigent 425 LC system to perform a peptide map on a series of samples that demonstrated increasing depth of coverage. To mimic a real situation of final product HCP levels, six unrelated proteins were spiked into a sample of a purified IgG of high concentration (10ug).

To prove that the analysis is both quantitative, and appropriate for unambiguous identification of proteins, the spiked in proteins were analyzed at a series of points reflecting a wide range of concentrations in a realistic study (Table 1). Figure 1 shows how the identification of proteins is robust and reproducible despite the decreasing concentration of proteins unrelated to the biotherapeutic. It must be noted that this was achieved without complex chromatographic means, and without interfering with the peptide mapping of the biotherapeutic protein itself. In addition, when using the amount of biotherapeutic as a measure against which to normalize, the identified contaminants

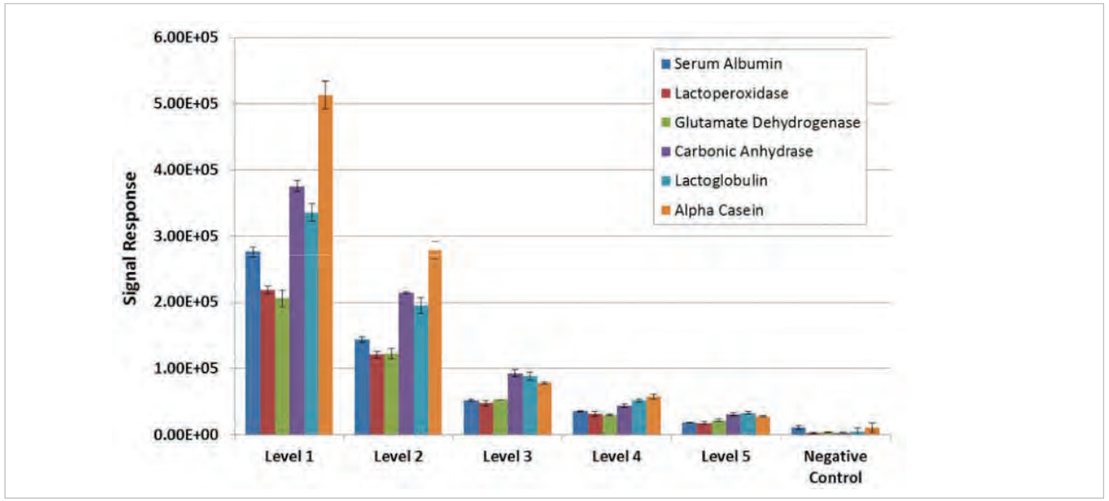


Figure 1: Dilution series of contaminant proteins showing wide dynamic range in a complex mixture with product. The error bars indicate the excellent reproducibility across 5 levels of dilution.

PPM levels of model proteins in 10ug of antibody (30' LC runs at 20 uL/min):						
	Serum Albumin	Lactoperoxidase	Carbonic Anhydrase	Glutamate Dehydrogenase	Alpha Casein	Lactoglobulin
Level 1	415	485	182	350	148	114
Level 2	207	242	91	175	74	57
Level 3	104	121	46	88	37	29
Level 4	52	61	23	44	18	14
Level 5	26	30	11	22	9	7

Table 1: Dilution series of contaminant proteins showing wide dynamic range in a complex mixture with product.

were consistent, and the coefficients of variation in the repeat analyses were consistently low. This is reflected in Figure 2 where a wide variety of peptides with and without modifications were simultaneously identified and quantified. The efficiency gains for an organization and the enhancements to monitoring by using this approach can therefore be very large.

The use of SWATH Acquisition provides the sequences of the peptides being analyzed and can therefore provide better proof of the identity of the proteins. SWATH Acquisition has been developed to provide MS/MS information throughout an analytical run and does not require pre-knowledge of the sample by the user, reducing the technical burden, as well as providing a level of automation that is welcome to any lab requiring improved throughput.

Conclusions

By improving their analytical strategies, organizations can save significant amounts of money by monitoring Host Cell Proteins at all stages of development. The use of LCMS on a SCIEX QTOF System has long been shown as an effective technique that can identify and quantify individual proteins to provide a detailed catalogue of contaminants[8]. In this new work, a comprehensive method of analysis has been developed that provide a straight forward platform to use. The simultaneous detection and quantification of HCPs in a sample contributes to the greater safety of the product, and has the potential to save significant time and costs in HCP analysis.

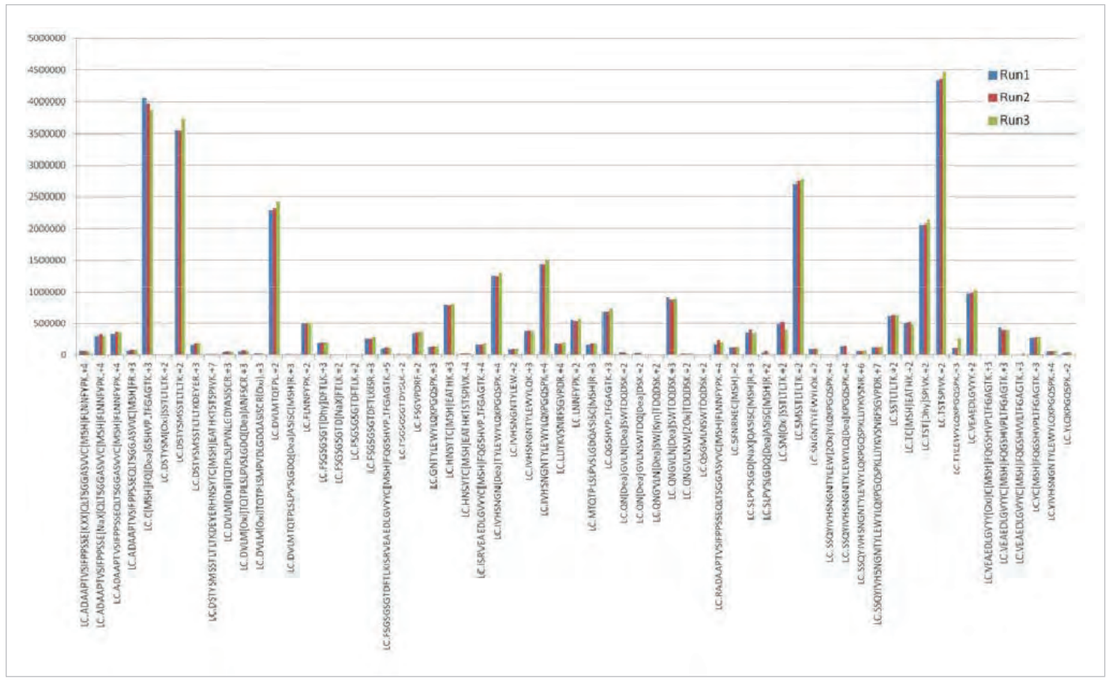


Figure 2: Reproducibility and Dynamic range are demonstrated by the ability to quantify specific peptides and modified peptides as part of a routine peptide map. All CVs are below 10 in this graph over a wide range of peptide sizes and modifications.

Appendix: Instrument Conditions

TripleTOF® 5600 System with the Turbo V™ Source.

- 65 micron Probe

Eksigent ekspert™ 425 System

- 5-50ul Gradient Flow module in Direct Inject mode

Column: 0.5mm X 100mm, 3µm, 120Å ChromXP™ C-18

- Flow: 15 µL/min; Column Oven at 40° C

Solvent A: 2%ACN 0.1% FA

Solvent B: 98%ACN 0.1% FA

TripleTOF System Parameters:

- One 0.16 second MS scan
- 30 0.08 second MSMS SWATH scans, 20 m/z width
- Total Cycle Time 2.56 seconds

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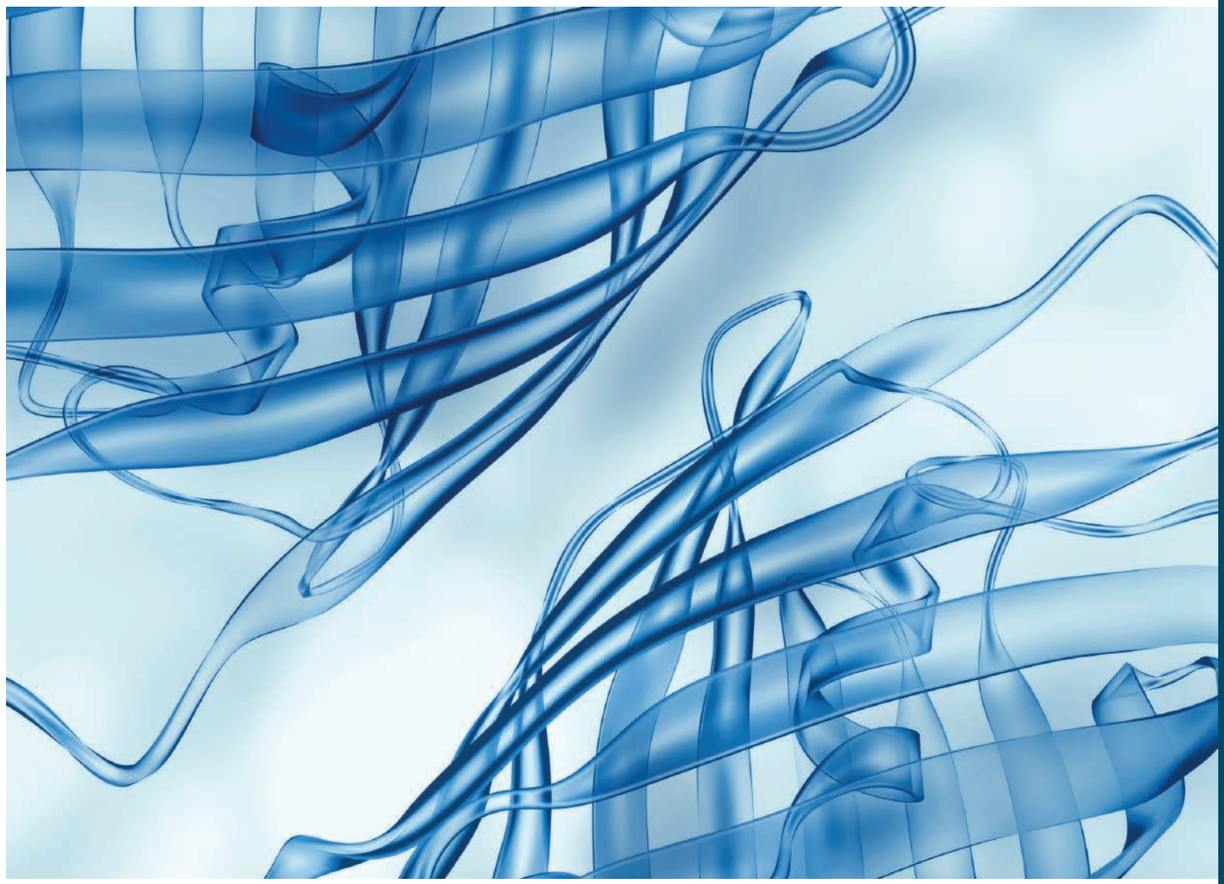

Quantitation of Low Level Host Cell Proteins in an IgG1 Monoclonal Antibody Preparation via Data-Independent Acquisition with a TripleTOF® System

Justin Blethrow and Eric Johansen
SCIEX, USA

Monoclonal antibodies (mAb) are major target-oriented biotherapeutics that are used to treat an array of human diseases. Current therapeutic monoclonal antibodies are immunoglobulin G (IgG) 1 derivatives, typically produced in mammalian cell culture using Chinese hamster ovary (CHO) or other cells[1]. Process-related impurities and other trace contaminants that co-purify during downstream processing must be characterized and monitored to control product quality, potency, and safety.

The detection and quantitation of host cell protein (HCP) contaminants is an area of particular concern, as these contaminants can elicit an adverse response in patients. EMA (European Union) regulations have been in effect since 1997 and reflect ICH guidelines that the removal of HCPs should be proven:

"6.2 Validation of the purification procedure – …The ability of the purification process to remove other specific contaminants such as host-cell proteins … should also be demonstrated."

Historically, HCPs have often been monitored using immuno-affinity assays (typically ELISA) based on polyclonal serum gene rated against the whole proteome of the host cell culture, or against a subset of the proteome obtained by enrichment under conditions similar to the product purification process. This approach is inherently biased toward the detection of highly-expressed HCPs, and HCPs that are highly immunogenic in the animal species used in reagent generation. Results of these tests can be misleading, as low-level HCPs are equally dangerous to the patient, and the human immune system might react differently to that of the animal. Failure to detect an immunogenic contaminant leads to product intolerance or adverse reactions in patients, which threatens patient health as well as clinical trial outcomes. Biopharmaceutical developers have substantial motivation to ensure that they have unbiased analysis of the HCP complement of a product. Modern mass spectrometry has emerged as an attractive tool to improve the analysis of HCPs in a rapid and efficient manner that also provides greater certainty. It would also be advantageous to identify the specific proteins individually, as well as quantify them. However, until recently it was unclear whether or not available mass spectrometry solutions could provide the sensitivity and dynamic range necessary to detect and quantify trace HCP contaminants within an enormous excess of biotherapeutic protein, and at speeds to complete confident analyses in a reasonable time. Moreover, conventional data-dependent acquisition methods adopted from shotgun proteomics for the detection of peptides from FASTA database searching techniques are inherently stochastic, leading to poor run-to-run reproducibility, particularly at the MS/MS level. A commercially viable MS-based solution for HCP analysis must avoid biases, operate without needing to know the contaminant proteins prior to establishing the assay (e.g. ELISA), and retain the beneficial aspects of speed, sensitivity, and consistency.

Here we leverage the high speed and sensitivity of a SCIEX TripleTOF® mass spectrometry system, combined with powerful data-independent SWATH® Acquisition to demonstrate unbiased, highly-reproducible quantitation of model host cell protein contaminants in an IgG1 sample down to the level of ten parts per million (ppm, wt/wt contaminant/product). Method fidelity and throughput were maintained via the use of micro-scale LC (20 µL/min) and short analytical runs (less than 45 minutes).

Advantages of the TripleTOF System with SWATH Acquisition for Host Cell Protein Analysis

- Comprehensive, unbiased quantitation with simultaneous MS/MS information: Amounts of any number of host cell proteins can be quantified in a single run.
- Sensitivity and speed: Host cell proteins can be quantified at low PPM levels, using LC gradients as short as thirty minutes.
- Throughput and reliability: The speed and sensitivity of the TripleTOF System allow the use of microflow LC for this assay, eliminating the problems with reliability and throughput that are typically seen with nano-scale LC.

Experimental Design

Sample Preparation: 1 mg of monoclonal mouse IgG1 isotype (Waters, Milford, MA) was denatured with urea, reduced with TCEP, and alkylated with MMTS. The resulting denatured protein was digested with trypsin at 37˚C for four hours. A constant concentration of this digest was spiked with a commercial six-protein digest mixture (Michrom, Auburn, CA), at a range of concentrations.

Chromatography: Samples were analyzed using the Eksigent ekspert™ 425 System and a ChromXP column (0.5 mm x 10 cm C18- CL, 3µm 120Å). The HPLC gradient is shown in Table 1. A flow rate of 20 µL/min was used. Solvent A consisted of 2% acetonitrile and 0.1% formic acid, solvent B consisted of 98% acetonitrile with 0.1% formic acid. 10µg of antibody were loaded per run. The column was maintained at 45° C.

Time	Solvent A	Solvent B
0	95	5
1	95	5
25	65	35
30	10	90
35	10	90
36	95	5
41	95	5

Table 1: LCMS Gradient Profile

Mass Spectrometry: Unmodified and spiked mAb digests were analyzed using a TripleTOF 5600+ system. An information dependent acquisition (IDA) LC-MS/MS method was used for initial peptide identification and to generate a peptide library for SWATH Acquisition data interrogation. This IDA method consisted of a high resolution TOF MS survey scan followed by 20 MS/MS per second with a minimum accumulation time of 50 msec. SWATH data-independent acquisition was subsequently performed in triplicate on each sample, using a 20 Da Q1 window width to obtain quantitative MS/MS chromatograms for every precursor ion between 400 and 1200 m/z.

Data Processing: IDA data files were searched using ProteinPilot™ Software against a database containing the sequence of the antibody and the sequences of the model "host" cell proteome. Quantitative analysis was performed using the search results as a peptide library to inform SWATH peptide fragment ion chromatogram extraction using the SWATH Acquisition tool inside the PeakView® Software.

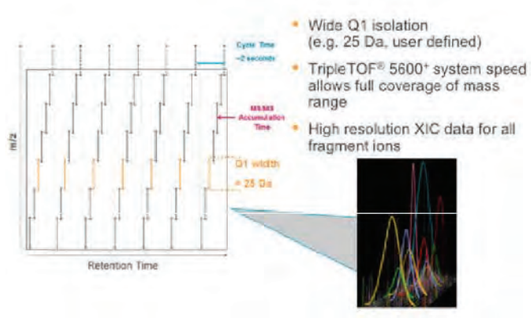

Figure 1: SWATH Acquisition Obtains MS/MS data on all ions. By rapidly stepping the MS/MS isolation window across the mass range, fragment ion chromatograms are collected for all observed ions within chromatographic peaks.

Peptide Mapping and Library Generation

The levels of each model host cell protein in our various spiked samples are shown in Table 2, expressed as parts per million (ppm). Qualitative characterization runs using IDA analysis were performed on the "Level 1" sample, where model HCPs ranged from approximately 100-500 ppm. These initial runs were used to catalog IgG1 and model HCP-derived peptides, and to generate a peptide library to inform subsequent SWATH analysis. Antibody sequence coverage was 100% for Light Chain and 99.5% for Heavy Chain (Figure 2). An advantage with this approach is that the peptide libraries produced in this step can be used indefinitely in any subsequent SWATH analysis. In general, the IDA runs should rarely need to be repeated, except for example, when making substantial changes to the protein expression or purification processes that are used. In a functional sense, this step can be considered analogous to the common practice of generating a polyclonal ELISA reagent against partially enriched HCPs, but the IDA method avoids the detection biases introduced with such a reagent.

Level	Serum Albumin	Lacto-peroxidase	Carbonic Anhydrase	Glutamate Dehydro-genase	Alpha Casein	Lacto-globulin
1	415	485	182	350	148	114
2	207	242	91	175	74	57
3	104	121	46	88	37	29
4	52	61	23	44	18	14
5	26	30	11	22	9	7

Table 2: Model HCP levels in test samples, expressed as parts per million.

Data-Independent Quantitation of Model Host Cell Proteins via SWATH Acquisition

Following IDA runs, samples were re-analyzed using data-independent SWATH Acquisition. Quantitative analysis was performed via peptide fragment ion chromatogram extraction using the SWATH Acquisition tool in PeakView Software, using the peptide library data to inform fragment ion selection. Figure 3 shows an example of SWATH Aquisition data representing a peptide from Serum Albumin. Aligned extracted ion chromatogram (XIC) traces for nine peptide fragment ions are shown, for each of the HCP spike levels. The peptide is easily detectable at the 10 PPM level, and visibly absent in the control sample lacking model HCPs (Figure 3, pane F).

For each of the model HCPs, three to four peptides were used for SWATH quantitation. It is important to note that the MS/MS information is used here for quantitation, rather than just confirmation of peptide identity, providing superior acuity compared to MS1-based quantitation. In all cases, protein-level quantitation reflects summation of the individual peptide signals. We also selected four peptides from the antibody itself for quantitation. Despite up to a 100,000 fold difference in

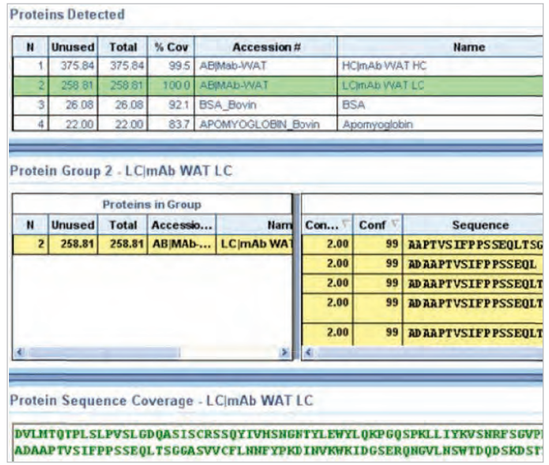

Figure 2: Antibody coverage in peptide mapping runs. Extremely high coverage of the heavy and light chains are obtained using ProteinPilot Software.

abundance between the product and the contaminants, the quantitation of both HCPs and antibody was highly reproducible. Further, quantitation of the antibody served as an internal loading control, allowing for normalization of the HCP signals, thereby eliminating minor differences in HCP signal response arising from variations in sample loading, etc. This made it possible to compare HCP levels in sets of runs performed many days apart with high quantitative accuracy, without undue concern for maintaining identical states with respect to the LC and MS systems. Such capability would also allow experiments to be run when needed, rather than having to dedicate a system to HCP analysis alone for a number of days. Variations in antibody response across samples run on different days were generally less than ten percent.

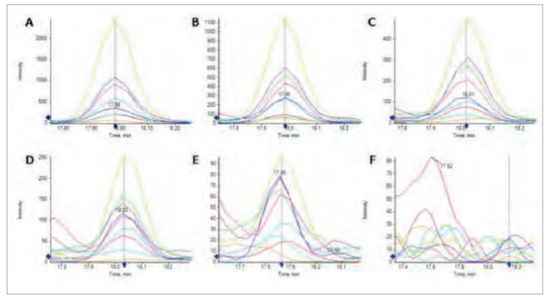

Figure 3: SWATH Acquisition XIC traces for a peptide from serum albumin. Fragmention XICs for the peptide SLHTLFGDELCK are shown. Traces correspond to protein levels of A: 415 ppm, B: 207 ppm, C: 104 ppm, D: 52 ppm, E: 26 ppm, and F: a negative control sample containing no model HCP peptides.

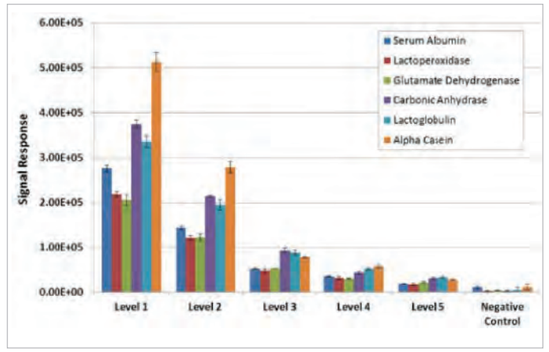

Figure 4: Protein level signal responses. Peptide level signals for each protein were summed, providing a direct measure of concentration. Values shown are the mean and standard deviations (error bars) of triplicate runs.

Quantitative Linearity and Detection Limits

Figure 4 shows the signal response for each model HCP across the sets of triplicate runs. Figure 5 highlights the coefficient of variance (CVs) observed for each protein and concentration level. At the higher load levels, CVs were all between 3-7%. Even at the lowest level, where the HCP proteins were present at 7-30 ppm, the average CV was only 7%, and the most variable protein had a CV of 12%. These results indicate extremely high quantitative reproducibility, independent of protein identity, suggesting that this approach should be broadly applicable for the unbiased quantitation of HCPs down to low ppm levels.

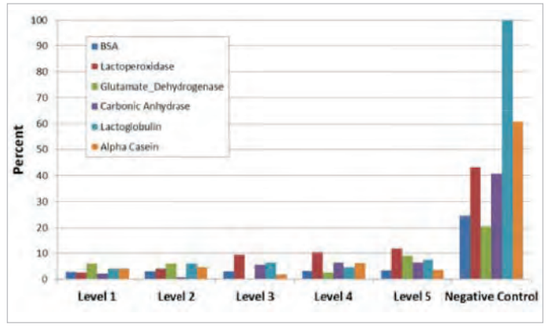

Figure 5: Protein level coefficients of variation across replicates. Consistently low CVs across the model HCP contamination levels indicate excellent quantitative accuracy down to the lowest tested level (protein present at 7-30 ppm). The very high CV values observed in the negative control confirm that the software could not find a reliable signal as expected.

Conclusions

Current analytical strategies for monitoring the levels of host cell protein contaminants in biotherapeutic preparations present an unpalatable choice. One can have sensitivity and speed at the cost of detection blind spots and biases (ELISA), or unbiased detection at the individual protein level, at the cost of extremely low throughput and poor reproducibility (typical MS approaches derived from proteomics workflows). The approach we present here, relying on the power of MS/MS^{ALL} via SWATH Acquisition, combines the key strengths of both of these approaches, while eliminating the liabilities. Because a single SWATH Acquisition method can target the entire precursor peptide mass range, any number of target proteins can be quantified in a single run. Further, this comprehensive quantitative profiling eliminates problems with run-to-run variability that are inevitable with conventional data-dependent acquisition strategies derived from shotgun proteomics. Critically, these advantages do not need to come at the cost of throughput, reliability, or sensitivity. As demonstrated here, SWATH Acquisition on the TripleTOF 5600+ system can deliver consistent, low ppm-level quantitation of host cell proteins in runs that are well under an hour, at flow rates in the tens of microliters per minute, thereby eliminating the complications and reliability issues associated with nano-scale LC.

Ultra-Sensitive Host Cell Protein Detection Using CESI-MS with SWATH® Acquisition

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Key Benefits

- Detection of host cell proteins from limited sample quantities in the early development phase.
- An orthogonal separation mechanism for additional method development or detection validation.
- Improvement in sensitivity through reduced ion suppression and higher ionization efficiency at ultra-low flow rates.

Introduction

Host cell proteins (HCPs) are undesired impurities in biologic preparation processes and can negatively affect biotherapeutic quality, potency, and safety. Identification and quantification of HCPs within therapeutic monoclonal antibody (mAb) preparations remain a challenge with a need for improved sensitivity and specificity. Mass spectrometry, particularly data-independent acquisition such as provided by SWATH Acquisition, offers a robust and sensitive means to quantify HCPs in the parts-per-million (ppm) range. The integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into one process (CESI) presents the possibility to improve the sensitivity of HCP quantitation through reduced ion suppression and improved ionization efficiency at ultralow nanoliter per minute flow rates.

We demonstrate the use of CESI-MS with SWATH Acquisition for the ultrasensitive detection of host cell proteins (HCPs) in a representative mAb preparation. To simulate HCPs, we spiked a mAb digest with digested protein standards over a concentration range of sub-ppm to parts per thousand (ppth) concentrations. HCP proteins at ppth concentrations were identified with a CESI-MS information-dependent acquisition (IDA) run to generate an ion library for proteins/peptides. Identical CESI separations were performed with the HCP dilution series using SWATH Acquisition. The ion library and SWATH Acquisition data were used to screen for peptides and fragment ions which best represent the HCP concentrations. The concentration-indicative peptides and ions were then used to generate calibration curves for the HCPs. HCPs were quantified down to the low ppm range and in some cases even into the sub-ppm range, representing detection of HCPs over three orders of magnitude.

Experimental

Sample Preparation: A representative mAb and 17 protein standard mixture were digested separately to peptides at 1 mg/mL using a 4-hour digestion protocol with RapiGest, DTT, iodoacetamide, and trypsin. The digested protein standard mixture was spiked into the digested mAb throughout the ppth, ppm, and ppb range to represent contaminant host cell proteins. The peptide preparations were then diluted to 250 µg/mL in 125 mM ammonium acetate, pH 4.

CESI Conditions: CESI experiments were carried out with a SCIEX CESI 8000 Plus system (P/N A98089) equipped with a temperature controlled auto sampler and a power supply with the ability to deliver up to 30 kV. An OptiMS Silica Surface Cartridge (P/N B07367) with a porous tip was used for infusion and peptide mapping experiments. Solutions of 10% acetic acid were employed as the background electrolyte (BGE) and conductive liquid. After pressure injection of ~25 ng peptides, sample stacking was performed using transient isotachophoresis (t-ITP). CESI separations were performed at 20 kV. The same CESI separation conditions are used for IDA identification and SWATH Acquisition quantitation runs.

MS/MS Conditions: A SCIEX TripleTOF® 5600+ system with a NanoSpray® III source and CESI adapter (P/N B07366) controlled by Analyst® TF 1.7 Software were used. HCP identification was performed with information dependent acquisition (IDA) with 15 MS/MS cycles (100 and 50 ms accumulation times for MS and MS/MS, respectively). HCP detection and quantification were performed using data-independent SWATH Acquisition with 30 constant window width scans (30 m/z with 1 m/z overlaps) from 300 – 1200 m/z using 150 and 50 ms accumulation times for MS and SWATH scans, respectively.

Data Analysis: High resolution MS and MS/MS spectra were analyzed using SCIEX ProteinPilot™, PeakView®, and MultiQuant™ Software.

Results

The first step for a host cell protein experiment is identifying proteins with an IDA experiment while present at higher concentrations. In practice, this experiment would be performed on a partially purified mAb preparation. Here we simulated

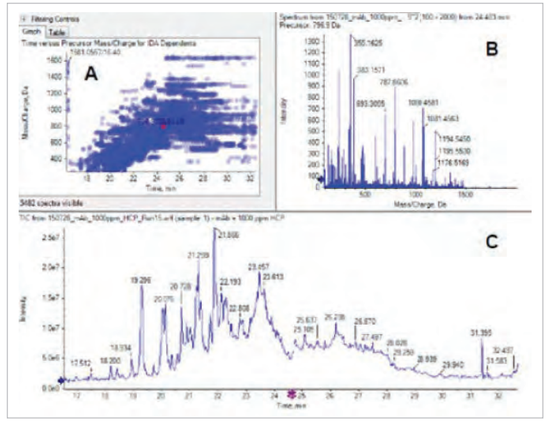


Figure 1: Raw data from a CESI-MS IDA run on the 1 parts per thousand protein standard and mAb sample preparation. The raw data is represented as (A) a heat map of the peptides selected for MS/MS, (B) a representative peptide MS/MS spectra, and (C) a total ion electropherogram (TIE) of the CESI peptide separation.

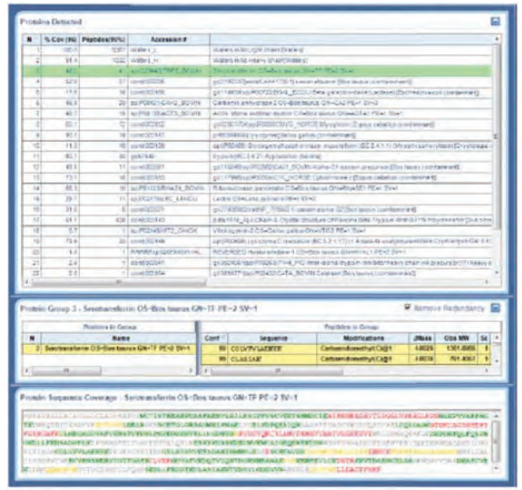


Figure 2: Identification of representative mAb and protein standards from CESI-MS IDA runs using ProteinPilot Software. Peptides shown in green within the protein sequence represent the best candidates for quantitation using SWATH Acquisition since they have the best MS/MS signals. These peptides are imported into the PeakView® Software SWATH microapp for evaluation.

the partially purified preparation with protein standards spiked in at 1 parts per thousand by mass relative to the mAb. Representative CESI-MS IDA data displayed by PeakView Software in IDA Explorer is shown in Figure 1. CESI-MS IDA runs allow for identification of peptides within representative host cell proteins which yield good MS/MS spectra at lower concentrations. ProteinPilot Software search results from the IDA experiment is shown in Figure 2. The peptide fragment ions from protein and peptide database matches can later be evaluated as good quantitative representatives of a HCP's concentration from the SWATH Acquisition data.

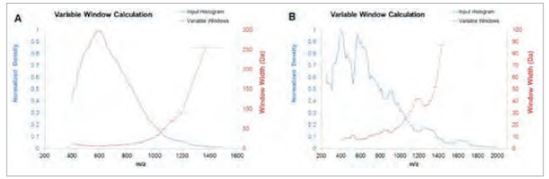


Figure 3: Calculation of variable SWATH™ acquisition window widths based on IDA runs of a (A) yeast lysate and (B) 1 parts per thousand HCP/mAb preparation.

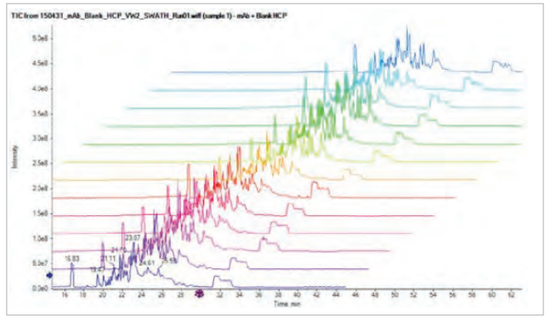


Figure 4: Representative total ion electropherograms (TIEs) from CESI-MS SWATH Acquisition runs including digested mAb alone and HCP-spiked runs in the ppb to ppth concentration range.

SWATH Acquisition can be performed with different m/z window configurations to focus on information-rich regions of the m/z domain. We investigated the use of constant and variable window widths on the detection of HCPs using CESI-MS. Peptides are generally most abundant in the 400 – 800 m/z range, so one of the variable window configurations tested was generated from LC-MS IDA analysis of a common peptide digest from yeast lysate. The other variable window configuration evaluated was generated from the replicate CESI-MS IDA runs from the HCP/mAb preparation as represented in Figure 1. Both variable window configurations are shown in Figure 3. Triplicate SWATH Acquisition runs were performed on the dilution series of demonstrative host cell proteins. Figure 4 shows representative total ion electropherograms from the replicate quantitation runs.

Candidate peptides and fragment ions can be selected for quantitation using SWATH Processing in PeakView software. Figure 5 shows representative SWATH Acquisition data selected for quantitation of HCPs. Two extracted fragment ion electropherograms for myoglobin peptide VEADIAGHGQEVLIR (Figure 5A) have the same migration profile indicating they are from the same peptide. These two ions were matched to those found in the peptide library from the IDA run (Figure 5B). Additionally, when the fragment ions are considered between triplicate runs of the calibration curve, the appropriate quantitative trend is observed (Figure 5C). Collectively these three pieces of data illustrate the capability of quantifying HCP surrogate peptides based on a few fragment ions.

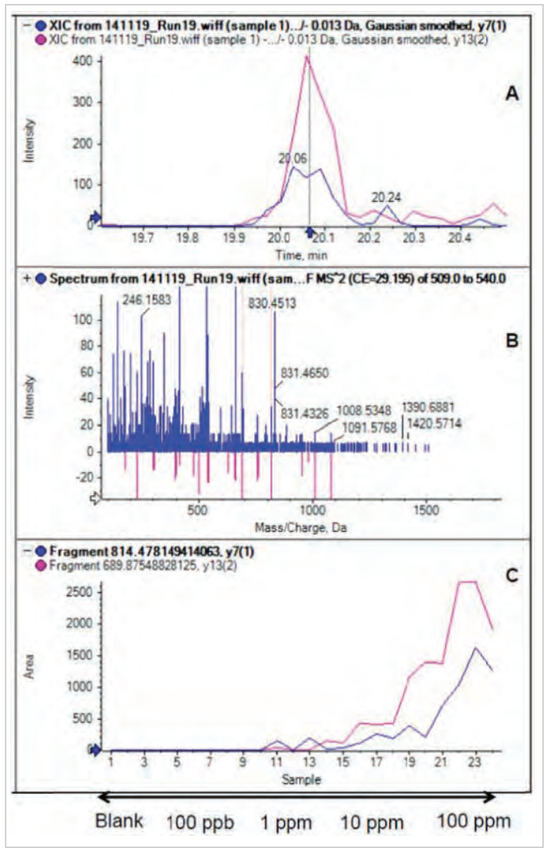


Figure 5: (A) Extracted fragment ion electropherograms for myoglobin peptide VEADIAGHGQEVLIR y7 and y13 ions. (B) Matching SWATH Acquisition MS spectra (blue) to previously identified myoglobin peptide fragment ions (pink). (C) Fragment ion peak area plot based on HCP concentration from triplicate runs.

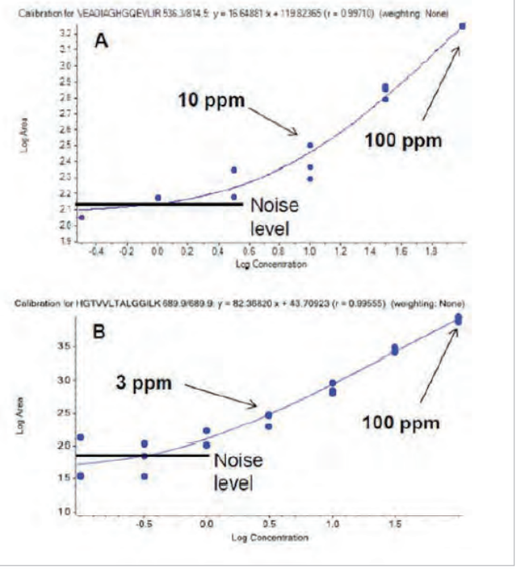


Figure 6: Calibration curves from triplicate CESI-SWATH Acquisition MS runs of myoglobin peptides (A) VEADIAGHGQEVLIR b2 ion and (B) HGTVVTALGGLK y7 ion.

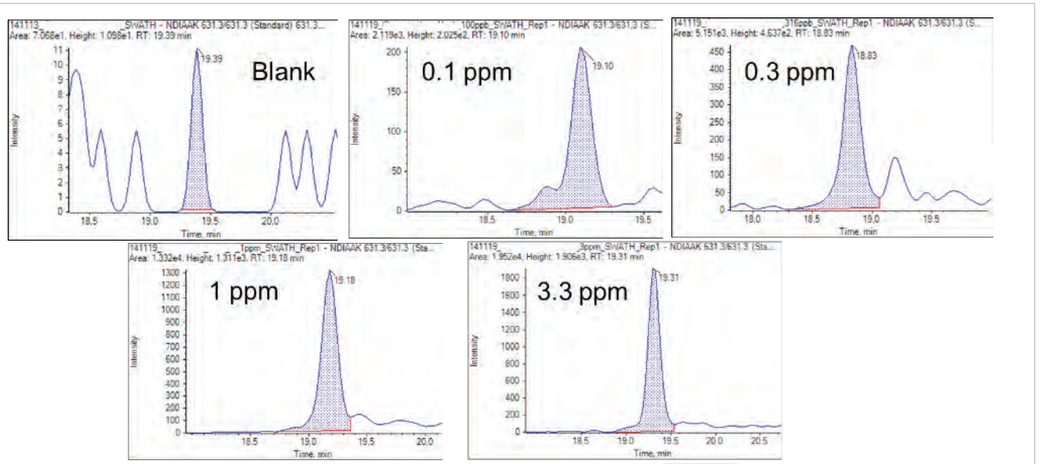


Figure 7: Extracted fragment ion electropherograms for myoglobin peptide NDIAAK in the concentration range from 0.1 ppm to 3.3 ppm using MultiQuant Software.

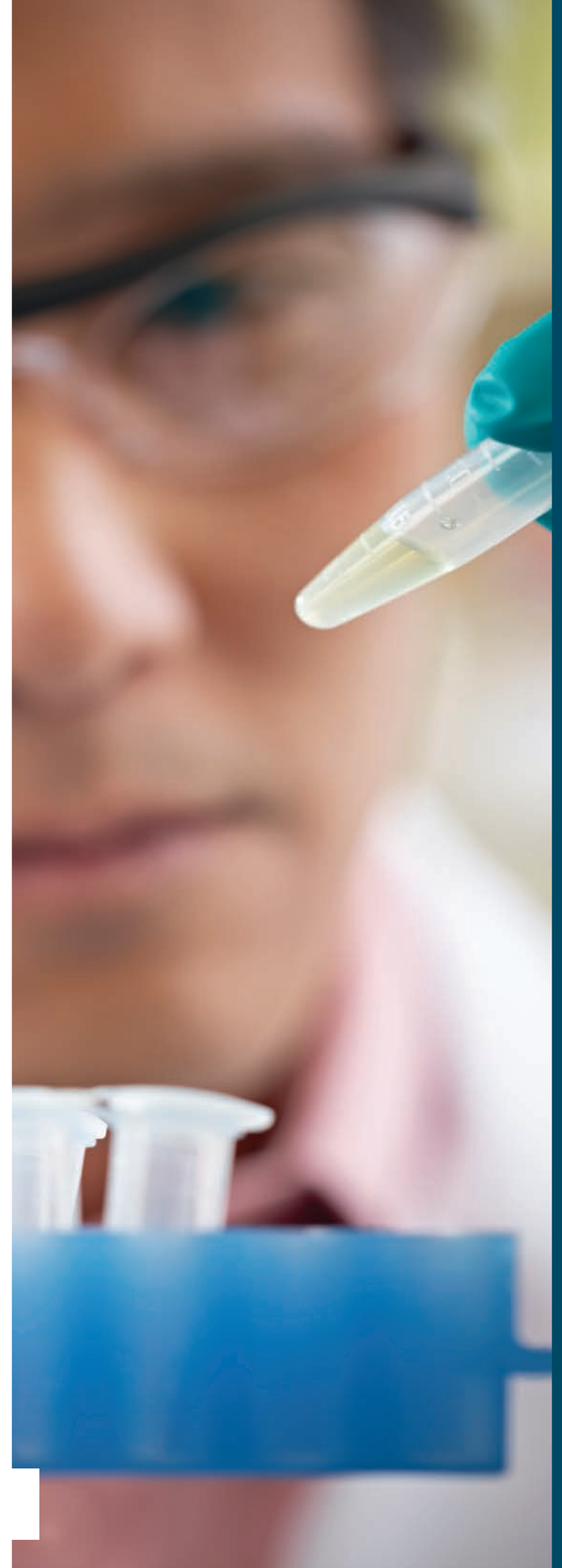
Due to the very low concentrations of host cell proteins within therapeutic mAb preparations, the sensitivity of mass spectrometric methods are generally pushed to the limit, potentially into the low S/N levels. With the reduced ion suppression of CESI run at ultra-low flow rates (~20 nL/min), it is possible to extend the dynamic range and sensitivity of HCP detection. Figures 6 illustrates the common, expected sensitivities and dynamic ranges for the peptides identified using CESI-SWATH Acquisition runs, similar to those, previously shown with LC-MS methods.[1] That is, from the candidate peptide ion surrogates for HCPs identified using SWATH Processing from CESI-MS runs, two unique myoglobin peptides could be quantified down to low ppm levels (Figure 6). However, there were examples of peptides with even greater sensitivity and dynamic range in our analysis. To specifically show the S/N ratios for one of these quantified peptides we generated extracted fragment ion electropherograms for myoglobin peptide NDIAAK shown in Figure 7. Even at 0.1 ppm peptide NDIAAK has a S/N ratio of ~20, facilitating detection of this representative HCP at the sub-ppm level. Notably, the blank sample run had an intensity of ~10 cps at the noise level, while the 0.1 ppm sample had an intensity of ~200 cps.

Conclusions

An integrated CESI-MS workflow is presented that facilitates the ultra-sensitive detection of representative host cell proteins in a mAb preparation. The combined use of CESI with SWATH Acquisition exploits reduced ion suppression and data-independent analysis to create a powerful tool for host cell protein quantitation. This new application adds to the versatility and efficiency of open tube capillary electrophoresis for biologics characterization and complements the detection of HCPs by our high- and micro-flow LC-MS methods.

References

[1] Justin Blethrow and Eric Johansen, High sensitivity host cell protein quantitation in an IgG1 monoclonal antibody preparation via data-independent acquisition with a TripleTOF® System, SCIEX Technical Note, Publication Number 7460213-01.



BIOPROCESSING

Biosimilarity and Comparability: LC- and CESI-MS/MS Tools that Provide Answers

Introduction

Biosimilars are one of the fastest growing sectors of biologics as a number of blockbuster biotherapeutic patents are set to expire. Many smaller proteins less than 40kDa have been off patent for a number of years (ex: Insulin, growth hormones). Over the next few years many monoclonal antibodies around 150kDa in size will go off patent, providing a rich source of income for pharmaceutical organizations. Pharmaceutical companies, smaller biotech organizations and CROs all need to expand their analytical capability. All of these organizations have analytical needs in common, but one predominates: analytical tools that are flexible and can be used in routine tasks day after day.

The heterogeneity of biologics means that assays developed for small molecule generics are unlikely to be appropriate. This is reflected in guidelines for Biologics that reflect the greater complexity of Biotherapeutics, as well as the requirement to provide greater levels of information. All major geographies now have guidelines for Biosimilars and all emphasize the need to apply the best technology possible in order to reduce the clinical burden of testing. Determining critical quality attributes (CQAs) is one of the early stages in development and this is an area where all relevant analytical tools are applied. Large molecule therapeutics require a number of analyses that are orthogonal: assays that may show different aspects of a test that provide more evidence of an appropriate identification or quantitation.

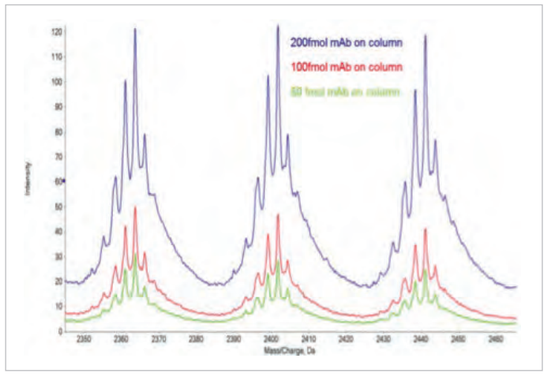


Figure 2: Intact mass spectra of a monoclonal antibody showing a zoomed-in view at around 39+ charge state. Even at the intact level, in the femtomolar range, QTOF instruments are able to distinguish low abundance species.

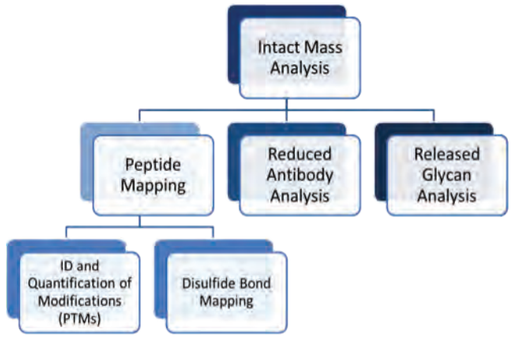


Figure 1: Diagrammatic representation of some of the routine tasks required for biosimilarity and comparability by mass spectrometry with LC or CE interfaces.

Biosimilar manufacturers find QTOF instruments particularly useful because of their ability to identify unknown analytes and quantify them, while easily coping with higher mass ranges. This article shows how a wide number of assays can be developed on QTOF instruments to determine CQAs or to determine appropriate profiles.

Intact Mass: Essential Capability Led by TOF Technology

For decades TOF technology has routinely analyzed intact proteins into the hundreds of thousands Daltons as a routine tool. Furthermore, TOF systems provide this capability while coping with small molecules equally well, allowing organizations to perform multiple tasks on the same instrument. Some of the commonly addressed issues in antibody development with intact mass include clone selection, molecular weight determination, overall glycosylation profile, assessment of light and heavy chains, and an indication of purity of the therapeutic protein (Figure 1). Biosimilar manufacturers may need to perform this task repeatedly and the ability to accurately compare runs despite great variability in concentration can prove an effective time and resource gain. Figure 2 shows how multiple intact mass analyses on a SCIEX TripleTOF® 5600 QTOF system at successive dilutions compare perfectly without any special conditions. Intact analyses benefit from deconvolution software to display the data as if there were no charge states present.

Reduced Antibody Analysis: More Features

Monoclonal antibodies are comprised of two heavy and two light chains, held together by disulfide bonds. Breaking these bonds allows analysis at a deeper level without difficult sample preparation. The ability to analyze these species routinely at high resolution on TripleTOF instruments can aid an organization in advancing method development without onerous sample preparation.

Light chain analyses can reveal terminal modifications which may not be easily apparent at the intact antibody level. The accompanying figure is an example of an anti-actin reduced monoclonal antibody with pyroglutamic acid modification (Figure 3). This mass difference is generally not visible at the intact level with any confidence, and ultra-high resolution of is of no help where individual isotopes are not identifiable with precision. Above 12kDa, dynamic range limitations mean that 12C isotopes are not generally detectable directly.

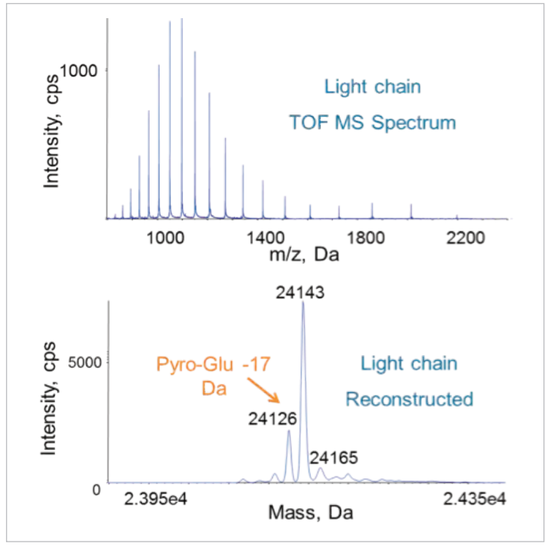


Figure 3: Light chain raw data and deconvoluted spectra showing Pyr-Glu terminal modification.

On the heavy chain, major glycosylations can be identified and assigned at the reduced level. The glycosylations are identifiable by differences in mass of various combinations of carbohydrate elements, such as illustrated in Figure 4 where differences from the average mass of the intact heavy chain are evident.

Peptide Mapping: Extending Gold Standard Assays

A default experiment for all biologics manufacturers is peptide mapping. Historically, peptide maps used HPLC with UV detection and may have taken 3 hours to perform. Applying mass spectrometry to this assay is not only sensible in terms of gains in time, but also increases information content. Using accurate

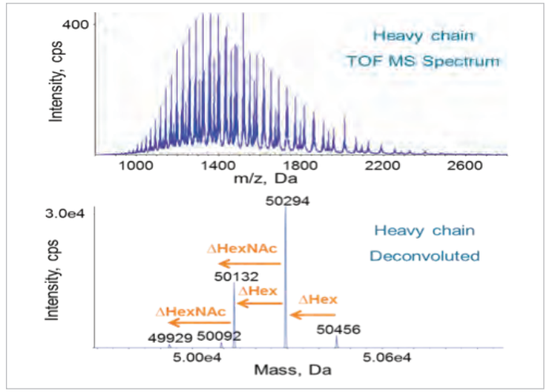


Figure 4: Heavy Chain Analysis - raw spectrum (top trace) and deconvoluted spectrum (bottom trace) showing how major glycoforms can easily be read off the spectrum.

mass to identify peptides is increasingly routine, and informatics packages such as BioPharmaView™ Software can automate the quantitation and presentation of peptide mapping data. Advances in detection systems on TripleTOF instrumentation mean that replicates show excellent reproducibility for a typical IgG (Figure 5).

TripleTOF systems can simultaneously provide MS/MS information as part of a peptide map to confirm sequence and to provide information on modifications. MS/MS information can be extremely valuable to confirm modifications that may be difficult to separate chromatographically, or close in mass but with different structures, or where the site of modification is not known. On the TripleTOF 5600+ system, a peptide map analysis applying a data independent methodology (SWATH® Acquisition), also allows a user to perform the analysis without pre-knowledge of the sample or modifications, to potentially shorten method development time, and has the ability to highlight problems far earlier in the development process; all of which conserve organizations' valuable analytical resources.

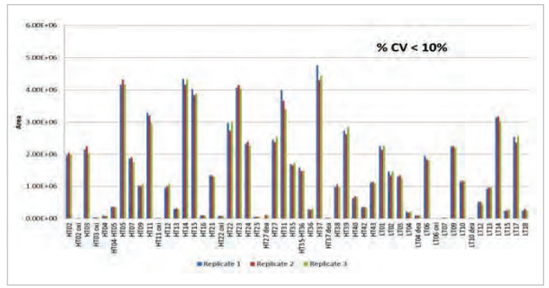


Figure 5: Quantitative mass spectrometric peptide map of an IgG showing CV of less than 10% on average for a peptide map in triplicate.

Modifications: Identifying and Quantifying Glycosylations, Deamidations and Oxidations in the Same Analysis

The ability of modern TOF technology to quantify and identify simultaneously is a key factor for success in peptide mapping with MS/MS. Historically, users had to perform multiple experiments to obtain accurate mass MS and MS/MS data, but this capability is a default feature of TripleTOF systems. SCIEX has built on previous generations of instruments by developing detector technology that can keep pace with modern demands and the fastest UHPLC speeds, irrespective of chromatographic inlet. Figure 6 shows an example of a deamidated peptide where the relative amount can be accurately measured, while the structure is confirmed. For applications such as Host Cell Proteins, or Sequence Variant Analysis, ProteinPilot™ Software can search for hundreds of biological modifications, sequence variants, and unexpected cleavages simultaneously without exponentially increasing the number of false positives.

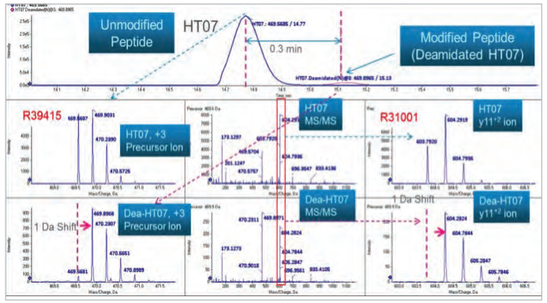


Figure 6: MS and MS/MS traces of a deamidated peptide compared to its unmodified form. The upper XIC trace shows the low abundance of the deamidated species, and the lower traces show the spectra of the relevant species.

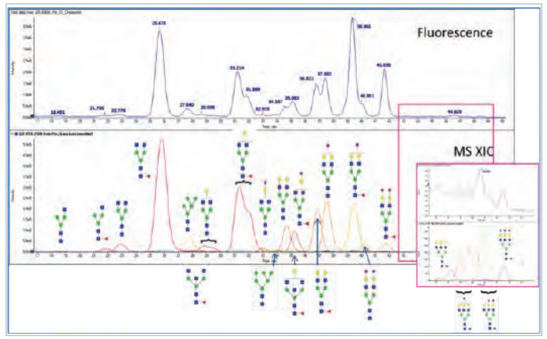


Figure 7: Stacked traces of released glycans with FL and MS chromatograms. The lower extracted ion chromatogram also has suggested structures as peak annotations. The inset picture shows how even complex species at low abundance can be confidently assigned.

Released Glycans: Routine Profiling with Accurate Mass

The profiling of all of the glycan species present in biotherapeutics can be used to aid clone selection, improve process understanding, inform cell line development, and confirm that the correct product has been made. The analysis of N-Linked glycosylations is a standard tool in the industry and quantitation is primarily performed by optical detection of chemically labeled species (e.g. 2AB for Fluorescence by LC and LCMS, and APTS for CE with LIF for example). Additional information can be obtained with MS detection and MS/MS or MS3 detection for structural information (Figure 7). Accurate mass is useful to better disambiguate species and to match the profiles of optical and MS detection, while MS3 (e.g. with QTrap® technology) can dig deeper into structure.

The ability to analyze peaks from CE systems with ultra fast separations is also one of the benefits of systems from SCIEX. Both LC and CE are used for impurity analysis as well as analyte specific studies. CE has long been used for peptide mapping and released glycan analysis, and the CESI interface has proven useful to identify unknown species by mass spectrometry in the event of queries. Figure 8 shows this capability for a glycopeptides from an IgG1.

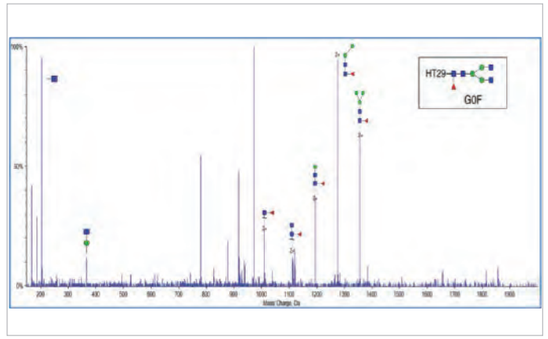


Figure 8: CESI MS/MS of G0F glycopeptide from an IgG (Image credit: Yannis Francois).

References and Further Reading

Separation and Quantitation of released glycans from IgGs on Beckmann PA800: https://www.beckmancoulter.com/wsrportal/bibliography?docname=P-14033.pdf

CESI for 100% coverage in a peptide map: Gahoual *et. al.*, Université de Strasbourg; Strasbourg, France; *mAbs 5:3, 479–490; May/June 2013*

Rapid and Efficient Comparability and Biosimilarity Studies

Introduction

Prior to reaching the market, a therapeutic product is filed with the FDA and undergoes clinical trials to prove its safety and efficacy. According to the well characterized biotherapeutic product (WCBP) model all subsequent batches of biotherapeutics in production must be comparable to the biotherapeutic product that succeeded it in clinical trials. The physicochemical testing of the product is an important aspect of this work. Comparability is enshrined in regulations for any manufacturing changes, and the FDA has indicated that comparability testing may enable a manufacturer to avoid clinical trials (Figure 1):

"Manufacturers should carefully assess manufacturing changes and evaluate the product resulting from these changes for comparability to the pre-existing product. Determinations of product comparability may be based on chemical, physical, and biological assays and, in some cases, other non-clinical data. If a sponsor can demonstrate comparability, additional clinical safety and/or efficacy trials with the new product will generally not be needed. FDA will determine if comparability data are sufficient to demonstrate that an additional clinical study(ies) is unnecessary".[2]

Therefore, the biotherapeutic industry needs fast, reliable and robust tools to assist in this task. BioPharmaView™ Software has been created directly from customer feedback with comparability in mind to help speed up the development and analysis of biotherapeutic products. For Biosimilars, the methodologies in analytical terms might be identical, in that multiple batches need to be analyzed with any differences highlighted. The number of relevant batches can be high if the developers of a biosimilar are to adequately show that they have made detailed comparisons to the innovator product. Regulators expect that the manufacturer has adequate analytical studies to demonstrate similarity. Furthermore, the analytical studies need to be rigorous, and regulators call out that detailed knowledge of the product will help prove that the manufacturing process is under control:

"Manufacturers should perform in-depth chemical, physical, and bioactivity comparisons with side-by-side analyses of an appropriate number of lots of the proposed biosimilar product and the reference product and, where available and appropriate, a comparison with the reference standard for specific suitable attributes".[3]

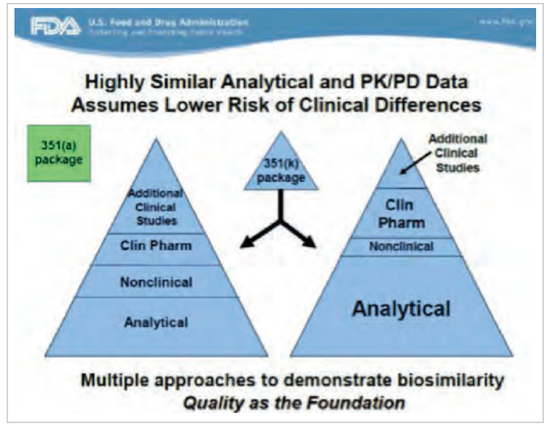


Figure 1: A 'Pyramid' representation of the potential for the reduction of Clinical Studies in favor of Increased Analytical Techniques to demonstrate Biosimilarity. BioPharmaView Software is a tool that facilitates the data processing for CESI-MS and LC-MS studies.[1]

Key Points

- Regulators recognize the need to efficiently move biosimilars through the pipeline and are willing to see a greater emphasis on analytical techniques to show that the products are similar to the original, innovator biotherapeutic.
- BioPharmaView Software has been created directly from customer feedback with comparability in mind to help speed up the development and analysis of biotherapeutic products.
- Detailed reporting directly from BioPharmaView Software can provide an unequivocal record of the attributes examined.

Discussion

Critical quality attributes (CQAs) are monitored to prove comparability through a variety of techniques. Intact molecular weight determination and peptide mapping by mass spectrometry are gold standard techniques that are used from early development to production to determine that quality attributes vary only within appropriate limits. Assigning and monitoring the CQAs can be a very onerous task because of the wide variety of types as well as the large number of attributes that are likely to be monitored in a heterogeneous product. BioPharmaView Software follows the model of comparability and automates the

Figure 2: The characterization stage in BioPharmaView Software for the analysis of a reduced monoclonal antibody. The light and heavy chains are matched automatically with a tabular display of expected major isoforms and spectral information.

assignments and display of the relevant analytes from sample to sample. In the setup stage a reference sample is analyzed so that all the relevant attributes are stored for later comparison. The user can enter variable modifications and choose from a drop down menu for those that are most common. Using the sequence information provided in the setup stage, BioPharmaView Software can automatically assign all species relevant to that protein therapeutic (Figure 2).

Intact molecular Weight

Adjustable panels in BioPharmaView Software display various elements of the analysis. In the example of a reduced monoclonal IgG1 in Figure 2, the total ion current (TIC) trace for the sample analyzed is shown above the spectral information. The bottom panels display the "raw" spectral information and the deconvoluted spectrum to return the average molecular weight of the reduced species. When subsequent batches are analyzed, they can then be compared directly to the reference ("Reference Standard") analysis. Figure 3 shows mirror plots where one batch of a monoclonal IgG1 is compared directly to a reference batch of the same molecule. Thanks to the ability to highlight multiple table entries, the differences can easily be visualized by the reviewer, or reported automatically. The report templates can be customized and the entries will automatically be generated for a given batch.

Peptide Mapping

A similar principle applies to the peptide mapping analysis. After the "Reference Standard" has been analyzed, detailed comparisons can be made of the variations in the attributes that are of interest. In Figure 4, a peptide is highlighted to indicate the information available at the touch of a button, by highlighting a line in the table (live linked). Multiple lines can be chosen, so that a peptide can be compared to its modified version, allowing the

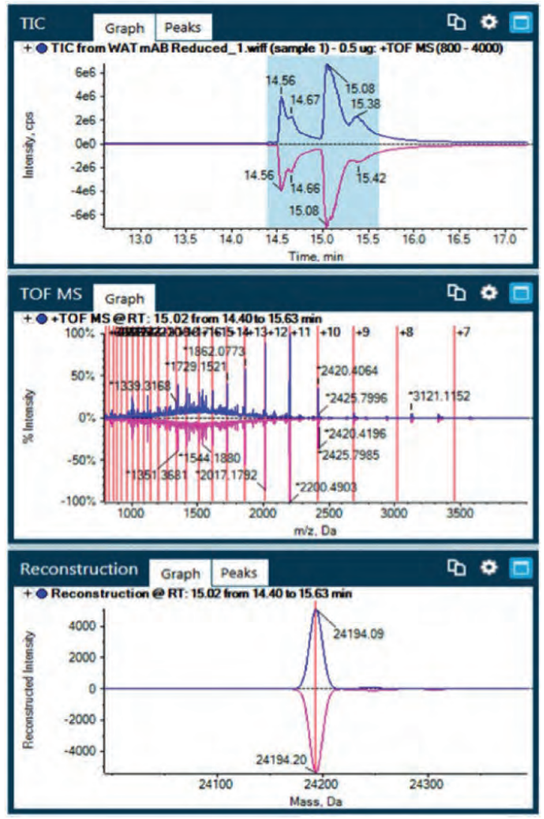


Figure 3: Mirror plots of subsequent batches of an IgG1, allow the analyst to rapidly view gross differences.

user to determine what proportion of that peptide is modified, and what fragment ions provide supporting evidence. Because both cases are automatically aligned in BioPharmaView Software view, a user can very easily determine the differences between the multiple components that are displayed.

Reports

A report (in .pdf or .doc format) can be automatically generated for this data table for any assignments made by BioPharmaView Software. In Figure 5, examples are shown of the different types of pages that can be generated for a report. Here, the intact molecular weight analysis and information for a specific peptide are shown for an IgG1.

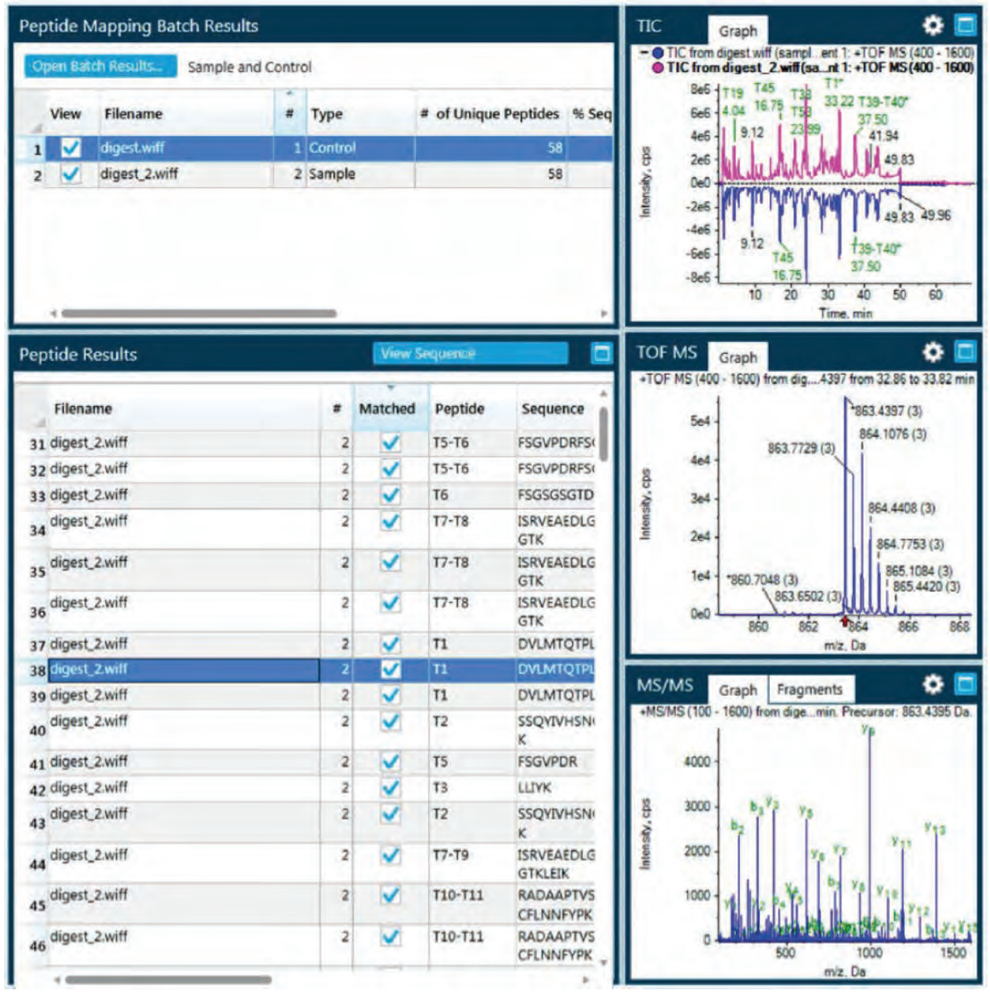


Figure 4: An example of the graphical user interface for a peptide map of an IgG1 mAb processed in BioPharmaView Software showing panels for tabular listing of the peptides, TIC, spectrum, and fragment information.

Conclusion

A productivity and efficiency enhancing tool has been created for accurate mass instruments by SCIEX in response to its customers' needs. Previously a user would have to perform analyses for each separate sample in turn, and report the information manually. In this latest generation software the processing and reporting is automated in BioPharmaView Software. Analysts can be secure in the knowledge that any differences are salient at a moment's notice, and that the rapid analysis will enable them to proceed to more scientifically valuable tasks in a shorter timeframe.

References

[1] FDA Biosimilars update (2012): *http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/AdvisoryCommitteeforPharmaceuticalScienceandClinicalPharmacology/UCM315764.pdf*

[2] FDA comparability testing: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122879.htm

[3] Quality considerations: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM291134.pdf

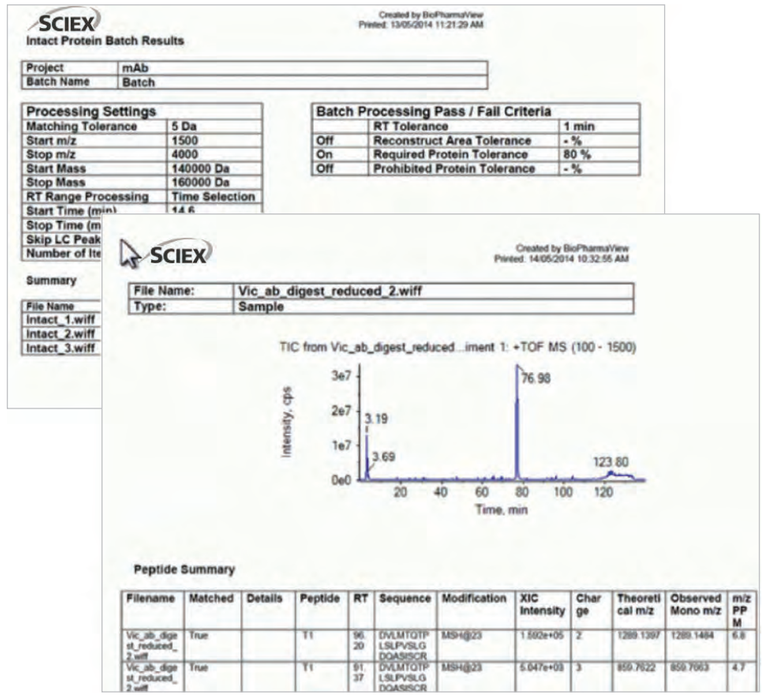


Figure 5: Examples of the types of report pages from BioPharmaView Software.

BioPharmaView™ Software for Efficient LC-MS and CESI-MS Data Processing for Biotherapeutics

Eric Johansen, St John Skilton, Justin Blethrow, Mark Lies, Rajeswari Lakshmanan, SCIEX

Introduction

Maturation of the biotherapeutics market is pressuring organizations to speed development, and to become more efficient in the production and monitoring of their products. SCIEX has responded to this need by developing tools that aid in significantly enhancing productivity. The combination of high resolution separations, unbiased MS/MS instruments and a post-processing informatics package provides the industry the ability to rapidly move molecules into production. Intact molecular weight determinations and peptide mapping for biotherapeutics in development are automated. Rigorous assignments of modifications, evidence of sequence, and direct comparability displayed to the user, are performed automatically and reported via standardized templates. SCIEX understands that orthogonal techniques like LC-MS/MS and CESI-MS/MS may be critical to thorough assessment and we therefore enable data processing for both types of acquisitions.

Basic Concepts of Accurate Mass Analysis

Accurate mass QTOF has become a gold standard technology in the biopharmaceutical industry. As its use increases, it has become the dominant mass spectrometry platform for aiding characterization of biologics' critical quality attributes (CQAs), assisting in process development analyses, and in troubleshooting. With the SCIEX TripleTOF® 6600 platform, therapeutic peptides and proteins of all sizes can be routinely analyzed. SCIEX BioPharmaView™ Software automates peak assignment thus minimizing human error, simplifying data processing and reporting, and therefore reducing the overall time to obtain results using the TripleTOF 6600 system, allowing users to increase their operational efficiency (Figure 1).

Figure 1: BioPharmaView Software user interface for a typical peptide map trace showing the TIC across the acquisition time.

Comparability and Orthogonality: Built In

Comparability is built into BioPharmaView Software by allowing the immediate and direct comparison of features within the data set to be highlighted and displayed on the same trace. Additionally, being able to analyze the data from two different techniques, CESI- and LC-MS on the same molecule, provides the user a capability and efficiency that will benefit their organization. Modern LC and high speed CE separations reduce analysis time, but the large amount of information generated from high efficiency separations requires the use of new generation high speed MS instruments. The TripleTOF 6600 system offers the necessary high acquisition rate and at the same time, providing high resolution and high mass accuracy in both MS and MS/MS modes (Figure 2). On the TripleTOF 6600 platform, with an isolating Q1 m/z range greater than 2000 m/z, no compromises on data are made, and all of the potential critical attributes of the therapeutic under development can be analyzed exhaustively.

BioPharmaView Software provides the user with an exhaustive set of details regarding their biotherapeutics, but can also simplify the information rapidly in the review stage. Figure 3 shows the panels of information available where an XIC trace is displayed above the

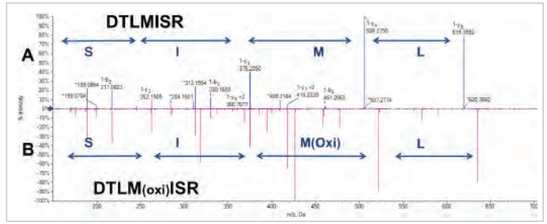


Figure 2: MS/MS identification of unmodified methionine (A) and oxidized methionine (B) at Met255 in the heavy chain of trastuzumab from CESI-MS acquisition.

Figure 3: The graphical user interface of BioPharmaView Software showing one of the peptide entries. As a user selects a table entry, the extracted ion chromatograms are automatically displayed in the upper panel, and spectrum and fragment ion information displayed in the lower panels.

tabular data. At the base are two panels depicting the spectrum (MS) and the fragment ions (MS/MS) for that peptide. The analyst only needs to glance at that data to know whether further investigation is needed.

BioPharmaView Software manages PTM characterization from LC-MS or CESI-MS analysis as a standard part of the automated analysis. The user is able to choose from a drop-down menu, or create custom modifications that are matched to peptides as part of the processing automatically.

Figure 4: All assigned peptides of a CESI-MS peptide map of a commercial IgG1 displayed in BioPharmaView Software as extracted ion chromatograms.

Conclusions

The BioPharmaView informatics post-processing package for data from SCIEX TripleTOF LC-MS systems greatly improves workflow efficiency by removing human bias, automating tedious procedures, systematically assigning variants for known therapeutic products (Figure 4). By building in comparability and orthogonality, SCIEX has responded to industry need, while providing best-in-class tools.

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