Waters Application Notes

Glycans

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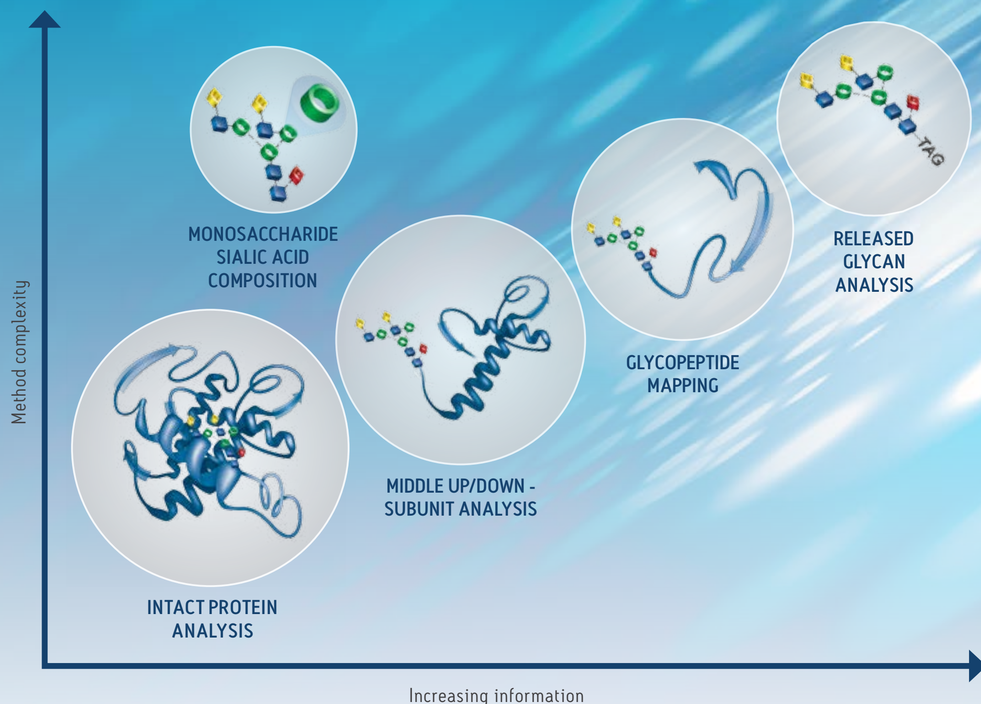
There are a variety of complementary techniques practiced to get the complete story about a glycoprotein. Each technique varies in complexity and provides a different layer of information.

This application notebook highlights a body of work that has been developed to detail applications of innovative chemistry solutions that provide complimentary techniques used to answer the what and why questions.



Our Scientists

Stephan M. Koza, Ph.D.

Stephan joined Waters Corporation in 2011. His research, development, and applications group has a primary focus on the use of UPLC, HPLC, LC-MS, and sample preparation technologies for the analysis of biomolecules. He received his B.A. in Chemistry from The Massachusetts College of Liberal Arts and his Ph.D. in Analytical Chemistry from The University of New Hampshire Prior to joining Waters he had nearly twenty years of experience with Genetics Institute, Wyeth, and Pfizer where he was primarily involved with biopharmaceutical characterization and analytical method development.

Matthew Lauber, Ph.D.

Matthew is a Principal Applications Chemist within the Consumables Business Unit at Waters Corporation. For the last 4 years, he has applied his expertise in protein chemistry and LC-MS based protein characterization methods toward the development and application of state-of-the-art reagents and separation chemistries. Starting with his graduate studies at Indiana University, Matthew has shown a penchant to pursue new methodologies for puzzling analytical challenges. Most recently, Matthew has helped lead investigations into the rapid and sensitive analyses of released N-glycans as well as the development of new strategies for characterizing protein glycosylation that are based on the use of a wide-pore HILIC column technology.

Kenneth J. Fountain III, M.S.

Kenneth received his B.S. in Biotechnology from the Worcester Polytechnic Institute and his M.S. in Chemistry from Tufts University. He began his career with Waters Corporation in 2001 as a Bioseparations Chemist. In this role, he developed LC and LC-MS methods for oligonucleotide, peptide, and intact protein analysis. In 2004, he joined the Analytical R&D group at Genzyme Corporation, where he implemented the use of UPLC Technology for the analysis of small molecule drugs and biomaterials. Ken returned to Waters Corporation in 2007, where he spent 7 years in the Consumables Business Unit managing the Chemistry Applied Technology Group and more recently, the Technology Advancement Group. Both of these groups focus on the evaluation, development, and application of chromatography columns, reagents, standards, and sample preparation devices for the pharmaceutical, biopharmaceutical, and food and environmental market areas. In January 2015, Ken accepted a position as the Director of the Biopharmaceutical Business Development Group in the worldwide marketing organization at Waters.

Erin E. Chambers, Ph.D.

As a principal scientist, Erin has been working almost exclusively on peptide and protein bioanalysis for the last seven years, while managing small and large molecule bioanalysis and clinical research applications for Waters' Consumables Business Unit. A conversation with her college dean helped put her on the path of scientific study. Ultimately, Erin graduated from Yale University with a degree in chemistry and earned her doctorate from Kings College London. Erin loves the dynamic nature of her job, helping to develop new methods and analytical systems that have a profound influence on illnesses like diabetes and Alzheimer's disease.

Scott McCall, M.S.

Scott is a Research Chemist within the Consumables Business Unit at Waters Corporation. During his 8 year career at Waters he has played a role in the development of numerous types of column chemistries. Most recently, his focus has been toward investigating biomolecule separations including peptides, proteins, and glycans. Scott has created methods for profiling released high mannose and complex N-glycan structures from monoclonal antibodies, and continues to work to answer complex questions regarding glycan analysis.

Rajiv Bharadwaj, Ph.D.

Functioning in the role of Application Specialist at Waters India Applications Laboratory, Rajiv is instrumental in the biopharmaceuticals and proteomics operations. His area of focus includes high resolution mass spectrometry based solutions, technical support, and application development. As a day-dreamer, he is influenced by fast technological turnover. Motivated by his profound experience with R&D's of Novozymes and Biocon, he aspires to create benchmarks in analytical sciences. He has been actively participating in scientific conferences/publications at organizations like Proteomics Society of India and IIT Bombay, to name a few. Trained at Indian Institute of Science (IISc), Bangalore, he also holds a university rank for post graduation in biotechnology. Being a gourmet food-enthusiast, Rajiv enjoys bringing new flavors to his dishes.

Jennifer Fournier, M.S.

Jennifer joined Waters Corporation in 2004. She has worked in many different parts of the organization. She started in Life Science Research and Development, then moved into the manufacturing group for the MassPREP line of standards, and now is a Product Manager for the same products she used to develop and manufacture. She received her B.S. in Biotechnology and M.S. in Biology from Worcester Polytechnic Institute. Prior to joining Waters she taught high school biology and also worked in manufacturing for a small pharmaceutical company that manufactures Albuterol for inhalers. In her free time, Jennifer enjoys spending time with her children and coaching.

Our Scientists *(Continued)*

Michael F. Morris M.S.

As a Senior Scientist with Waters Synthesis Group, Michael has worked on particle synthesis and chromatographic support development for the last 10 years. Mike has brought 30 years experience developing novel separations reagents for pharmaceutical, biopharma, and diagnostic markets to Waters' Consumables Business Unit. Prior to joining the synthesis group in 2006 he worked with VICAM, now a division of Waters Corp, developing diagnostic products for the food, agriculture, and dairy markets. Having grown up in Pennsylvania farm country, he was initially interested in studying Forestry but was convinced as an undergrad that Chemistry offered a better fit to his curiosity about biological systems. He went on to earn an M.S. in Biochemistry from Purdue University. Mike has always been an avid outdoors man and still spends most of his weekends in the woods and on the water.

Darryl Brousmiche, Ph.D.

Darryl is a Principal Scientist and Manager of the Synthesis Group in the Consumables Business Unit at Waters Corporation. Since joining Waters in 2003, he has been engaged in developing new sample prep and stationary phase materials for chromatographic separations, as well as novel tagging reagents. Darryl began his career studying Organic Chemistry at the University of Ottawa before completing his Ph.D. in Organic Photochemistry at the University of Victoria. He then spent two years as a postdoc at the University of California, Berkeley investigating light harvesting materials. In his free time, Darryl enjoys camping, hiking, and skiing.

Pamela Iraneta, B.S. Chemistry, B.S.E.E.

Pamela has worked for Waters Corporation for over 25 years. Her interest in all forms of chromatography started while characterizing silica sols using Sedimentation Field Flow Fraction (SF^3) – a technique based on some of the same principles used in chromatographic separations. She has experience in the following chromatographic techniques: mix-mode ion exchange, reversed-phase, HILIC, inverse-size exclusion, size exclusion chromatography (SEC), gel permeation chromatography (GPC), and supercritical fluid chromatography for both small molecule and large biomolecule separations. Throughout her career at Waters, she has made significant contributions to the Oasis family of solid phase extraction (SPE) products, as well as the Ostro protein precipitation and phospholipid removal plates, and PoraPak Rxn cartridges. For column chemistries, she has contributed to the success of ACQUITY BEH, XBridge, XSelect, UPC^2, BEH, to CORTECS, among others. Pam is a co-author on 26 peer-reviewed publications and inventor on 14 patents. The patents are diverse in their application and have included antimicrobial chromatographic systems, chromatographic materials using charged surface technology, hybrid monoliths, 96-well SPE extraction devices, dried biological matrix carriers and extraction devices, and chromatographic materials for the separation of glyco-proteins and -peptides, among others.

Sean M. McCarthy, Ph.D.

Sean joined Waters Corporation in 2008, and currently serves as a Principal Scientist and Senior Manager within the Biopharmaceutical Business Organization. During his tenure at Waters, he has led applications development teams for a variety of bioseparations application areas including protein and peptide chromatography, oligonucleotide analysis, and analytical method development/transfer. Sean received his Ph.D. in Chemistry from the University of Vermont in 2005, and prior to joining Waters completed postdoctoral training within the Department of Pathology at the same institution, where he focused on environmental oxidative stress related diseases using a variety of biochemical and mass spectrometric techniques.

Robert E. Birdsall, Ph.D.

As a Senior Scientist, Robert has an active interest in the application of instrumentation in the characterization of biopharmaceuticals. Robert has spearheaded several key initiatives since joining the Pharmaceutical Business Operations at Waters. He attributes his success, in the field of bioseparations, to his academic advisors who fueled his passion and curiosity in the sciences, the culmination of which resulted in a Doctorate in Analytical Chemistry from Purdue University. One of Robert's favorite aspects of his job is developing novel applications using cutting-edge instrumentation that can assist the biopharmaceutical industry in bringing safe and efficacious therapeutic products to market.

Ying Qing Yu, Ph.D.

Ying Qing is a Senior Science Manager in the Pharmaceutical Group at Waters Corporation. She joined Waters Corporation in 2001, shortly after she received her Ph.D. in Analytical Chemistry from Purdue University. Her group's focus is on protein biotherapeutics characterization using the UPLC/QTof MS platform. The projects she is currently working on range from glycan profiling, peptide mapping, and lately, the hydrogen/deuterium exchange mass spectrometry for higher order structural protein analysis. She has extensive experience in mass spectrometry, gas-phase ion chemistry, and LC separation techniques.

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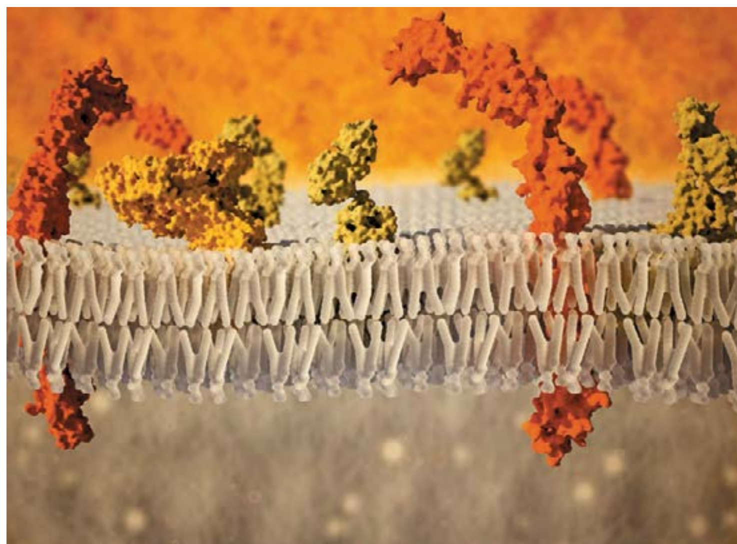
Written by Jennifer Fournier, Product Marketing Manager at Waters, and published in BioPharm International.

A Review of Glycan Analysis Requirements

Jennifer Fournier

The author explores the basic rationale and requirements for standardized glycan analysis.



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More than two-thirds of recombinant biopharmaceutical products on the market are glycoproteins, and every stage of their manufacture is carefully monitored and tested to ensure consistency in quality, safety, and effectiveness (1). Of the various aspects of biopharmaceutical production (such as yield, protein folding, and post-translational modifications), the host cell's biosynthesis of attached oligosaccharides (glycans) is often the most difficult to control. Selected expression systems and even slight changes in process conditions can alter the synthesis of glycans and as a consequence, the physicochemical properties (e.g., serum half-life), safety, efficacy, and immunogenicity of the end product. Regulatory agencies worldwide require state-of-the-art glycan analyses and the demands placed on these methods have steadily increased as better technologies have been developed. Ultimately, robust, information-rich, and reproducible methods for glycan analysis must be included in regulatory filings for glycoprotein-based biotherapeutics to ensure accuracy and consistency. Method simplification and standardization will provide additional assurance that the glycan-analysis methods used are transferrable between testing sites both within and outside (e.g., contract research organizations) of the organization, ensuring better quality and efficiency in manufacturing.

GLYCANS FACE NEW SCRUTINY

By 2008, the biotechnology company Genzyme had developed and marketed

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Table I: Common sample preparation methods in glycan analysis.

Sample preparation		Description	Application
PNGase F	Peptide-N-glycosidase F	Release of N-glycan chain except those with (∝1,3)-linked core fucose	Release of complex, hybrid, and oligomannose N-glycans
PNGase A	Peptide-N-glycosidase A	Release of N-glycan chain containing (∝1,3)-linked core fucose	Release of complex, hybrid, and oligomannose N-glycans
Proteolysis		The use of a protease to generate peptides (including glycopeptides) from a glycoprotein	The peptides are often analyzed to investigate glycosylation sites and occupancy
Alkaline beta elimination/ hydrazinolysis		Chemical cleavage of O-linked glycans from polypeptide chains	Primarily used in the analysis of O-linked glycans
Permethylation		The methylation of oligosaccharide hydroxyl groups to make glycans more amenable to mass spectrometric (MS) analysis	MS-based characterization of glycans including linkage analysis
Amine/ glycosylamine labeling		Modification of glycans to facilitate fluorescence detection	Detection of glycans and glycopeptides when native detection is not available. Increases options for chromatographic separation methods. May also enhance MS analysis

the drug Myozyme (alglucosidase alfa) for the treatment of Pompe disease, a rare and progressively debilitating disorder characterized by deficiency of lysosomal enzyme alpha-glucosidase (GAA). The company was preparing to expand the targeted treatment population from primarily children to adults. Its 160-L production facility was working at capacity, so $53 million was invested to build a 2000-L facility for Myozyme in Allston, MA (2). The company was ready to launch, but FDA rejected Genzyme's application to sell the drug from the 2000-L plant. According to regulators, the version made in the 2000-L tank was no longer the same drug as the one produced in the 160-L tank. FDA argued that the differences in glycosylation—specifically in this case, the composition of mannose-6-phosphate—meant that the drug was no longer the biological equivalent of the original material produced in the 160-L bioreactor, and may in fact introduce unknown clinical variables. Genzyme argued that it had already conducted a clinical trial on the larger batch material, demonstrating safety and effectiveness. Ultimately, Genzyme had to market the product from the larger bioreactor under a different name.

The incident was a watershed moment in the biopharmaceutical industry, marking the emergence of new challenges (1). First, regulatory authorities were beginning to scrutinize the glycan structures of biopharmaceutical products more carefully based on established technical guidelines (e.g., ICH Q5E, ICH Q6B, and FDA's *Guidance for Industry, PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance*), yet there remained inconsistencies in how FDA, the European Medicines Agency (EMA), and Japanese regulators determined what is "biosimilar". Second, products with complex glycosylation patterns have the potential to easily fall out of specification with changes in biomanufacturing processes and scale-up, so to meet the new regulatory demands, manufacturers had to start carefully characterizing product glycosylation and its relation to the biological and clinical activity of a medication, and begin monitoring relevant glycan characteristics during production (3, 4, 5).

In the years following FDA's decision on Myozyme, the attention given to glycan structure in biopharmaceuticals has only increased, reflecting improvement in analytical technology and a greater understanding of the role these structures play in the physical characteristics, stability, biological activity, and the clinical safety and effectiveness of a drug (6, 7). The technical guidelines for characterizing and monitoring glycans have changed little since 2008; manufacturers refer mainly to International Conference on Harmonization (ICH) documents Q5E and Q6B (3, 4). These documents list the following recommendations on characterizing glycans:

"For glycoproteins, the carbohydrate content (neutral sugars, amino sugars, and sialic acids) is determined. In addition, the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile), and the glycosylation site(s) of the polypeptide chain is analyzed, to the extent possible."

Other guidelines exist, setting expectations for glycan analysis, such as FDA's *Guidance for Industry, Immunogenicity Assessment for Therapeutic Protein Products*, and EMA's 2007 monograph on the characterization of

Table II: Common separation methods in glycan analysis.

Separation method		Description	Application
HPAEC	High-pH anion-exchange chromatography	Liquid chromatographic separation of negatively charged (acidic) molecules carried out at high pH	Separation, identification, and quantification of glycans and glycopeptides. Analysis of monosaccharide and/or sialic acid composition. Often coupled with pulsed amperometric detection (PAD) for detection of underivatized molecules
HPCE/CZE	High-performance capillary electrophoresis/capillary-zone electrophoresis	Separation of molecules by charge using an electric field in a narrow capillary channel	Separation, identification, and quantification of charged glycans. Analysis and quantification of sialylation
HILIC	Hydrophilic-interaction, high-performance liquid chromatography	A variation of high-performance liquid chromatography (HPLC) that separates molecules using a hydrophilic stationary phase and an organic-yet-water-miscible liquid phase	Separation, identification, and quantification of glycans and glycopeptides
WAX–HPLC	Weak-anion exchange–high-performance liquid chromatography	Separates anionic molecules based on their degree of charge	Separation, identification, and quantification of glycans and glycopeptides
RP–HPLC	Reverse-phase–high-performance liquid chromatography	Separates molecules on the basis of differences in the strength of their interaction with a hydrophobic stationary phase	Separation, identification, and quantification of glycans and glycopeptides

monoclonal antibodies (8). The monograph says the following on glycans:

"Glycan structures should be characterized, and particular attention should be paid to their degree of mannosylation, galactosylation, fucosylation, and sialylation. The distribution of the main glycan structures present (often G0, G1, and G2) should be determined."

These documents, however, present few details on how to set specification limits on glycans, or recommend technologies and procedures for consistent analytical results. The consequences for this long-standing ambiguity are that manufacturers and regulators sometimes end up with different ideas as to what constitutes a necessary specification for a glycan structure. Furthermore, companies submit reports to regulatory authorities with widely different analytical approaches. Procedures may vary even within the same organization, potentially leading to inconsistent results, analytical testing failures, and ultimately, regulatory delays.

QUALITY BY DESIGN VS. QUALITY IN PRACTICE

In 2002, in response to an increasing burden on FDA of regulating product manufacturing, and a perception among companies that regulatory requirements were limiting flexibility in process optimization, FDA implemented changes through its Pharmaceutical cGMP 21st Century Initiative and the release of FDA's process analytical technology guidance (PAT) (5). The new approach placed greater responsibility on the manufacturers to monitor quality control through timely measurements and corrections during processing.

Around the same time, ICH published two guidance documents: ICH Q8 *Pharmaceutical Development* (7), ICH Q9 *Quality Risk Management* (8), and ICH Q10 *Quality Systems Approach to Pharmaceutical cGMP Regulations* (9, 10, 11). These documents helped to further define current scientific and risk-based approaches to pharmaceutical quality control.

The concept of quality by design (QbD) was incorporated into FDA review in 2004, which together with the aforementioned guidelines, emphasized a greater understanding of the product and its manufacturing process, and designing quality control into the process, rather than testing it after the fact (12). This approach is particularly well-suited to glycan analysis, which is typically associated with a complex set of critical quality attributes (CQAs) (such as sialylation, antennary structure, or glycan structure heterogeneity) that are important to the biological or clinical activity of the drug. The CQAs must be identified, measured during process development, and maintained within required parameters (i.e., the design space) during production.

In the case of glycans, the measurement itself may introduce uncertainty and risk, due to a high variability of outcomes when characterizing oligosaccharide chains. An interlaboratory study presenting 11 industrial, regulatory, and academic labs with the same set of four released N-glycans demonstrated that results were not consistent between the laboratories when

Table III: Common detection methods in glycan analysis.

Detection method		Description	Application
PAD	Pulsed amperometric detection	Permits detection without fouling electrodes	Detection of non-derivatized glycans (most sugars do not absorb UV). Frequently linked to high-pH anion-exchange chromatography (HPAEC)
FD	Fluoresence detection	Selective fluorescent labeling and detection, which may use derivatizing agents	For the analysis of derivatized glycans and glycopeptides when native detection does not offer sufficient sensitivity. Increases options for chromatographic separation methods. May also enhance mass spectrometric (MS) analysis
ESI–MS	Electrospray ionization–mass spectrometry	Mass measurement of gas-phase ionized molecular species, where ions are generated by applying a high voltage to a liquid to create an aerosol, with little fragmentation of molecules. Can be directly integrated with liquid chromatography	Mass mapping of glycans and glycopeptides (including non-derivatized) for identification of sequence, antennary pattern, modifications, and heterogeneity, etc.
MALDI–TOF MS	Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry	Mass measurement of gas-phase ionized molecular species, where ions are generated by embedding molecules in a solid matrix, and releasing them as ions via laser ablation	Mass mapping of glycans and glycopeptides (including non-derivatized) for identification of sequence, antennary pattern, modifications, and heterogeneity, etc.

comparing analyses of sialylation and antennary structure (13). This particular study did not address the potentially added variability caused by sample preparation. The variability in outcomes may be due in part to the availability of numerous analytical approaches and differences between labs as to the selection of approach and limitations of available equipment. Inconsistent levels of training and expertise in glycan analysis may also have had an impact.

BASIC REQUIREMENTS FOR STANDARDIZED PROTOCOLS OF GLYCAN ANALYSIS

The establishment of a robust protocol for glycan analysis can help extract the maximum benefit from QbD practice; give manufacturers greater control over product quality and comparability between batches and process modifications; and ensure consistency and quality in regulatory submissions. Such a protocol should have the following features.

Well-characterized reference standards

A selection of known glycoproteins, glycopeptides, released glycans, and monosaccharides will help calibrate and validate any system of glycoprofiling used in the initial characterization of the product or monitoring of the manufacturing process.

Well-characterized sample standards

Isolated product with a known clinical safety and efficacy profile provides a reference point for comparing glycan structure of batch products under different process conditions and times.

Comprehensive identification of critical glycan attributes

Structural features of glycans have been linked to circulating half-life of the glycoprotein in the blood (sialylation); placental transport (galactosylation); antibody-dependent cell-mediated cytotoxicity (core fucosylation); and a wide range of effector functions, bioavailability, and safety characteristics (14, 15). Critical attributes may include:

- Antennary profile
- Sialylation state, including degree and linkage type (∝2-3 vs. ∝2-6)
- Site-specific glycosylation profiles and occupancy
- Fucosylation
- Galactosylation
- N-acetyl-lactosamine repeats
- High mannose residues composition
- Absence of immunogenic elements such as *N*-glycolylneuraminic acid (Neu5Gc), deacetylated *N*-acetylneuraminic acid (Neu5Ac), and Gal∝(1-3)Gal.

Variations in these CQAs introduced by manufacturing can originate from selection of cell line, bioreactor conditions such as nutrient levels, pH or oxygen content, as well as inadvertent modifications during downstream purification.

Establishment of ranges of acceptable variation in complex glycosylation patterns

Many glycoproteins, particularly those with multiple glycosylation sites, do not exist as a single species, but as a mixture of glycoforms. The natural complexity and heterogeneity of glycan structures can have important functional relevance for a protein, and even minor, low-abundance glycoform species can be crucial. For clinical purposes, each product may have a different tolerance or requirement for glycoform distribution. In particular, clarity on the extent to which low-abundance glycoforms should be identified and monitored is essential.

Adherence to best practices in sample preparation

Selecting the most appropriate method from the wide range of published and commercial sample preparation methods can be daunting. For example, purification of glycans after release from protein may be performed by solvent precipitation, solid-phase extraction, or size-exclusion, hydrophobic-interaction, or hydrophilic-interaction chromatography. Some methods may lead to a non-stoichiometric recovery of oligosaccharides, skewing the results of glycan profiling. Recent developments in sample preparation have allowed for a reduction in preparation times and improved quantitative yields of both high- and low-abundance glycoforms (16).

Selection of glycoanalysis technologies, methods, and strategy

There is a wide array of technologies that can be applied to glycan analysis (see **Tables I–III**). A series of detailed optimal workflows and best practices would help to harmonize analytical procedures between and within organizations that submit regulatory reports. Workflows would cover initial characterization through to routine monitoring and quality control. Considerations should be made with respect to the simplicity and time of analysis, as long as the required levels of accuracy and reproducibility are not compromised.

The use of orthogonal and complementary methods of analysis help compensate for systematic errors in measurement. These methods typically isolate molecules and their fragments based on different physical properties (e.g., high-performance capillary electrophoresis [HPCE] vs. hydrophilic interaction liquid chromatography [HILIC]) or analytical treatment (e.g., electrospray ionization–mass spectrometry [ESI–MS] vs. matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry [MALDI–TOF–MS]), and are compared to compensate for potential bias introduced by each analytical method.

CONCLUSION

Pharmaceutical regulatory agencies worldwide have laid out the general principles of quality control and risk management in biopharmaceutical manufacturing. Of the many CQAs that require consideration, the variation of the N-linked and O-linked glycosylation profiles of biotherapeutic glycoproteins is one of the most complex to assess. Currently, there are numerous methods used to elucidate these structures with varying degrees of accuracy and precision. In addition, the use of these somewhat disparate methodologies makes it not always possible to directly compare results between laboratories. To meet regulatory requirements for consistent process and quality control, it would be beneficial to establish more specific and standardized guidelines for glycan analysis performance with respect to reproducibility, accuracy, and sensitivity for the characterization and routine monitoring of critical glycoforms, including those of low abundance. While such guidelines are within purview of national regulatory bodies and international consensus organizations (such as ICH), no such guidelines have been released to date. The requirements for glycan analysis described in this article could address many of the issues related to process and quality control in glycoprotein manufacturing.

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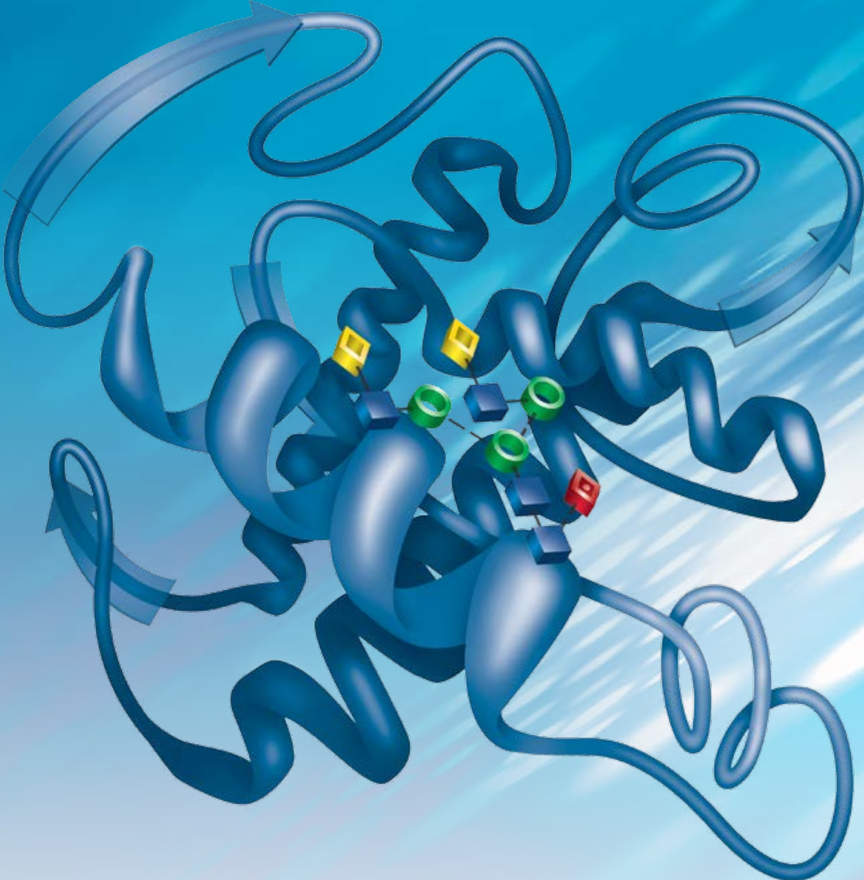
INTACT PROTEINS



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Developing High Resolution HILIC Separations of Intact Glycosylated Proteins Using a Wide-Pore Amide-Bonded Stationary Phase

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved HILIC separations of intact protein glycoforms through optimization of stationary phase (bonded phase and pore size), ion pairing, column pressurization, and injection approaches.
- MS-compatible HILIC to enable detailed investigations of sample constituents.
- Orthogonal selectivity to conventional reversed-phase (RP) separations for enhanced characterization of glycoprotein samples.
- Glycoprotein BEH Amide, 300Å, 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility.

WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide, 300Å Column

Glycoprotein Performance Test Standard

ACQUITY UPLC H-Class Bio System

Xevo® G2 QTof Mass Spectrometer

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated proteins, glycosylation, HILIC

INTRODUCTION

Hydrophilic interaction chromatography (HILIC) has been widely adopted as a tool for separating highly polar compounds. In fact, it has become a relatively widespread technique for small molecule separations. By comparison, the application of HILIC to large biomolecules has been comparatively limited even though there are instances in which the separation selectivity of HILIC would be highly valuable, for example during the characterization of protein glycosylation. A standard approach to the analysis of glycans involves their enzymatic or chemical release from their counterpart protein followed by their chromatographic separation using HILIC. UPLC®-based separations founded upon an optimized, sub-2-µm amide-bonded stationary phase has transformed HILIC separations of released glycans by facilitating faster, higher resolution separations.[1-2] Although released glycan analysis is a gold-standard approach, it has historically required lengthy and at times cumbersome sample preparation techniques. And while the recent introduction of the GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit alleviates many of these shortcomings,[3] alternative means of characterizing protein glycosylation must sometimes be investigated,[4-6] for instance when it is of interest to elucidate sites of modification.[7]

To enable the complementary analysis of glycans as they are still attached to their counterpart proteins, we present an optimized HILIC stationary phase and corresponding methods for resolving the glycoforms of intact and digested glycoproteins. A wide-pore (300Å) amide-bonded, organosilica (ethylene bridged hybrid; BEH)[8] stationary phase is employed along with rigorously developed methods to achieve unprecedented separations of the glycoforms of intact proteins ranging in mass from 10 to 150 kDa.

EXPERIMENTAL

Sample description

Glycoprotein Performance Test Standard (a formulation of bovine RNase A and RNase B, p/n 186008010) and RNase B (Sigma R7884) were reconstituted in 18.2 MΩ water to a concentration of 2 mg/mL. Trastuzumab was diluted with water from its formulated concentration of 21 mg/mL to a concentration of 2 mg/mL.

For column conditioning, the components of a vial of Glycoprotein Performance Test Standard (100 µg) were dissolved in 25 µL of 0.1% trifluoroacetic acid (TFA), 80% acetonitrile (ACN) to create a 4 mg/mL protein solution.

To investigate the resolution of glycan occupancy isoforms, Intact mAb Mass Check Standard (p/n 186006552) was deglycosylated using the following techniques. The glycoprotein (15 µg) was reconstituted to a concentration of 0.52 mg/mL into a 28.2 µL solution of 1% (w/v) *Rapi*Gest™ SF Surfactant and 50 mM HEPES (pH 7.9). This solution was heated to 90 °C over 3 minutes, allowed to cool to 50 °C, and mixed with 1.2 µL of GlycoWorks Rapid PNGase F solution. Deglycosylation was completed by incubating the samples at 50 °C for 5 minutes. To produce partial deglycosylation, Intact mAb Mass Check Standard was deglycosylated using only a 5 minute, 50 °C incubation with PNGase F without a heat-assisted pre-denaturation.

Method conditions

(unless otherwise noted)

Column conditioning

New (previously unused) ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns should be conditioned, before actual test sample analyses, via two sequential injections and separations of 40 µg Glycoprotein Performance Test Standard (10 µL injections of 4 mg/mL in 0.1% TFA, 80% ACN) or with equivalent loads of a test sample for which the column has been acquired. The separation outlined in Figure 2 can be employed for conditioning with the Glycoprotein Performance Test Standard.

LC conditions

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 5 °C

Analytical column temp.: 30 °C (unless noted otherwise in the caption)

UV detection: 214/280 nm, 2 Hz

Fluorescence detection: Ex 280/Em 320 nm, 10 Hz

Flow rate: 0.2 mL/min

Injection volume: ≤1 µL (aqueous diluents). Note: It might be necessary to avoid high organic diluents for some samples due to the propensity for proteins to precipitate under ambient conditions. A 2.1 mm I.D. column can accommodate up to a 1.2 µL aqueous injection before chromatographic performance is negatively affected.

Columns:

ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard);

ACQUITY UPLC Glycoprotein BEH Amide 300Å, 1.7 µm, 2.1 x 100 mm (p/n 176003701, with Glycoprotein Performance Test Standard);

ACQUITY UPLC BEH HILIC, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186003462)

XBridge BEH HILIC, 130Å, 5 µm, 2.1 x 150 mm (p/n 186004446)

ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742);

ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 100 mm (p/n 186004741);

Competitor columns: PolyHYDROXYETHYL A™, 300Å, 3 µm, 2.1 x 100 mm;

Glycoplex® A, 3 µm, 2.1 x 100 mm;
ZORBAX® RRHD 300-HILIC, 300Å, 1.8 µm, 2.1 x 100 mm;

Halo® PentaHILIC, 90Å, 2.7 µm, 2.1 x 100 mm;

SeQuant® ZIC-HILIC, 200Å, 3.5 µm, 2.1 x 100 mm;

Accucore™ Amide, 150Å, 2.6 µm, 2.1 x 100 mm;

TSKgel® Amide-80, 80Å, 3 µm, 2.0 x 100 mm

Column connector (for coupling 150 mm columns): 0.005 x 1.75 mm UPLC SEC Connection Tubing (p/n 186006613)

Vials: Polypropylene 12 x 32 mm , 300 µL Screw Neck Vial, (p/n 186002640)

Gradient used to demonstrate the progression of HILIC separation technologies (Figure 1):

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	20.0	80.0	6
20	80.0	20.0	6
21	20.0	80.0	6
30	20.0	80.0	6

Focused gradient for RNase B HILIC separations (Figures 2 and 5)

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	20.0	80.0	6
1	34.0	66.0	6
21	41.0	59.0	6
22	100.0	0.0	6
24	100.0	0.0	6
25	20.0	80.0	6
35	20.0	80.0	6

Gradient for benchmarking/evaluations (Figure 3)

Column dimension: 2.1 x 100 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0.0	20.0	80.0	6
0.7	30.0	70.0	6
29.3	45.0	55.0	6
30.0	80.0	20.0	6
31.3	80.0	20.0	6
32.0	20.0	80.0	6
40.0	20.0	80.0	6

Gradient employed to select a mobile phase additive (Figure 4):

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water or 50 mM ammonium formate, pH 4.4 or 0.5% (w/v) formic acid, water

Mobile phase B: ACN

Time	%A	%B	Curve
0	20.0	80.0	6
20	80.0	20.0	6
21	20.0	80.0	6
30	20.0	80.0	6

Focused gradient for reversed phase of RNase B (Figure 6):

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	95.0	5.0	6
1	74.5	25.5	6
21	67.5	32.5	6
22	10.0	90.0	6
24	10.0	90.0	6
25	95.0	5.0	6
35	95.0	5.0	6

Focused gradient for intact trastuzumab (Figures 7 and 8)

Column dimension: 2.1 x 150 mm, with varying lengths 25 µm I.D. PEEK post-column tubing

Or two coupled 2.1 x 150 mm columns

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	20.0	80.0	6
1	30.0	70.0	6
21	37.0	63.0	6
22	70.0	30.0	6
24	70.0	30.0	6
25	20.0	80.0	6
45	20.0	80.0	6

Conditions for resolving glycan occupancy isoforms of an IgG (Figure 9):

Column dimension: Two coupled 2.1 x 150 mm or a single 2.1 x 150 mm

Column temp.: 80 °C

Mobile phase A: 0.1% TFA, 0.3% HFIP in water

Mobile phase B: 0.1% TFA, 0.3% HFIP in ACN

Time	%A	%B	Curve
0.0	20	80	6
10.0	50	50	6
11.0	100	0	6
14.0	100	0	6
15.0	20	80	6
25.0	20	80	6

MS conditions

MS system: Xevo G2 QTof

Ionization mode: ESI+

Analyzer mode: Resolution (~20 K)

Capillary voltage: 3.0 kV

Cone voltage: 45 V

Source temp.: 150 °C

Desolvation temp.: 350 °C

Desolvation gas flow: 800 L/Hr

Calibration: NaI, 2 µg/µL from 100–2000 *m/z*

Acquisition: 500–4000 *m/z*, 0.5 sec scan rate

Data management: MassLynx® Software (v4.1)

RESULTS AND DISCUSSION

Progression of HILIC technology for glycoprotein separations

HILIC originated in the early 1990s as a separation technique to resolve highly polar molecules using mobile phases adapted from reversed phase chromatography.[9] The HILIC separation mechanism is largely believed to be dependent on a polar stationary phase that adopts an immobilized water layer.[9] Hydrophilic analytes partition into this immobilized water layer and undergo interaction with the phase via a combination of hydrogen bonding, dipole-dipole, and ionic interactions. In this way, hydrophilic analytes will be retained on the HILIC phase under apolar initial mobile phase conditions and later eluted by increasing polar mobile phase concentration via use of an LC gradient.[9]

Numerous HILIC or HILIC-like stationary phases have been developed in the last two decades. Many based solely on unbonded silica particles are widely available, so too are HILIC phases based on polyalcohol bondings or charge bearing surfaces, such as those with zwitterionic bondings. For the enhanced retention and selectivity of glycans, amide bonded phases have become increasingly popular. The ACQUITY UPLC Glycan BEH Amide stationary phase found in Waters Glycan Column has, for instance, found wide-spread use for high resolution released glycan separations.

As mentioned before, HILIC has, however, not seen wide-spread use in intact large molecule applications. Concerns that high organic solvent concentrations can result in protein precipitation have most likely discouraged many from attempting to develop HILIC-based, protein separation methods. Endeavoring beyond these perceptions, we have developed a new amide-bonded stationary phase based on a wide-pore, organosilica (ethylene bridged hybrid; BEH) particle that was specifically designed to facilitate large molecule separations. It exhibits a porous network accessible to most proteins and an average pore diameter that does not impart significant peak broadening due to restricted diffusion, which can occur when protein analytes are too close in size to the average pore diameter of a stationary phase (e.g. within a factor of 3).

The progression of HILIC technology culminating in this new stationary phase is remarkable. The emerging technology of large molecule HILIC can be captured by separations of bovine ribonuclease B (RNase B), a 13 kDa protein comprised of several high mannose (Man5 to Man9) glycoforms. Figure 1 shows RNase B separated by several different stationary phases. From bottom to top, increasingly better separations of RNase B were achieved as increasingly newer chromatographic technologies were adopted, from 5 µm to 1.7 µm particles, from unbonded to amide bonded particles, and from standard pore diameter (130Å) to wide-pore diameter (300Å) particles. It is with BEH Amide, 300Å, 1.7 µm particles that RNase B glycoforms are best separated. The use of a wide-pore stationary phase plays a significant role in achieving optimal resolution. This is highlighted in Figure 2 wherein benchmarking results are presented from the use of a newly developed test mixture, called Glycoprotein Performance Test Standard, which contains bovine RNase B, its corresponding glycoforms and its aglycosylated isoform (RNase A). Example separations are provided for this standard wherein a focused gradient has been used with the wide-pore (300Å) BEH Amide as well as the standard pore size (130Å) BEH Amide stationary phase. Notice that the widepore amide column affords a measurable (24%) increase in the resolution between the aglycosylated RNase A isoform and the Man5 glycoform of RNase B, in addition to sizeable increases in resolution throughout the separation.

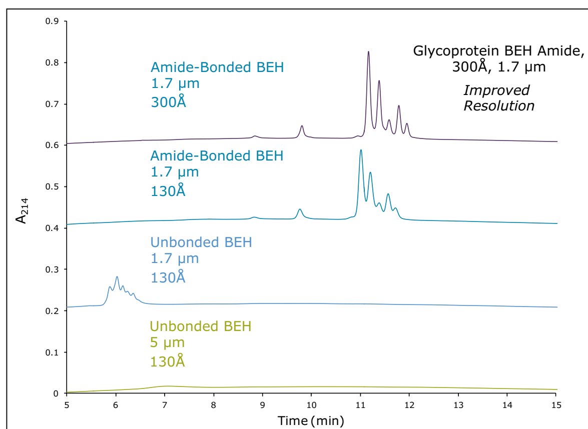
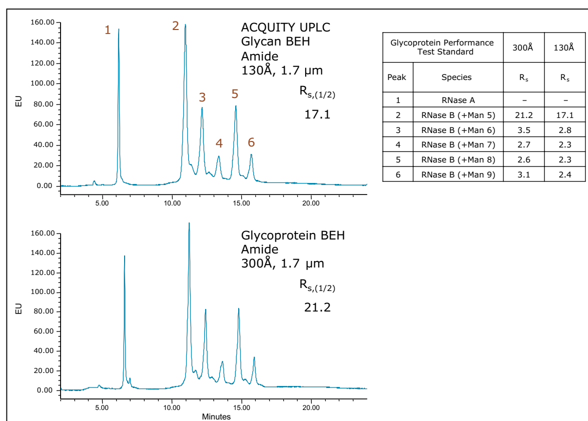


Figure 1. Progression of HILIC stationary-phase technologies for intact glycoprotein separations. Separation of 1 µg of RNase B using 2.1 x 150 mm columns packed with stationary phases ranging from HPLC-size unbonded organosilica (XBridge BEH HILIC, 130Å, 5 µm) to sub-2-µm amide-bonded organosilica 300Å, 1.7 µm particles (ACQUITY UPLC Glycoprotein BEH Amide 300Å, 1.7 µm).



Glycoprotein Performance Test Standard		300Å	130Å
Peak	Species	R_s	R_s
1	RNase A	–	–
2	RNase B (+Man 5)	21.2	17.1
3	RNase B (+Man 6)	3.5	2.8
4	RNase B (+Man 7)	2.7	2.3
5	RNase B (+Man 8)	2.6	2.3
6	RNase B (+Man 9)	3.1	2.4

Figure 2. Separations of the Glycoprotein Performance Test Standard (RNase A + RNase B glycoforms) using a Glycoprotein BEH Amide 300Å, 1.7 µm Column versus a BEH Amide, 130Å, 1.7 µm Column. The reported resolution values were calculated using the half-height peak widths of species 1 and 2 (RNase A and RNase B Man5 glycoforms, respectively). Fluorescence detection at Ex 280 nm and Em 320 nm and a column temperature of 45 °C were employed in this example.

The significance of these recent developments becomes more apparent when benchmarked against other commercially available HILIC phases. RNase B separations resulting from an evaluation of 10 different HILIC stationary phases are shown in Figure 3. It can be seen that 6 out of the 10 evaluated materials showed undesirable characteristics, including poor recovery and poor retention. It was only with the amide bonded stationary phases and particle technologies based on 100Å or greater pore diameters that reasonable separations of RNase B glycoforms could be achieved.

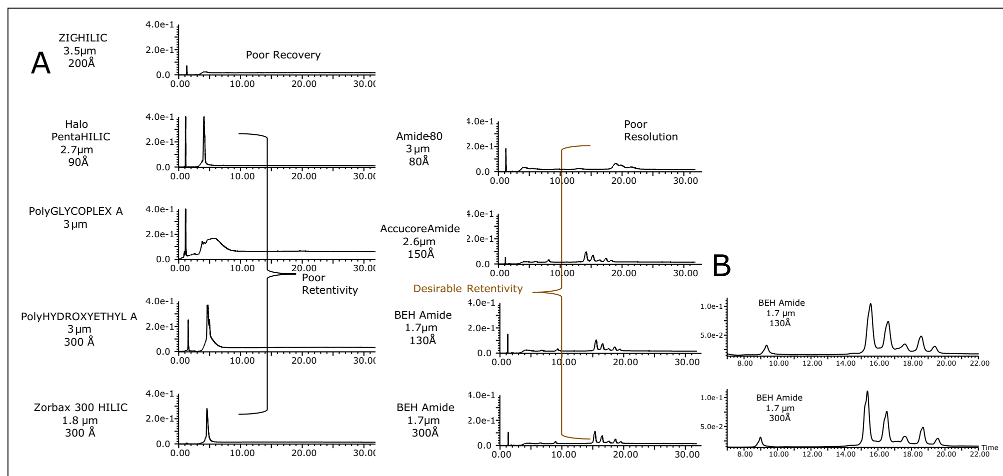


Figure 3. Evaluation of commercially available HILIC columns for intact glycoprotein separations. (A) UV chromatograms obtained for RNase B using 10 different stationary phases. (B) Zoomed HILIC UV chromatograms for the highest resolution separations.

Mobile phase optimization, MS compatibility, and orthogonality to reversed phase

High resolution HILIC separations of protein glycoforms require that mobile phase selection be given significant consideration. Most HILIC separations have been developed so as to rely on ammonium salts (formate or acetate) to mitigate significant ionic interactions and to control mobile phase pH. The suitability of such mobile phase systems to glycoproteins was evaluated using RNase B.

Figure 4 shows the corresponding RNase B chromatogram obtained when 0.1% TFA is used as the mobile phase modifier instead of 50 mM ammonium formate or 0.5% formic acid, two mobile phase compositions more frequently used for HILIC separations.[2,7] It is with 0.1% TFA that glycoforms are best resolved. Along with enhancing glycoform resolution, the TFA-modified mobile phase reduced the retention of RNase B. Together these observations highlight the significance of acidic, ion pairing mobile phases to being able to achieve high resolution glycoprotein separations using HILIC. It is proposed that the acidic condition imparted by the TFA ensures that acidic residues of the protein are fully protonated and thus present in their more hydrophobic state. In addition, the ion pairing of the TFA counter ion to basic residues, ensures that cationic residues will also be separated in a more hydrophobic form. In this way, retention of a glycoprotein onto a HILIC phase is driven primarily by the glycans and a separation more selective to resolving differences in the glycan modification is achieved.

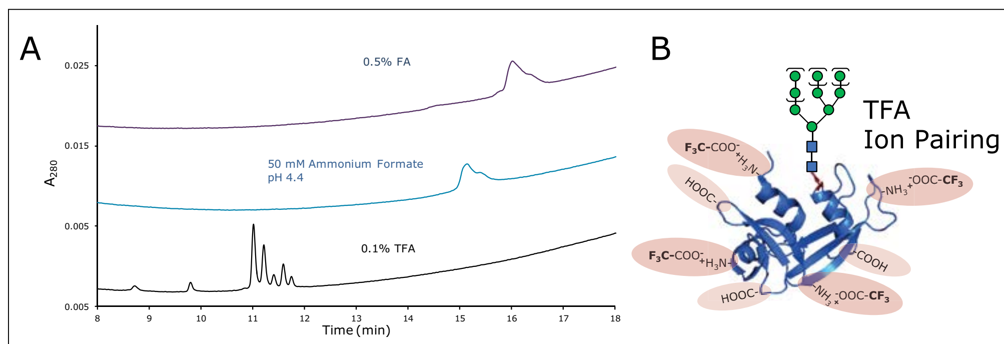
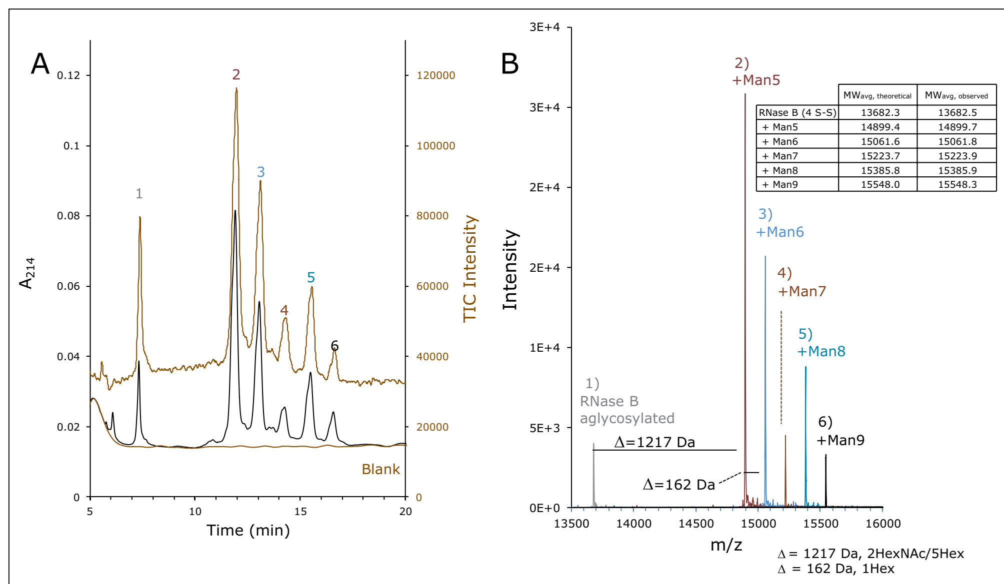


Figure 4. Optimization of mobile phase conditions for separations of intact and digested glycoproteins. (A) UV chromatograms obtained for RNase B when using various mobile phases and a Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Schematic portraying the utility of ion pairing for glycoprotein HILIC separations. Reduced hydrophilicity imparted via ion pairing with a hydrophobic, strong acid is displayed with shading. [PDB:1RBB]

Fortunately, TFA-modified mobile phases can be readily coupled to ESI-MS, due to their volatility. This aspect of the developed HILIC methods enables on-line characterization of the resolved glycoforms and presents a new option for profiling a sample containing glycosylated protein. To this end, the peaks resolved from RNase B using a BEH Amide, 300Å, 1.7 µm column were subjected to interrogation by ESI-MS.

Figure 5 shows both a UV chromatogram and a corresponding total ion chromatogram (TIC) obtained when separating RNase B. By summing and deconvoluting (MaxEnt™ 1) the mass spectra obtained for the six labeled peaks, it was confirmed that RNase B glycoforms were being detected. In fact, the observed deconvoluted masses support identifications of aglycosylated RNase B (RNase A) along with RNase B modified by Man5 through Man9.



	$MW_{avg, theoretical}$	$MW_{avg, observed}$
RNase B (4 S-S)	13682.3	13682.5
+ Man5	14899.4	14899.7
+ Man6	15061.6	15061.8
+ Man7	15223.7	15223.9
+ Man8	15385.8	15385.9
+ Man9	15548.0	15548.3

Figure 5. HILIC-MS of RNase B. (A) UV (bottom) and TIC (top) chromatograms obtained for RNase B when using a focused gradient and a Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Deconvoluted mass spectra obtained for each labeled peak along with corresponding glycoform identifications.

Finally, it should be pointed out that the newly developed stationary phase and the demonstrated methodologies provide new separation selectivity, one that is orthogonal and complementary to conventional reversed phase separations. Figure 6A shows that RNase B can, for instance, be separated by reversed-phase chromatography using a BEH C4, 300Å, 1.7 µm column so as to produce a high resolution separation of aglyocosylated RNAse B (RNase B) from its glycosylated isoforms. By reversed phase, however, none of high mannose glycoforms of RNase B can be resolved from one another. In contrast, a BEH Amide, 300Å, 1.7 µm column yields baseline resolution of each major glycoform (Figure 6B).

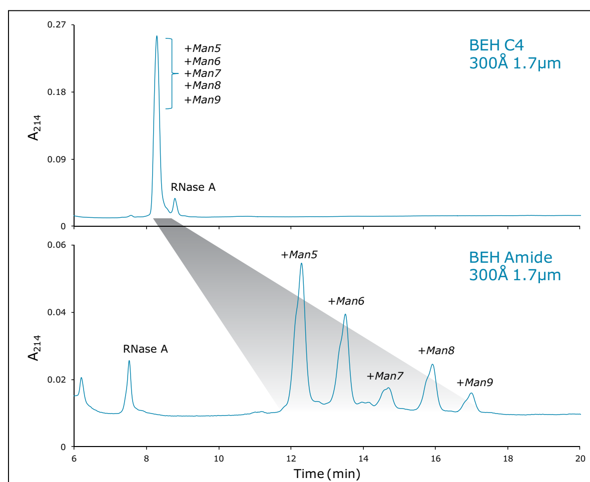


Figure 6. Orthogonality of reversed phase with BEH C4, 300Å, 1.7 µm and HILIC with BEH Amide, 300Å, 1.7 µm Columns. (A) Separation of RNase B (1 µg) using an ACQUITY UPLC Protein BEH C4, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Separation of RNase B (1 µg) using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column.

Separation of the heterogeneous glycoforms of an intact mAb

To explore the limits of this new technology, we have investigated the capabilities of resolving the glycoforms of intact mAbs. Specifically, separations of trastuzumab have been explored. These experiments required special considerations regarding sample injection, primarily because trastuzumab and numerous other glycoproteins are not readily soluble in high organic concentrations. In fact, 70–80% ACN is generally a solution condition that initiates the precipitation of proteins, such as an IgG. Accordingly, conditions for the optimal injection of aqueous diluents were developed. It has been found that a 2.1 mm I.D. column can accommodate an injection of aqueous sample up to 1 µL. From a 2 mg/mL aqueous sample of trastuzumab, appropriate sample mass loads could thus be injected and HILIC separations of the IgG could be performed. It should be mentioned that high ACN diluents can be used in intact protein HILIC, but care must be taken to enhance the solubility of the protein sample through either the use of TFA ion pairing at concentrations between 0.2–1.0%, the combined application of TFA and hexafluoroisopropanol (HFIP), or by use of co-solvents, such as dimethylsulfoxide (DMSO) (data not shown).

As shown in Figure 7, trastuzumab can indeed be separated into multiple chromatographic peaks using a BEH Amide, 300Å, 1.7 µm column and an injection from a simple 100% aqueous diluent. However, at the backpressures produced from just a 150 mm length column, a noticeably tailing profile was observed. MS analysis indicated that the first set of peaks could be accurately assigned as the G0F/G0F, G0F/G1F, G1F/G1F, and G1F/G2F glycoforms of intact trastuzumab. An intact IgG is a dimeric structure, with a minimum of two N-glycan sites on two heavy chains, explaining the observation of combinatorially formed glycoforms. This is consistent with observations by intact mass analysis of IgGs.[10] The tailing component of the chromatographic profile was in contrast found by MS to correspond to multiple, co-eluting trastuzumab glycoforms. With this result, we proposed that on-column aggregation was occurring and that increased column pressure could be a solution to HILIC of intact immunoglobulins, specifically since it had previously been reported that ultrahigh pressures can be beneficial to limiting carryover and ghosting during reversed phase of intact proteins.[11] The effects of introducing additional column pressure was investigated by means of introducing varying lengths of narrow I.D., post-column PEEK tubing. Figure 7 (darker traces) displays the effects of introducing increasingly higher column pressure. By doubling the column pressure so that trastuzumab would elute under conditions of approximately 7,500 psi, the putative, aggregate peaks in the chromatographic profile were eliminated. It is encouraging that under these conditions the resulting chromatographic profile is represented by 5 major glycoforms, which again is consistent with ESI-MS of intact trastuzumab.[10] It is interesting to additionally note that retention decreases as column pressure increases. This is a phenomenon that has been described previously for HILIC separations of monosaccharides.[12] It has been proposed that increasingly higher pressures result in less coordination of water to the analyte and in turn reduced retention, an opposite effect to that observed during reversed phase chromatography.[12]

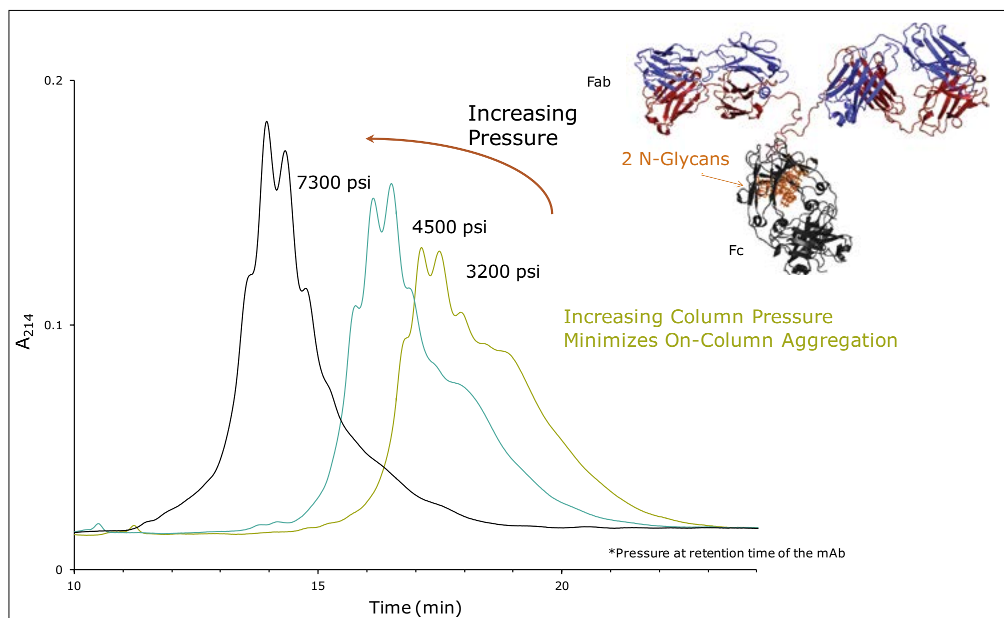


Figure 7. Effect of column pressure on the HILIC separation of an IgG. Trastuzumab (1 µg) was separated on Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column with and without flow restriction. [PDB:1IGT]

Given that intact IgGs benefit from separations at ultrahigh pressures, we pursued separations based on the use of two BEH amide 300Å, 1.7 µm, 2.1 x 150 mm columns coupled with a low volume, high pressure column connector. The separation for intact trastuzumab obtained with these coupled columns is displayed in Figure 8, along with extracted ion chromatograms that provide evidence to achieving separations of the glycoforms. This 300 mm configuration provided the requisite column pressures for an optimal HILIC separation and additionally produced greater resolution between glycoforms. Clearly, additional theoretical plates are therefore advantageous during HILIC of even very high molecular weight species, which supports the significance of partitioning for such separations.

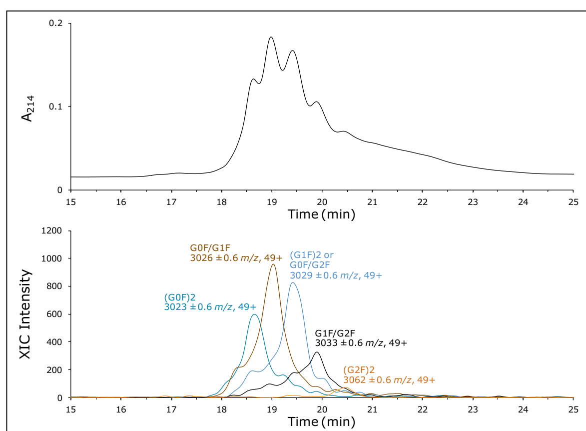


Figure 8. Separation of intact trastuzumab glycoforms using coupled ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Columns. A UV chromatogram and extracted ion chromatograms for each of the major heterogenous glycoforms of trastuzumab are displayed. The column pressure at the retention time of the mAb was approximately 7,000 psi.

An LC method for glycan occupancy

A UPLC HILIC separation of an intact IgG can be used for more than just an attempt to separate individual glycoforms. Equally interesting is the use of these new separation capabilities to resolve information about glycan occupancy. To this end, we evaluated the capabilities of the BEH Amide, 300Å column to assess the glycan occupancy of an IgG. This was exemplified by a study of reaction products resulting from various PNGase F deglycosylation treatments. Using an elevated 80 °C column temperature, TFA ion pairing, and an HFIP mobile phase additive, we have been successful in enhancing the solubility of IgGs and collapsing the fine structure otherwise captured for the individual, heterogenous intact IgG glycoforms (i.e. G0F/G0F versus G0F/G1F). Figure 9 presents HILIC fluorescence chromatograms resulting from such a separation of native Intact mAb Mass Check Standard (a murine IgG1 mAb) and its partially as well as completely deglycosylated isoforms. As can be seen, HILIC fluorescence profiles for these three samples are dramatically different. On-line mass spectrometric detection has confirmed that the peaks in these profiles correspond to different states of glycan occupancy.

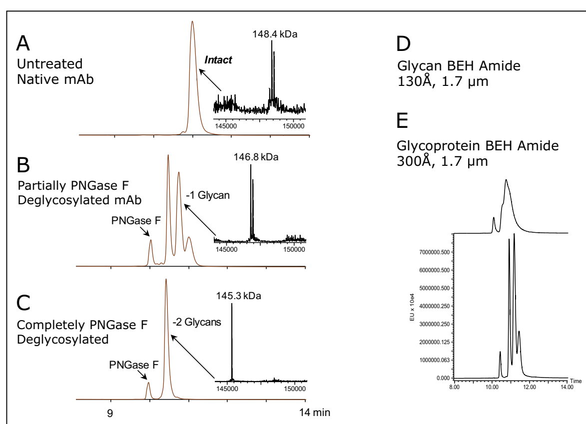


Figure 9. Assaying glycan occupancy and deglycosylation by intact protein HILIC-FLR-MS. HILIC fluorescence profiles obtained for three different samples are shown: (A) native, (B) partially deglycosylated, and (C) completely deglycosylated Intact mAb Mass Check Standard. Samples of this mAb (1.5 µg) were separated using two coupled Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Columns. HILIC fluorescence profiles of partially deglycosylated Intact mAb Mass Check Standard using a (D) ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column versus a (E) Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column.

The most strongly retained species, represented by the native mAb sample, corresponds to the doubly (fully) glycosylated form of the intact mAb. The partially deglycosylated mAb sample meanwhile yielded several additional peaks with lower HILIC retention, two of which with corresponding detected molecular weights that are indicative of once deglycosylated and fully deglycosylated mAb species and a third with a corresponding detected molecular weight consistent with PNGase F. In contrast, the completely deglycosylated mAb sample presented a homogenous fluorescence profile along with an observed molecular weight for the mAb that is in agreement with the predicted molecular weight of the deglycosylated mAb (145.3 kDa). It is worth noting that when attempting to use the BEH Amide, 130Å, 1.7 µm stationary phase, none of the above peaks could be resolved (Figures 9D and 9E). So indeed, the widepore phase facilitates the development of previously unobtainable separations.

In our hands, the above assay has been used to develop rapid enzymatic deglycosylation protocols.[3] However, it is natural to suggest that these same methods could be applied to measure the glycan occupancy of an intact therapeutic mAb, in which case the relative abundance of aglycosylated forms (-2 and -1 N-glycans) could potentially be monitored by fluorescence and corroborated by LC-MS.

CONCLUSIONS

HILIC of small molecules has garnered wide-spread attention and use. In contrast, the application of the technique to large biomolecule separations has been limited. With the development of the above mentioned amide-bonded, wide-pore HILIC stationary phase and corresponding methods, it is now possible to resolve the glycoforms of intact glycosylated proteins, as has been exemplified by the resolution of the heterogenous glycoforms on intact trastuzumab. Alternatively, the described techniques can be applied to studies of glycan occupancy. Just as reversed phase separations are employed for resolving protein isoforms that have varying hydrophobicities, HILIC separations with BEH Amide 300Å can be explored for resolving protein isoforms that exhibit varying hydrophilicities, such as isoforms differing with respect to glycan occupancy. With the availability of these new separation capabilities, it will be possible to perform more detailed characterization of intact glycoproteins, whether by means of combining HILIC with optical detection or with ESI-MS.

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Measuring the Glycan Occupancy of Intact mAbs using HILIC and Detection by Intrinsic Fluorescence

Authors: Matthew A. Lauber and Stephan M. Koza

GOAL

To demonstrate the use of HILIC with an ACQUITY UPLC® Glycoprotein BEH Amide 300Å Column to assay the glycan occupancy of intact mAbs.

BACKGROUND

Monoclonal antibodies (mAbs) have emerged as some of the most important therapeutics on the market. These mAbs that are prescribed for therapeutic use are most often expressed from eukaryotic cell lines, such as CHO, and, as a result, are N-glycosylated at two consensus site asparagine residues in the Fc portion of their heavy chains. Since glycosylation can be a measure of efficacy, safety and manufacturing conditions, it is often critical to characterize and routinely monitor the N-glycan profile of a mAb drug substance.[1-2] The recent introduction of the GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit has made it significantly easier to perform highly detailed HILIC-based, released N-glycan analyses and to thereby elucidate the heterogeneity of *N*-glycosylation.[3-4] However, it is also critical to determine the extent to which the asparagine linkage sites are occupied with N-glycans, particularly since incomplete glycosylation dramatically changes the effector functions of a mAb.[5] Traditionally, this assessment has been performed by a sized-based Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS) separation of the heavy chains resulting from reduction of a mAb.[6,7] To instead directly assess glycan

Unprecedented Hydrophilic Interaction Chromatography (HILIC) separations of intact mAb glycan occupancy variants using the ACQUITY UPLC Glycoprotein BEH Amide 300A 1.7µm Column.

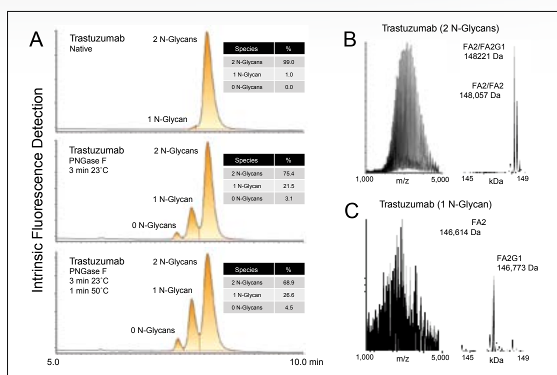

Figure 1. HILIC analysis of intact trastuzumab. (A) HILIC fluorescence chromatograms for native and partially deglycosylated trastuzumab. (B) Raw and deconvoluted ESI mass spectra for the major species resolved in the native trastuzumab sample. (C) Raw and deconvoluted ESI mass spectra for the low abundance species resolved in the native trastuzumab sample. HILIC was performed on 1 µL aqueous injections of 5 mg/mL trastuzumab using an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column heated to 80˚C. Sample was separated at a flow rate of 0.4 mL/min with aqueous (A) and acetonitrile (B) mobile phases containing 0.1% (v/v) TFA and 0.3% (v/v) HFIP. A linear gradient was applied as follows: hold at 20% A for 0.5 min, 20 to 25% in 0.5 min, 25% to 40% A in 9 min, 40% to 100% A in 0.5 min, hold at 100% A for 0.5 min, 100% to 20% A in 0.5 min, and hold at 20% A for 3.5 min for re-equilibration. Eluting proteins were detected by intrinsic fluorescence (Ex 280 nm/Em 320 nm).

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occupancy variants for intact mAbs, we have developed an LC separation based on HILIC. To achieve these unprecedented separations, a new, purposefully designed HILIC column was employed. This new column, the ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column, contains a wide-pore amide bonded stationary phase that ensures that mAb species have access to the porous network of the stationary phase and are not significantly impaired by restricted diffusion.[8]

THE SOLUTION

A high-throughput, high resolution HILIC separation was established for intact mAbs using a 2.1 x 150 mm wide-pore BEH amide column, a 0.4 mL/min flow rate, and a column temperature of 80 °C. In addition, two mobile phase additives, 0.1% trifluoroacetic acid (TFA) and 0.3% hexafluoroisopropanol (HFIP) were employed to improve the solubility of intact mAbs in the high organic, initial mobile phase conditions of the HILIC gradient. To enhance the sensitivity of this LC method, the intact proteins were detected by means of their intrinsic fluorescence. Excitation and emission wavelengths of 280 and 320 nm were found to provide optimal signal-to-noise and consistently flat chromatographic baselines that are desirable for peak integration. A representative set of chromatograms resulting from this 15 minute LC method is shown in Figure 1A. Three chromatograms are displayed. The top chromatogram shows trastuzumab as injected from a dilution of its formulation, while the other two chromatograms show samples of trastuzumab after being subjected to partial PNGase F deglycosylation. Deglycosylated samples of trastuzumab clearly showed three distinct peaks in their HILIC profiles, as was predicted if the glycan occupancy variants of a mAb were to be resolved. The unadulterated sample of trastuzumab contained measurable levels of only the fully occupied and singly occupied forms (1%) as confirmed by online mass analysis. The deconvoluted mass spectrum corresponding to the main LC peak exhibited several masses, such as 148,057 Da and 148,221 Da,

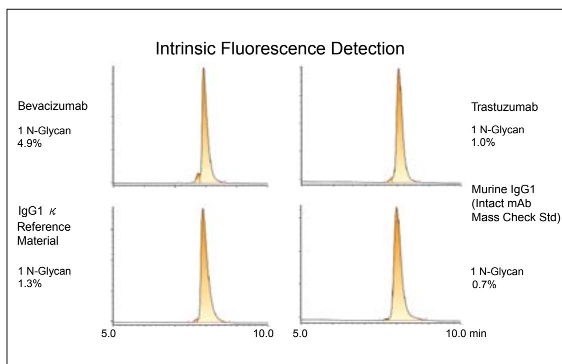

Figure 2. HILIC analysis of four intact mAbs. Bevacizumab, NIST IgG1κ candidate reference material, and trastuzumab were injected without any sample preparation except for dilution to 5 mg/mL from their respective formulations. Prior to HILIC analysis, Intact mAb Mass Check Standard (1 mg) was reconstituted in 500 µL of 6M guanidine HCl, filtered via 3 passes with a 100KDa MWCO polyethersulfone membrane (GE Healthcare Life Sciences, Vivaspin 500), and concentrated to 5 mg/mL.

that are within 2 Da of the theoretical molecular weights for the predominant glycoforms of trastuzumab (Figure 1B).[9] In contrast, the deconvoluted mass spectrum for the smaller, less strongly retained peak showed reduced heterogeneity and masses that were lighter by approximately 1000 to 2000 Da, consistent with the single occupancy form (Figure 2B). Fully aglycosylated species were not detected in this sample. An interesting observation from these data is that the levels of fully deglycosylated forms are lower than would be predicted if both sites were deglycosylated at the same rate. In these examples, the observed level of fully deglycosylated forms is approximately one-third lower than would be predicted. This may indicate that either the digestion rate for one of the N-glycans is slower than the other, or that upon removal of the first N-glycan, the digestion rate of the remaining N-glycan is reduced.

To assess the applicability of this technique to other mAbs, we analyzed three additional IgG samples. Results on trastuzumab, bevacizumab, a candidate IgG1κ reference material, and a murine IgG1 (Intact mAb Mass Check Standard, p/n 186006552) are shown in Figure 2. Integrations on the HILIC-fluorescence chromatograms indicated that these samples contain 1 N-glycan (incomplete glycosylation) variants at relative abundances ranging from 0.7 to 4.9%. These observations suggest that this HILIC method could be an attractive technique for assaying incomplete glycosylation of mAbs down to levels well below 1% for the 1 N-glycan form. Detection limits for the 0 N-glycan form may perhaps be even lower. Of these samples, bevacizumab might be predicted to have the highest abundance of the 0 N-glycan form. Indeed, the bevacizumab profile presented a peak, albeit very minor, with a retention time consistent with a 0 N-glycan species, the area of which would contribute to the overall profile

at a level of only 0.05%. Such an observation suggests that the 0 N-glycan level is lower than would be statistically predicted; however, rigorous determination of the quantitative limits of this analysis and the identity of this putative 0 N-glycan peak would need to be evaluated to confirm that this is indeed the case.

This strategy for measuring mAb glycan occupancy is most appealing in that it requires minimal, if any, sample preparation. We have observed that some interferences can be encountered that are due to partially fragmented and/or reduced mAb species. In which case, as with the Intact mAb Mass Check Standard, a simple, centrifugal filtration clean-up step with a 100kDa MWCO polyethersulfone membrane was sufficient to minimize such interferences. Given the unique selectivity of the HILIC separation, it should also be possible to address potential interferences by performing offline or online 2D-LC, wherein a size exclusion or reversed phase separation could be coupled to the wide-pore amide HILIC separation. Future investigations could also include reducing glycan heterogeneity (via a sialidase or other exoglycosidase) to allow the mAb glycan occupancy variants to be more discretely resolved. Similarly, there is an opportunity to use this separation in combination with enzymes that generate Fc subunits.

SUMMARY

In addition to profiling the heterogeneity of glycosylation, it is also critical to assay glycan occupancy. Here, we demonstrate that a Glycoprotein BEH Amide column, purposefully designed for large molecule HILIC separations, can be used to directly quantify incomplete N-glycan occupancy in intact mAb samples. Unlike a conventional CE-SDS separation of reduced mAbs, this technique provides a non-inferred assessment on the nature (i.e. 1 N-glycan versus 0 N-glycans) of glycan occupancy for the intact mAb. The proposed HILIC methodology is also MS-compatible, making it possible to readily confirm the assignments of observed peaks.

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Intact protein characterization: Waters Biopharmaceutical System Solution with UNIFI

GOAL

To demonstrate the capabilities of the integrated UPLC®-MS analysis of an intact monoclonal antibody with a comprehensive platform for accurate mass measurement, data processing, and reporting with UNIFI® Scientific Information System.

This UNIFI-based platform addresses previous limitations with a comprehensively integrated platform for data acquisition by chromatography and mass spectrometry, with automated reporting.

BACKGROUND

The growing biotherapeutic pipeline means that the efficient characterization of monoclonal antibodies (mAb) is of growing importance, both to regulatory authorities and to pharmaceutical companies. Being able to perform acquisition and processing within the same platform, complete with an audit trail, is an important goal for regulated environments.

Accurately identifying post-translational modifications such as protein glycosylation is required as part of guidelines as they play several key roles in biological systems. Fast and accurate analysis of the glycoproteins is required in order to ensure the safety and efficacy of the biotherapeutic.

The ACQUITY UPLC® H-Class Bio's high-resolution bioseparations combined with high mass accuracy mass spectrometry detection with the Xevo® G2 Tof provides routine UPLC-MS applications for biopharmaceutical laboratories.

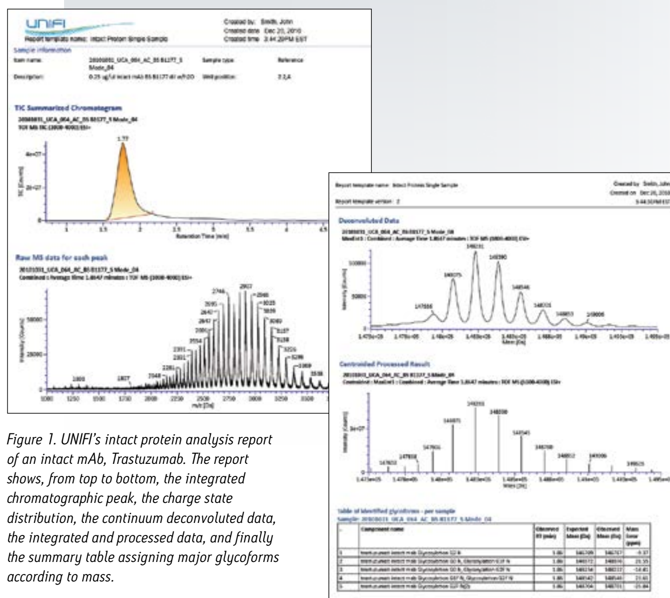

Figure 1. UNIFI's intact protein analysis report of an intact mAb, Trastuzumab. The report shows, from top to bottom, the integrated chromatographic peak, the charge state distribution, the continuum deconvoluted data, the integrated and processed data, and finally the summary table assigning major glycoforms according to mass.

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There is a large set of data generated during each mAb analysis requiring interpretation of a variety of glycosylated forms and comprehensive characterization of the final product. This step sets productivity limits to otherwise high-throughput procedures and hinders automation of the process.

The UNIFI-based platform addresses these limitations with a comprehensively integrated platform for data acquisition by chromatography and mass spectrometry, with automated reporting.

THE SOLUTION

To solve the problem of time-consuming data analysis and facilitate data processing of therapeutic mAb, the Biopharmaceutical Platform Solution with UNIFI was configured for the study of intact proteins. This represents a holistic approach of UPLC-MS data acquisition followed by automatic processing and annotation of the data in a high-throughput manner, which are further exported for data management.

UPLC-MS analysis of the mAb Trastuzumab was performed automatically. Aqueous solutions of 0.1% FA and 0.1% FA solution in acetonitrile were used as eluents A and B, respectively. Column temperature set to 80 °C is critical for successful chromatographic separation. The system included an ACQUITY UPLC H-Class Bio, an ACQUITY UPLC Protein BEH C_4 Column, and a Xevo G2 Tof. The UNIFI Scientific Information System for acquisition, data processing, and reporting completes this comprehensive Biopharmaceutical Platform Solution.

Table of identified glycoforms - per sample
Sample: 20101031_UCA_064_AC_BS B1177_5 Mode_04

.	Component name	Observed RT (min)	Expected Mass (Da)	Observed Mass (Da)	Mass Error (ppm)
1	trastuzumab intact mab Glycosylation G2 N	1.86	146789	146787	-9.37
2	trastuzumab intact mab Glycosylation G0 N, Glycosylation G1F N	1.86	148072	148076	26.55
3	trastuzumab intact mab Glycosylation G0 N, Glycosylation G2F N	1.86	148234	148232	-14.41
4	trastuzumab intact mab Glycosylation G1F N, Glycosylation G2F N	1.86	148542	148546	23.61
5	trastuzumab intact mab Glycosylation G2F N(2)	1.86	148704	148701	-25.84

Figure 2. Zoomed in view of the table in Figure 1 summarizing mass measurement of the intact mAb and accurately assigned mAb glycan variants.

The intact protein analysis report demonstrates the report objects, which can be entirely configured by the user: TIC summarized chromatogram; raw, deconvoluted, and centroid mass spectra; and tabulated summary of the interpreted LC-MS data (Figure 1). This detailed view shows an example of a deconvoluted spectrum within a specified mass range and parameter settings defined in the method. Deconvolution reveals several core glycosylated species which match the number of glucose residues and level of fucosylation. Another report object is a table with mass measurement of the intact mAb and accurately assigned mAb glycan variants (Figure 2). Mass errors were reported for each Trastuzumab MS peak with a corresponding retention time entry from the TIC chromatogram.

Such an integrated LC-MS approach provides the user flexibility to work with both raw and processed data followed by quick and efficient data management.

SUMMARY

The capabilities of the Biopharmaceutical Platform Solution with UNIFI have been successfully demonstrated with the example of an intact biotherapeutic mAb.

Modern instrumentation and evolving analytical techniques extend the limits of the biopharmaceutical industry and consequently impose strict control of manufacturing processes.

Highly efficient and cost-effective integrated UPLC-MS approaches with the UNIFI Scientific Information System for data processing and reporting satisfies regulatory requirements and facilitates intact protein characterization. This technology covers the range from detailed structural protein characterization to sophisticated data management with UPLC-MS platforms.

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[APPLICATION NOTE]

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Streamlining Compliant and Non-Compliant Intact Mass Analysis of Biotherapeutic mAbs with the Biopharmaceutical Platform Solution with UNIFI

Henry Shion and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Biopharmaceutical Platform Solution with UNIFI® enables a fully integrated workflow for intact mass analysis, including acquisition, processing, and reporting, for organizations in early development and those operating under regulatory compliant environments. The ability to automate and standardize intact mass analysis enables laboratories to deploy their scientific resources with greater efficiency and effect.

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI

ACQUITY UPLC® H-Class Bio System

Xevo® G2 QTof

ACQUITY UPLC Protein BEH C4 Column

Xevo G2-S QTof

ACQUITY UPLC Tunable UV Detector

KEY WORDS

Intact mass analysis, mAb, biotherapeutic characterization

INTRODUCTION

Intact mass analysis is a rapid and convenient method for confirming protein identity and profiling product-related variants. In conjunction with other analytical techniques, such as peptide mapping and released glycan analysis, intact mass analysis can help determine if the biomolecule had been correctly cloned, expressed, purified, and formulated during the biopharmaceutical drug development process.

Intact mass analysis can provide a semi-quantitative view of product heterogeneity and is often employed to determine relative composition of product glycoforms. As a lot release test, intact protein mass analysis often provides a quick identity test using the mass of a major variant, sometimes in conjunction with a purity test with defined product variation for peaks corresponding to variants displaying critical product attributes. Demonstration of process consistency through such comparability exercises is critical to obtain initial regulatory approval and for later process improvement studies.

Data processing and report generation often become productivity-limiting tasks for organizations responsible for biotherapeutic protein characterization and analysis. It is still common for LC-MS intact protein data to be manually processed, an inefficient process that lacks standardization and is prone to human error. Further inefficiency and sources of error result from scientists having to reformat results into graphical and tabular formats suitable for communicating information to their organizations.

The ability to automate and standardize data acquisition, processing, and reporting for intact mass analysis allows laboratories to deploy their scientific resources with greater efficiency and effect. The Waters® UNIFI Scientific Information System enables these benefits, as well as regulatory compliance, to be realized throughout discovery, development, and quality management organizations.

In this application note, an integrated and compliant-ready solution for intact mass analysis is described. The combination of UPLC® separations, optimized application-tested protein column chemistries, the Xevo G2-S QTof for mass detection, all used under control of the UNIFI Scientific Information System, achieves the goal of total workflow automation and standardization.

EXPERIMENTAL

LC conditions

System: ACQUITY UPLC H-Class Bio System

Detector: ACQUITY UPLC Tunable UV Detector

Column: ACQUITY UPLC Protein BEH C4 Column, 300Å, 1.7 µm, 2.1 mm X 50 mm (p/n 186004495)

Column temp.: 80 °C

Mobile phase A: Water

Mobile phase B: Acetonitrile

Mobile phase C: 1% formic acid

Optical detection: UV 280 nm

LC gradient table:

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.40	85.0	5.0	10.0	0	Initial
1.00	0.40	85.0	5.0	10.0	0	6
1.01	0.20	85.0	5.0	10.0	0	6
3.50	0.20	5.0	95.0	0.0	0	6
3.70	0.40	5.0	95.0	0.0	0	6
4.00	0.40	10.0	80.0	10.0	0	6
4.50	0.40	10.0	80.0	10.0	0	6
5.00	0.40	85.0	5.0	10.0	0	6
5.50	0.40	85.5	5.0	10.0	0	6

Total run time: 6.5 min

MS conditions

Mass spectrometer: Xevo G2-S QTof

Capillary: 2.5 kV

Sampling cone: 80 V

Extraction cone: 4 V

Source temp.: 150 °C

Desolvation temp.: 350 °C

Cone gas flow: 0 L/h

Desolvation gas flow: 800 L/h

Informatics

UNIFI Scientific Information System

Results derived from an intact IgG1 mAb mass analysis are used to illustrate how this integrated system solution can help the biopharmaceutical laboratories to streamline a common analytical workflow, shown in Figure 1, and more quickly and efficiently communicate key information needed to bring better molecules to market faster.

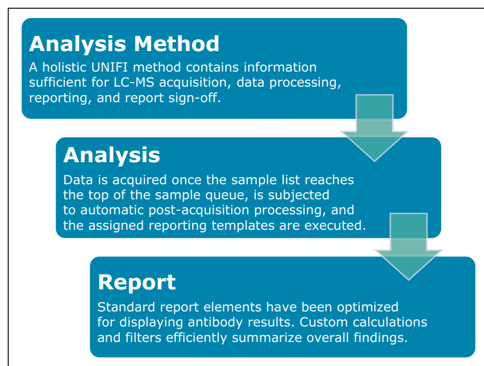


Figure 1. Intact mass analysis workflow with the UNIFI Scientific Information System.

Biopharmaceutical System Solution with UNIFI

- ACQUITY UPLC H-Class Bio System
- Protein Separation Technology (PrST) Columns
- Xevo G2-S QTof with an ACQUITY UPLC Tunable UV Detector
- UNIFI Scientific Information System

Sample preparation

Waters Intact mAb Mass Check Standard (p/n 186006552) was analyzed by solubilizing the standard (10 mg/mL or 67 µM, 100 µL DI water to standard vial, 5 min sonication), and diluting 20X (Final 3.3 µM, 0.50 µg/µL) with eluent A for Xevo G2 QTof analysis or 200X (0.33 µM, 0.05 µg/µL) for Xevo G2-S analysis.

RESULTS AND DISCUSSION

An automated mAb LC-MS analysis set of 11 injections was automatically acquired, processed, and reported as specified in a single UNIFI method. Data are representative of a simple method development set, where the goal of the researcher is to assess the extent of product glycovariation and determine analytical reproducibility.

For the processed results, a single injection is represented in the review panel of the UNIFI analysis center, shown in Figure 2. This panel is configured to convey chromatographic information (integrated total ion chromatogram), the MaxEnt™ deconvoluted MS spectrum corresponding to the summed spectra under the detected peak, and a component summary window filtered to display the top five most intensely assigned glycoforms (G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, or G2F/G2F).

This combined panel enables a researcher to assess chromatographic quality, the quality of MS data processing, and the quality of glycoform assignments in a single display. Closer examination also reveals the relative abundance of each glycoform was automatically calculated as part of the processing.

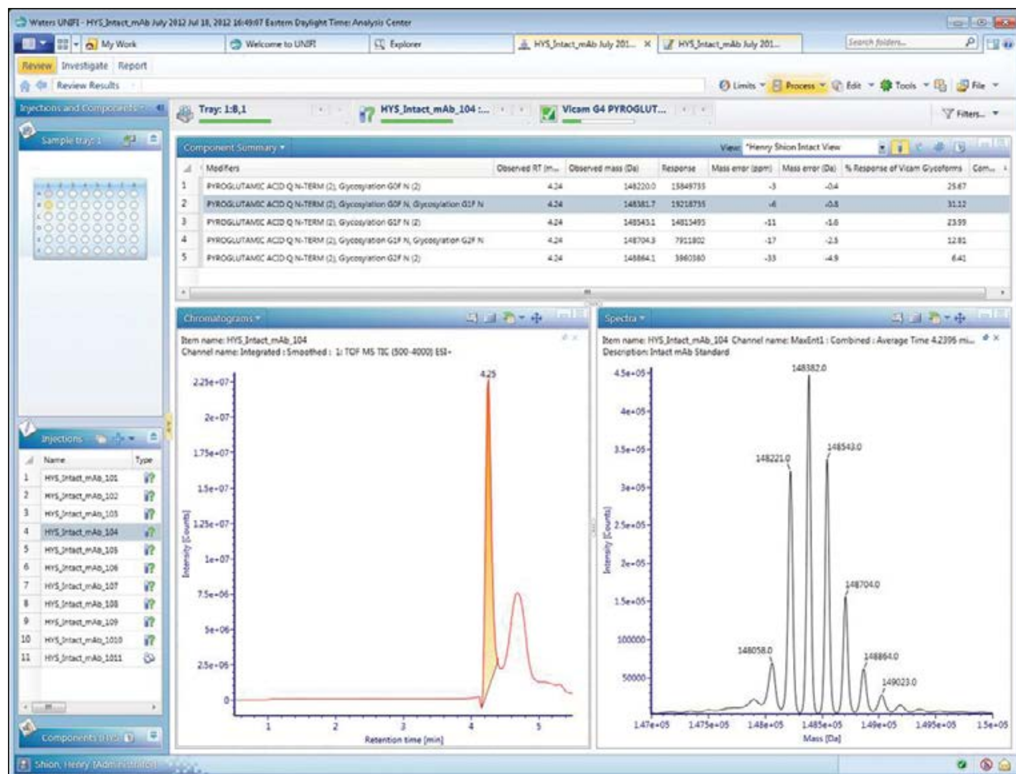


Figure 2. The review panel in UNIFI displays automatically processed experimental results.

Having designated one sample as the reference enables a researcher to select the comparative mode display of the review tab. The binary comparison display, shown in Figure 3, provides a means to visually examine the differences between the two samples, thus revealing the extent of variation between samples. In this display, comparative chromatograms and spectra (A280 and summed *m/z* spectra) are depicted, along with the component summary, now reformatted to address comparative questions. Since both injections were from the same sample, minimal experimental result differences are predictably observed.

Figure 3. UNIFI's review panel (compare mode) displays automatically processed experiment results, with a focus on identifying similarities and differences between a reference sample and unknown samples.

The summary plot tool within the review tab enables researchers to quickly compare trends and differences within the larger data set. The variation of mAb glycoform MS response, as shown in Figure 4, would be a common application of this capability, as would comparisons of observed retention time or mass error across the sample set. The consistent MS response of glycoforms across all injections illustrates the expected reproducibility of the intact mass analysis of replicated injections.

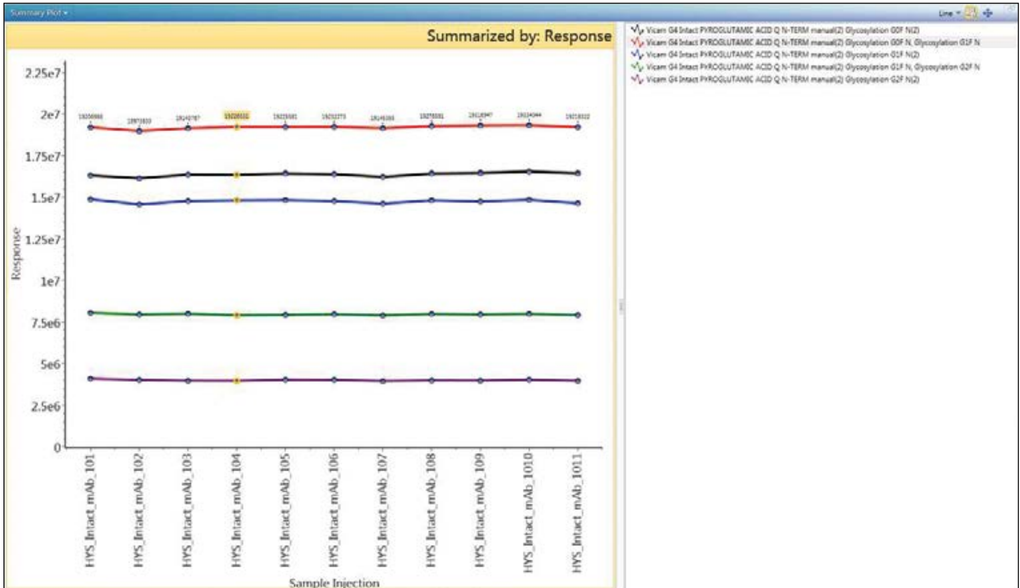


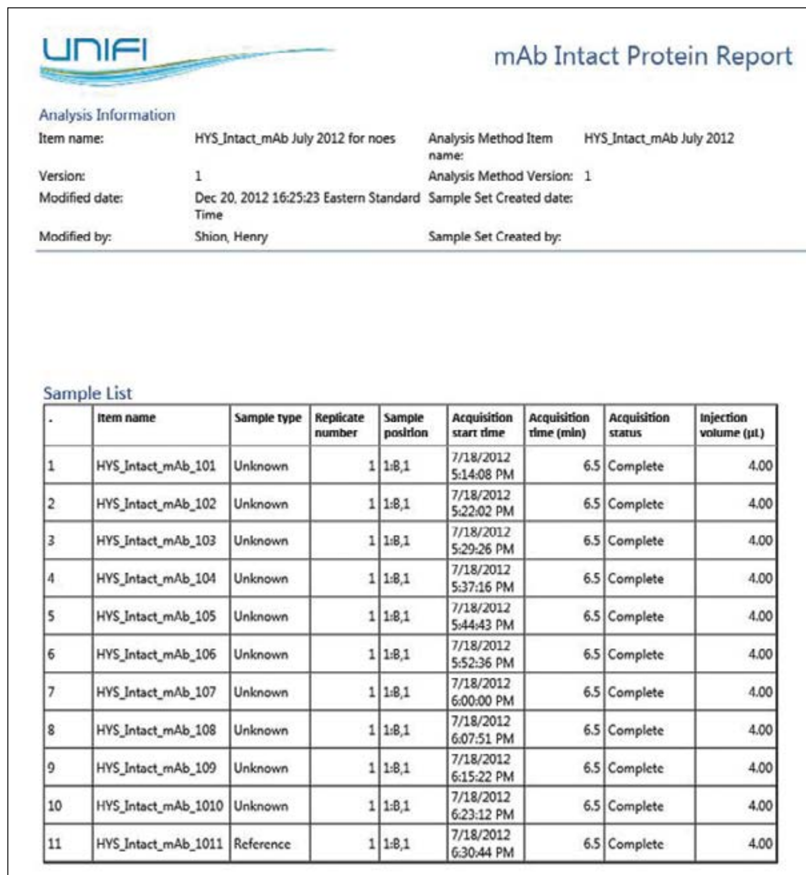
Figure 4. summary plot view of MS response for 5 mAb glycoforms (n = 11 injections).

UNIFI reporting

The reporting functionality within UNIFI Software is powerful, addressing one of the common bottlenecks encountered by organizations when generating and managing large volumes of complex scientific data. The ability to customize common report objects by means of filters, formatting, and the use of custom fields and calculations enables report content to be automatically generated by an entire organization with high quality on a consistent basis. Based on the analytical objectives, one or more report templates can be attached to the analysis method.

The first page of a typical intact mass analysis experimental report contains a summary of sample information and acquisition status, as shown in Figure 5. More detailed experimental results (such as TUV and TIC chromatograms, raw and deconvoluted MS spectra, and identified component response summary table) are often grouped for each injection, as shown in Figure 6.

In the case of mAbs, generic report objects were tuned to account for the rapid desalting LC-MS method that was used, the acquisition of UV and MS data, and the typical input *m/z* and output mass ranges encountered during antibody ESI mass analysis.



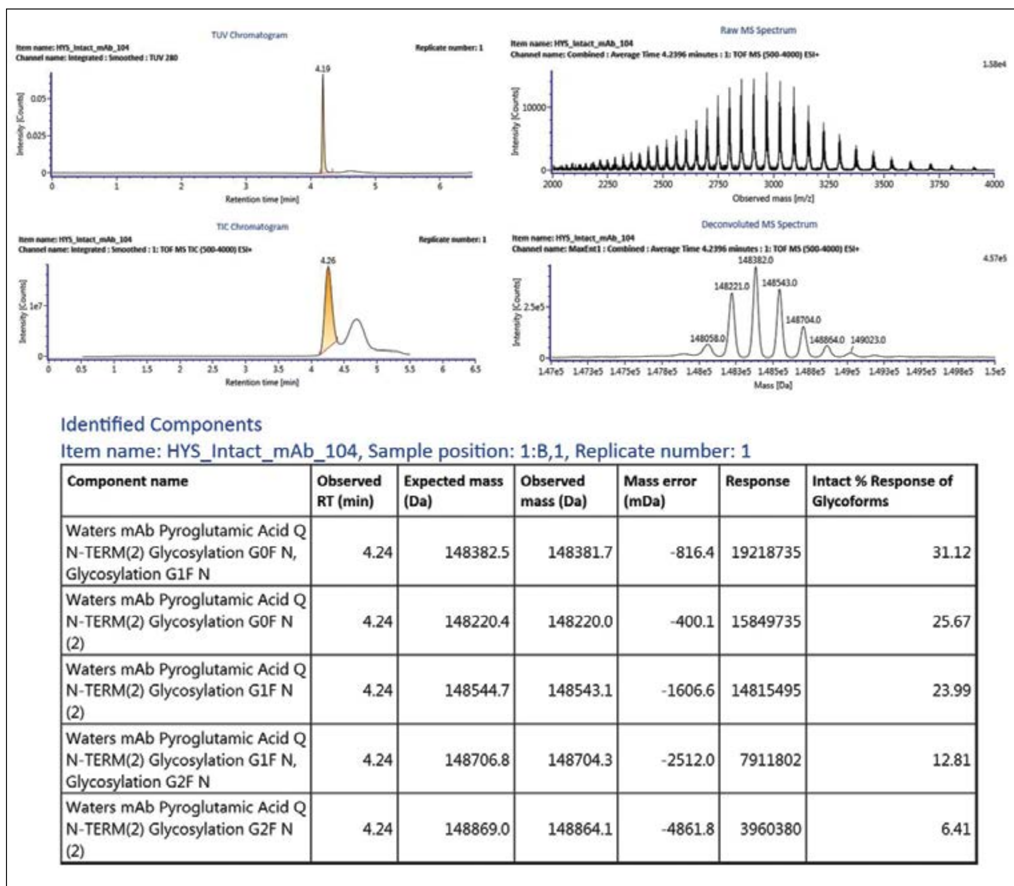
Analysis Information

Item name:	HYS_Intact_mAb July 2012 for noes	Analysis Method Item name:	HYS_Intact_mAb July 2012
Version:	1	Analysis Method Version:	1
Modified date:	Dec 20, 2012 16:25:23 Eastern Standard Time	Sample Set Created date:	
Modified by:	Shion, Henry	Sample Set Created by:	

Sample List

.	Item name	Sample type	Replicate number	Sample position	Acquisition start time	Acquisition time (min)	Acquisition status	Injection volume (µL)
1	HYS_Intact_mAb_101	Unknown	1	1:B,1	7/18/2012 5:14:08 PM	6.5	Complete	4.00
2	HYS_Intact_mAb_102	Unknown	1	1:B,1	7/18/2012 5:22:02 PM	6.5	Complete	4.00
3	HYS_Intact_mAb_103	Unknown	1	1:B,1	7/18/2012 5:29:26 PM	6.5	Complete	4.00
4	HYS_Intact_mAb_104	Unknown	1	1:B,1	7/18/2012 5:37:16 PM	6.5	Complete	4.00
5	HYS_Intact_mAb_105	Unknown	1	1:B,1	7/18/2012 5:44:43 PM	6.5	Complete	4.00
6	HYS_Intact_mAb_106	Unknown	1	1:B,1	7/18/2012 5:52:36 PM	6.5	Complete	4.00
7	HYS_Intact_mAb_107	Unknown	1	1:B,1	7/18/2012 6:00:00 PM	6.5	Complete	4.00
8	HYS_Intact_mAb_108	Unknown	1	1:B,1	7/18/2012 6:07:51 PM	6.5	Complete	4.00
9	HYS_Intact_mAb_109	Unknown	1	1:B,1	7/18/2012 6:15:22 PM	6.5	Complete	4.00
10	HYS_Intact_mAb_1010	Unknown	1	1:B,1	7/18/2012 6:23:12 PM	6.5	Complete	4.00
11	HYS_Intact_mAb_1011	Reference	1	1:B,1	7/18/2012 6:30:44 PM	6.5	Complete	4.00

Figure 5. Typical first page of an intact protein LC-MS report in UNIFI Software that summarizes sample and acquisition details.



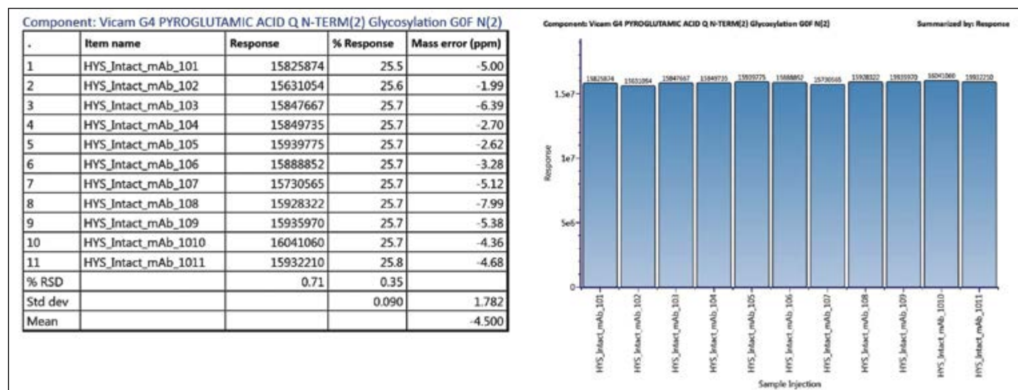
Identified Components

Item name: HYS_Intact_mAb_104, Sample position: 1:B,1, Replicate number: 1

Component name	Observed RT (min)	Expected mass (Da)	Observed mass (Da)	Mass error (mDa)	Response	Intact % Response of Glycoforms
Waters mAb Pyroglutamic Acid Q N-TERM(2) Glycosylation G0F N, Glycosylation G1F N	4.24	148382.5	148381.7	-816.4	19218735	31.12
Waters mAb Pyroglutamic Acid Q N-TERM(2) Glycosylation G0F N (2)	4.24	148220.4	148220.0	-400.1	15849735	25.67
Waters mAb Pyroglutamic Acid Q N-TERM(2) Glycosylation G1F N (2)	4.24	148544.7	148543.1	-1606.6	14815495	23.99
Waters mAb Pyroglutamic Acid Q N-TERM(2) Glycosylation G1F N, Glycosylation G2F N	4.24	148706.8	148704.3	-2512.0	7911802	12.81
Waters mAb Pyroglutamic Acid Q N-TERM(2) Glycosylation G2F N (2)	4.24	148869.0	148864.1	-4861.8	3960380	6.41

Figure 6. Example report object grouping (TUV and TIC chromatograms, raw and deconvoluted MS spectra, and identified component response summary table) from a single injection within the analysis.

In addition, the ability to automate reporting summary results across the sample sets eliminates the use of external software for data aggregation, as shown in Figure 7. This not only greatly increases the timeliness of communicating results, but avoids the human errors and validation efforts that cost analytical organizations time and money. In the case of this typical method validation injection set, the precision of MS response and mass accuracy is reported for one of the observed glycoforms.



Component: Vicam G4 PYROGLUTAMIC ACID Q N-TERM(2) Glycosylation G0F N(2)

.	Item name	Response	% Response	Mass error (ppm)
1	HYS_Intact_mAb_101	15825874	25.5	-5.00
2	HYS_Intact_mAb_102	15631054	25.6	-1.99
3	HYS_Intact_mAb_103	15847667	25.7	-6.39
4	HYS_Intact_mAb_104	15849735	25.7	-2.70
5	HYS_Intact_mAb_105	15939775	25.7	-2.62
6	HYS_Intact_mAb_106	15888852	25.7	-3.28
7	HYS_Intact_mAb_107	15730565	25.7	-5.12
8	HYS_Intact_mAb_108	15928322	25.7	-7.99
9	HYS_Intact_mAb_109	15935970	25.7	-5.38
10	HYS_Intact_mAb_1010	16041060	25.7	-4.36
11	HYS_Intact_mAb_1011	15932210	25.8	-4.68
% RSD		0.71	0.35	
Std dev			0.090	1.782
Mean				-4.500

Figure 7. The report object summarizes MS response and mass accuracy/precision across all injections within the sample set in table and bar chart formats.

CONCLUSIONS

The intact mass analysis workflows within the Biopharamceutical Platform Solution with UNIFI enable automated data acquisition, processing, and reporting of a typical method validation sample set. This demonstrates UNIFI Software's ability to facilitate robust glycoform profiling of a recombinant mAb, removes the necessity of manual data processing, and improves the process of data review and reporting. The implementation of such highly automated workflows should enable biotherapeutic development and quality organizations to handle larger volumes of sample requests with the same resources, while improving the quality of the information they provide.

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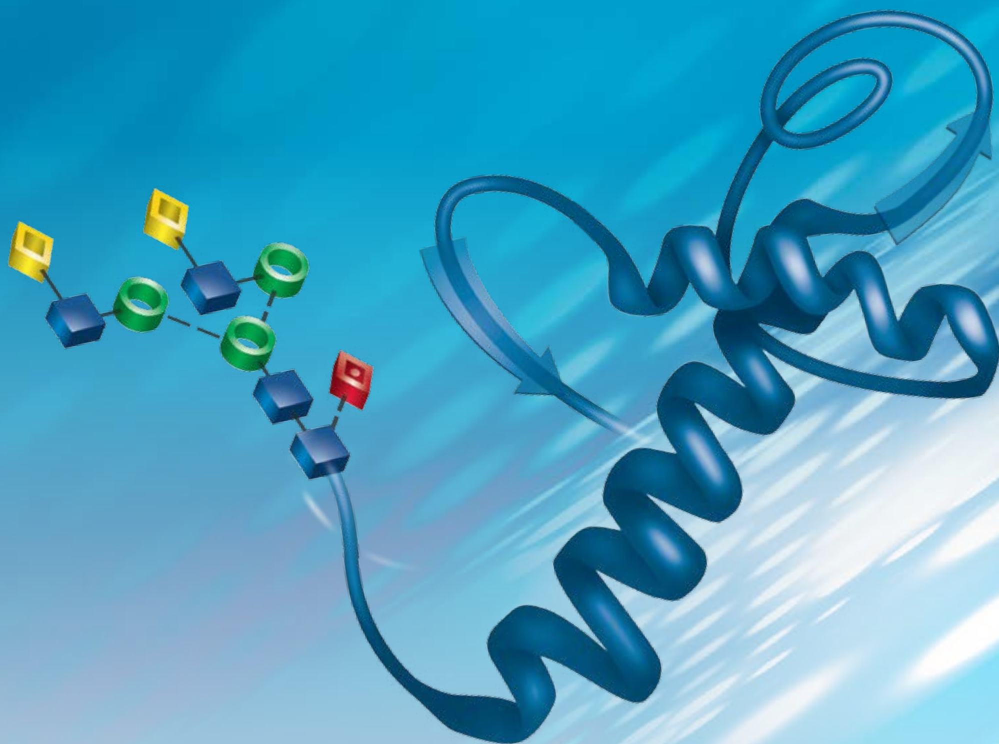
GLYCOPEPTIDE MAPPING AND SUBUNIT ANALYSIS



[APPLICATION NOTE]

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HILIC Glycopeptide Mapping with a Wide-Pore Amide Stationary Phase

Matthew A. Lauber and Stephan M. Koza
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Orthogonal selectivity to conventional reversed phase (RP) peptide mapping for enhanced characterization of hydrophilic protein modifications, such as glycosylation
- Class-leading HILIC separations of IgG glycopeptides to interrogate sites of modification
- MS compatible HILIC to enable detailed investigations of sample constituents
- Enhanced glycan information that complements *Rapi*Fluor-MS released N-glycan analyses
- Glycoprotein BEH Amide 300Å 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility

WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide 300Å Column (patent pending)

Glycoprotein Performance Test Standard

ACQUITY UPLC H-Class Bio System

Waters SYNAPT® G2-S HDMS

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated proteins, glycosylation, HILIC, mab, glycopeptide, glycoprotein

INTRODUCTION

Peptide mapping of biopharmaceuticals has longed been used as a tool for identity tests and for monitoring residue-specific modifications.[1-2] In a traditional analysis, peptides resulting from the use of high fidelity proteases, like trypsin and Lys-C, are separated with very high peak capacities by reversed phase (RP) separations with C_{18} bonded stationary phases using ion-pairing reagents. Separations such as these are able to resolve peptides with single amino acid differences such as asparagine; and the two potential products of asparagine deamidation, aspartic acid and isoaspartic acid.[3-4]

Nevertheless, not all protein modifications are so easily resolved by RP separations. Glycosylated peptides, in comparison, are often separated with relatively poor selectivity, particularly if one considers that glycopeptide isoforms usually differ in their glycan mass by about 10 to 2,000 Da. So, while RP separations are advantageous for generic peptide mapping, they are limited in their ability to resolve hydrophilic modifications. Previous studies have demonstrated that hydrophilic interaction chromatography (HILIC) with an amide-bonded stationary phase can provide complementary and highly resolving separations of glycosylated peptides.[5-6] These studies have demonstrated that amide-bonded stationary phases are particularly effective for these separations, because they afford high retentivity as a consequence of their hydrophilicity and propensity for hydrogen bonding.[7]

Expanding upon this technology, we have developed an amide-bonded stationary phase with a nominally larger pore diameter, a so-called "wide-pore" material, such that amide HILIC separations can be universally applied to separating the glycoforms of both intact and digested glycoproteins. This stationary phase found in ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Columns ensures that glycopeptides, regardless of their size, will have access to the majority of the porous network and be less prone to restricted diffusion.[8-9] In previous work, we have demonstrated the use of this HILIC column to assay the glycan occupancy of an intact monoclonal antibody (mAb),[10] to map the domain-specific glycosylation of IgG subunits,[11] and to improve the resolution of tri- and tetra-antennary GlycoWorks,™ *Rapi*Fluor-MS™ labeled N-glycans.[12] Here, we explore the use of the Glycoprotein BEH Amide 300Å 1.7 µm Column to produce high resolution HILIC separations of glycopeptides from three different monoclonal antibodies: trastuzumab, cetuximab and an IgG1K candidate reference material from NIST.

EXPERIMENTAL

Sample description

Lys-C digest of trastuzumab and NIST candidate reference material

An adaptation of a previously published single reaction vial, overnight (16+ hours) procedure[4] was employed to prepare non-reduced Lys-C digests of trastuzumab and a IgG1K monoclonal antibody candidate reference material obtained from NIST (#8670, lot# 3F1b). TFA quenched digests were stored at -80 °C until analyzed. In preparation for HILIC chromatography, aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide and were then centrifuged at 16 x 1000 g for 10 minutes to remove any insoluble composition. Supernatant from the centrifuged digest was thereafter injected.

Lys-C/tryptic digest of cetuximab

Reduced and alkylated cetuximab was digested with a combination of Achromobacter protease I (Lys-C) and trypsin. Formulated cetuximab was concentrated to 10 mg/mL and buffer exchanged with a 10 kDa MWCO centrifugal filter (Millipore, Billerica, MA) into a solution of 6 M GuHCl, 50 mM DTT, and 0.2 M phosphate (pH 8.1), then incubated at 37 °C for 2 hours. Thereafter, the sample was diluted with a solution of iodoacetamide, bringing the antibody concentration to 8 mg/mL and the buffer composition to 4.8 M GuHCl, 40 mM DTT, 50 mM iodoacetamide, and 0.17 M phosphate (pH 8.1). Alkylation with iodoacetamide was allowed to proceed under these conditions for 10 min in the dark at 37 °C, before being quenched by the addition of cysteine, diluted with a urea-containing buffer, and mixed with Achromobacter protease I (Lys-C) at a 4:1 w/w ratio. The resulting digest solution of 0.8 mg/mL cetuximab, 0.5 M GuHCl, 3 M Urea, 40 mM NH_2OH, 4 mM DTT, 5 mM iodoacetamide, 6 mM cysteine, and 0.1 M phosphate (pH ~7.1) was incubated at 37 °C. After 2 hours of incubation, this digest solution was diluted two fold with water and an aliquot of trypsin (Sigma T6567), such that the protein:trypsin ratio was 4:1 (w/w). After incubation at 37 °C for another 2 hours, the digest solution was again diluted two fold with water and a fresh aliquot of trypsin. With a total protein:trypsin ratio of 2:1 (w/w), the digest was left to incubate at 37 °C for 16 hours. Following this incubation, the digest was quenched by acidification with TFA and stored at -80 °C until analyzed. In preparation for HILIC chromatography, aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide and were then centrifuged at 16 x 1000 g for 10 minutes to remove any insoluble composition. Supernatant from the centrifuged digest was thereafter injected.

Method conditions

(unless otherwise noted):

Column conditioning

ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Columns (as well as other amide columns intended for glycoprotein or glycopeptide separations) should be conditioned via two sequential injections and separations of 40 µg Glycoprotein Performance Test Standard (p/n 186008010; 10 µL injections of 4 mg/mL in 0.1% TFA, 80% ACN) or with equivalent loads of a test sample for which the column has been acquired. The separation outlined by the following method can be employed for conditioning with the Glycoprotein Performance Test Standard.

Column conditioning gradient

2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, H2O

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	33.0	67.0	6
21.0	40.0	60.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

LC conditions for LC-UV-MS of mAb glycopeptides (Figures 1–6):

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 30 °C (trastuzumab Lys-C digest HILIC separations)

60 °C (cetuximab Lys-C/tryptic digest HILIC separations)

60 °C (trastuzumab Lys-C reversed phase separations)

Flow Rate: 0.2 mL/min

Mobile phase A: 0.1% (v/v) TFA, H_2O

Mobile phase B: 0.1% (v/v) TFA, ACN

HILIC injection volume: 100–250 µL (Aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide to obtain a miscible, HILIC compatible diluent.)

Reversed phase injection volume: 24.2 µL (Aqueous digest)

Columns: ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard)

ACQUITY UPLC Peptide BEH C_{18} 300 Å 1.7 µm, 2.1 x 150 mm (p/n 186003687)

Vials: Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

Gradient used for reversed phase separations of trastuzumab Lys-C digests (Figure 1A):

Time	%A	%B	Curve
0.0	98.0	2.0	6
96.0	50.0	50.0	6
99.0	20.0	80.0	6
101.0	20.0	80.0	6
102.0	98.0	2.0	6
113.0	98.0	2.0	6

Gradient used for HILIC separations of trastuzumab Lys-C digests and Lys-C/tryptic digests of cetuximab (Figures 1B-6):

Time	%A	%B	Curve
0.0	20.0	80.0	6
60.0	50.0	50.0	6
61.0	80.0	20.0	6
63.0	80.0	20.0	6
64.0	20.0	80.0	6
75.0	20.0	80.0	6

MS conditions for IgG subunit separations

MS system: SYNAPT G2-S HDMS

Ionization mode: ESI+

Analyzer mode: Resolution (~20 K)

Capillary voltage: 3.0 kV

Cone voltage: 25 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Desolvation gas flow: 800 L/Hr

Calibration: NaI, 1 µg/µL from 100–2000 *m/z*

Lockspray: 300 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds

Acquisition: 50–2500 *m/z*, 0.1 sec scan rate

Data management: MassLynx Software (V4.1) / UNIFI V1.7

LC Conditions for a Glycopeptide Mapping of an IgG1K with Fluorescence Detection (Figure 7):

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 45 °C

Fluorescence detection: Ex 280/Em 320 nm (10 Hz scan rate, Gain =1)

Injection volume: 100 µL (DMF/ACN diluted sample)

Mobile phase A: 0.1% TFA in water

Mobile phase B: 0.1% TFA in ACN

Columns: ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm, 2.1 x 50 mm (p/n 176003702, with Glycoprotein Performance Test Standard)

Other columns: Column A: 2.6 µm, 2.1 x 150 mm

Column B: 1.8 µm, 2.1 x 150 mm

Vials: Polypropylene 12 x 32mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

Gradient (Figure 7):

Time (min)	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	30.0	70.0	6
21.0	37.0	63.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

Data management: UNIFI v1.7

RESULTS AND DISCUSSION

Orthogonal and complementary glycopeptide mapping separations

To demonstrate a conventional approach to peptide mapping, we first performed LC-UV-MS analysis on a Lys-C digest of a mAb using a RP chromatographic separation with a wide-pore C_{18} bonded stationary phase (Peptide BEH C_{18} 300Å 1.7 µm). Trastuzumab was selected for this study, given its prominence as a first generation mAb drug product and a potential target for biosimilar development.[13] Figure 1A shows a UPLC chromatogram that is typical for a Lys-C digest of trastuzumab, wherein peptides are broadly resolved across a separation with a gradient corresponding to a change of 0.5% acetonitrile per minute. The non-glycosylated peptides of the digest spread across the extremes of the chromatogram while the glycopeptides elute in an approximately one minute wide window at a retention time of about 60 minutes. The conditions to produce this high resolution separation involve the use of mobile phases modified with trifluoroacetic acid (TFA); the same mobile phases that have proven to be optimal for HILIC of proteinaceous analytes.[10-11]

Accordingly, an orthogonal method to the RP separation can be achieved via HILIC by simply reversing the gradient and using the newly developed wide-pore amide bonded stationary phase (Glycoprotein BEH Amide 300Å 1.7 µm). An example of a chromatogram obtained from a column packed with this wide-pore amide material and a gradient ramp of 0.5% acetonitrile per minute is shown in Figure 1B. Here, the peptides from the Lys-C digested trastuzumab are very clearly segregated into early and late eluting species, corresponding to the non-glycosylated and glycosylated species, respectively. The use of TFA ion pairing facilitates obtaining this separation, as it masks the hydrophilicity of the peptide residues and provides improved selectivity for the hydrophilic modifications. Also note that the glycopeptides have not only been class separated with the amide column, but the selectivity of the peptide glycoforms is remarkably improved over the analogous RP separation.

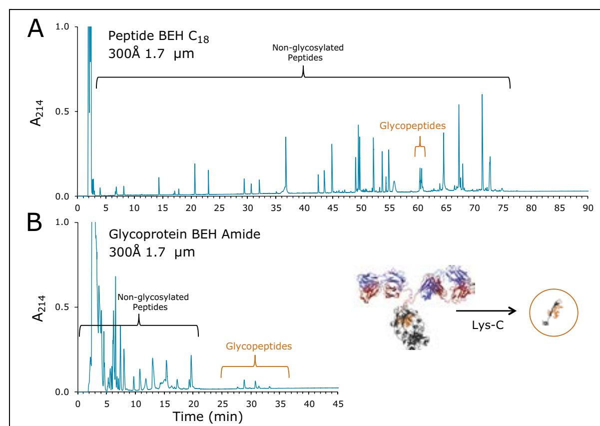


Figure 1. Lys-C glycopeptide mapping of trastuzumab. (A) A traditional reversed phase separation of the Lys-C digest using a 2.1 x 150 mm ACQUITY UPLC Peptide BEH C_{18} 300Å 1.7 µm Column. (B) A HILIC separation of the Lys-C digest using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column. In each analysis, 9.2 µg of the Lys-C digest was separated using the same gradient slope and injecting sample from a diluent comprised of either approximately 0.2% TFA in 80:20 ACN/water (HILIC) or 100% water (reversed phase).

By focusing on the strongly retained peaks, one can begin to interrogate the glycosylation of the trastuzumab molecule (Figure 2A). In particular, MS data acquired from online mass detection and a total ion chromatogram (TIC) can be applied to identify the peptide species and its corresponding glycoforms, as shown in Figure 2B. This Lys-C glycopeptide map presents a 29 amino acid residue peptide (K16) from the Fc domain of trastuzumab. From an analysis of the MS data, many biantennary structures typical found on mAbs in relatively high abundance can be readily identified (Figure 3A). Further interrogation of the MS data, also shows that low abundance N-glycan species can likewise be detected. Figure 3B, for instance, provides MS data supporting the identification of monosialylated and disialylated glycoforms at retention times of approximately 34.7 minutes and 36.4 minutes, respectively. These identifications correlate extremely well with the released N-glycan profiles of trastuzumab that have been previously reported.[14-15]

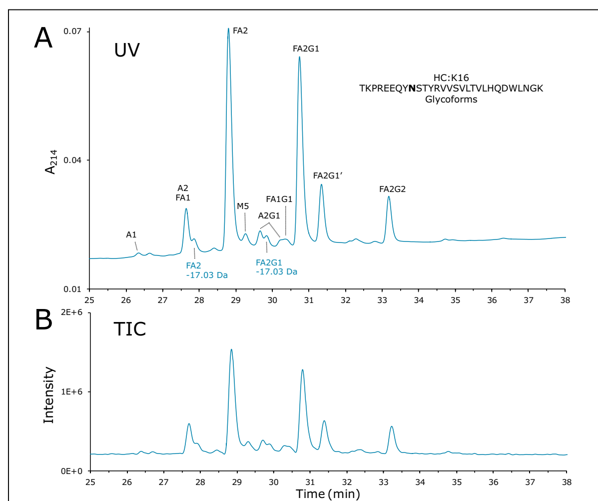
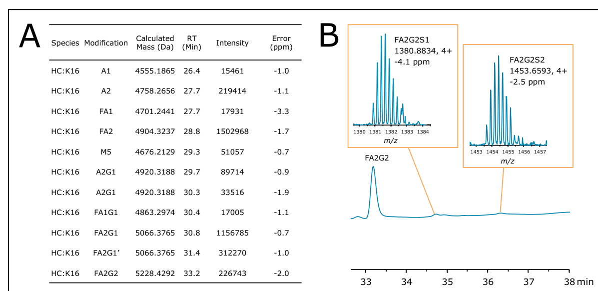


Figure 2. Lys-C glycopeptide mapping of trastuzumab with HILIC and an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column. (A) UV chromatogram for the Lys-C glycopeptide retention window. (B) Total ion chromatogram (TIC) for the same retention window.



Species	Modification	Calculated Mass (Da)	RT (Min)	Intensity	Error (ppm)
HC:K16	A1	4555.1865	26.4	15461	-1.0
HC:K16	A2	4758.2656	27.7	219414	-1.1
HC:K16	FA1	4701.2441	27.7	17931	-3.3
HC:K16	FA2	4904.3237	28.8	1502968	-1.7
HC:K16	M5	4676.2129	29.3	51057	-0.7
HC:K16	A2G1	4920.3188	29.7	89714	-0.9
HC:K16	A2G1	4920.3188	30.3	33516	-1.9
HC:K16	FA1G1	4863.2974	30.4	17005	-1.1
HC:K16	FA2G1	5066.3765	30.8	1156785	-0.7
HC:K16	FA2G1′	5066.3765	31.4	312270	-1.0
HC:K16	FA2G2	5228.4292	33.2	226743	-2.0

Figure 3. Mass spectrometric data supporting trastuzumab Lys-C glycopeptide identifications. (A) Retention times, MS intensities and mass errors for assignments labeled in Figure 2. (B) MS spectra supporting the identification of low abundance Lys-C glycopeptides modified with mono and di-sialylated N-glycans.

Lot-to-lot analysis of trastuzumab glycosylation via HILIC-UV glycopeptide mapping

HILIC-MS based glycopeptide mapping clearly yields information-rich data. However, these HILIC glycopeptide mapping separations also lend themselves to methods based only on optical detection. We have, for example, applied a HILIC-UV method to perform lot-to-lot analysis of trastuzumab glycosylation for two drug product samples. Representative HILIC chromatograms for glycopeptide K16 obtained from two different lots of trastuzumab are shown in Figure 4A. Previous released glycan analyses on these lots have shown there to be differences in glycosylation.[14] Through comparison of peak areas across the glycopeptide profile, we have found that these two lots of trastuzumab indeed differ with respect to their glycosylation. Specifically, these lots of trastuzumab appear to have different extents of terminal galactosylation, as can be seen in the differing abundances of FA2, FA2G1 and FA2G2 glycoforms (Figure 4B). This observation was consistent with data obtained from previous released glycan analyses and previous HILIC based profiling of trastuzumab subunits.[11]

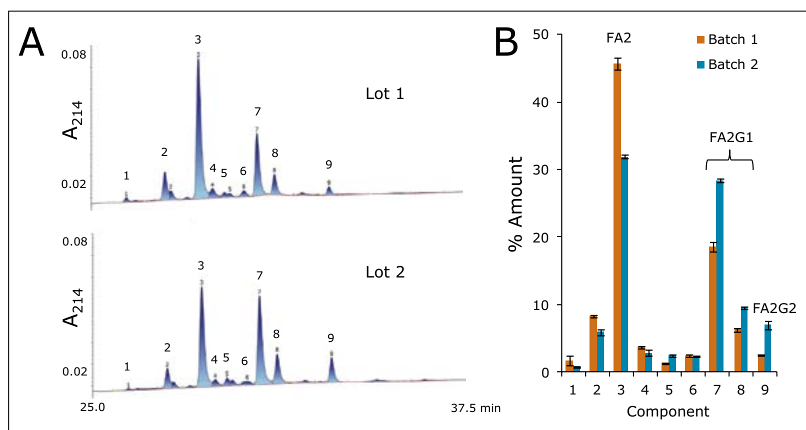


Figure 4. Lot-to-lot profiling of trastuzumab Lys-C peptide glycoforms. (A) HILIC chromatograms of trastuzumab Lys-C glycopeptides from two different lots of drug product. (B) Relative abundances of the major sample components. Analyses were performed in triplicate using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column.

Complementing GlycoWorks *Rapi*Fluor-MS N-glycan analyses with domain and peptide specific information about mAb glycosylation

An appealing aspect of glycopeptide mapping is that it can be applied to the elucidation of domain and peptide specific information. By inference or ETD fragmentation analyses, or both, glycopeptide mapping can also be used to detail the exact sites of glycosylation.[16] As we have noted before,[11] IgGs contain one conserved N-glycosylation site at Asn297 of the heavy chain, meaning they will be modified with two glycans in their Fc subunit. In addition, some IgGs and even some mAb IgG therapeutics exhibit multi-domain glycosylation. Cetuximab, for instance, is glycosylated in both its Fc and Fab domains,[17] making it a very interesting case study for this work.

A HILIC glycopeptide map of a Lys-C/tryptic digest of cetuximab provides a clear indication of the complicated glycan profile of this molecule (Figure 5). In the presented chromatogram, approximately thirty chromatographic peaks are observed. Furthermore, a cursory analysis of the MS data has shown there to be at a minimum twenty five different glycoform species with rather high relative abundances of greater than 1–2%. Figure 6 provides the MS data supporting these assignments. As can be seen, 9 unique glycoforms could be assigned to tryptic peptide T22 from the Fc domain, while the other 16 glycoforms could be assigned to tryptic peptide T8 from the Fab domain of cetuximab. It is interesting to note that the majority of the Fab domain (T8) glycans contain immunogenic epitopes, such as non-human alpa-1,3-galactose or non-human N-glycolyl-neuraminic acid moieties.[18] In previous work, these glycan species were identified through complementary subunit mapping and *Rapi*Fluor-MS released N-glycan analyses.[11] With these results on glycopeptide mapping, we show yet another complementary technique for assessing protein glycosylation.

Figure 5. Assaying the N-linked glycan sites of cetuximab and their microheterogeneity using a combined Lys-C/tryptic digest. Assignments for glycoforms of peptide T22 from the Fc domain of cetuximab are shown in dark gray, while assignments to the glycoforms of peptide T8 from the Fab domain are shown in red. Analyses were performed on 9.2 µg of Lys-C/tryptic digest using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column and a sample diluent of approximately 0.2% TFA in 80:20 ACN/water.

Species	Modification	Calculated Mass (Da)	RT (Min)	Intensity	Error (ppm)
HC:T22	FA1	2429.9592	32.2	9047	1.5
HC:T22	FA2	2633.0386	33.6	263523	1.4
HC:T22	M5	2404.9275	35.2	78988	0.0
HC:T22	FA2G1	2795.0913	36.1	186156	1.2
HC:T22	FA2G1'	2795.0913	36.7	57185	0.7
HC:T22	[Hex5HexNAc3DHex1]	2754.0647	37.8	7783	1.0
HC:T22	M6	2566.9805	38.1	5432	-1.8
HC:T22	FA2G2	2957.1443	38.9	38257	1.1
HC:T22	[Hex6HexNAc3DHex1]	2916.1177	40.5	12246	-1.3
HC:T8	FA2	3350.3689	29.4	5688	4.9
HC:T8	M5	3122.2578	30.4	6437	2.7
HC:T8	FA2G1	3512.4216	31.7	8925	3.2
HC:T8	FA2G2	3674.4746	34.1	11695	8.2
HC:T8	FA2G2Ga1	3836.5273	36.7	9657	-0.6
HC:T8	FA2G2Ga1'	3836.5273	37.0	15605	0.3
HC:T8	FA2G2Sg1	3981.5649	37.5	25532	1.9
HC:T8	FA2G2Ga2	3998.5801	39.1	212644	-0.7
HC:T8	A2G2	3471.3950	39.1	21542	2.0
HC:T8	[Hex7HexNAc5DHex1]	4144.6377	39.2	6944	-10.8
HC:T8	FA2G2Ga1Sg1	4143.6177	39.6	90905	-0.7
HC:T8	FA2G2Sg2	4288.6553	40.2	7428	-2.5
HC:T8	[Hex8HexNAc5DHex1]	4363.7124	41.8	5280	1.7
HC:T8	[Hex7HexNAc5DHex1NGNA1]	4508.7498	42.4	7114	-3.6
HC:T8	[Hex9HexNAc5DHex1]	4525.7651	43.8	20104	4.3
HC:T8	[Hex8HexNAc5DHex1NGNA1]	4670.8027	44.2	7640	1.2

*Hex: hexose, HexNAc: N-acetylate hexosamine, DHex: deoxyhexose, NGNA: N-glycoyl neuraminic acid / ('): structural isomer

Figure 6. Mass spectrometic data supporting glycopeptide assignments from the HILIC-UV-MS analysis of Lys-C/trypsin digested cetuximab. From a cursory analysis of MS data, 9 unique glycoforms could be assigned to the Fc domain, and 16 unique glycoforms could be assigned to the Fab domain.

Benchmarking the Capabilities of the Glycoprotein BEH Amide 300Å 1.7 µm Column

The peak capacities obtained in these example glycopeptide separations are particularly noteworthy when a comparison is made to otherwise available column technologies. To benchmark the performance of the Glycoprotein BEH Amide 300Å 1.7 µm Column, we have analyzed a Lys-C digest of a NIST candidate reference material, an IgG1K mAb. In this testing, a focused gradient was used along with intrinsic peptide fluorescence instead of low wavelength UV detection so that higher signal-to-noise could be achieved in the obtained chromatograms. The glycopeptide that originates from the Fc domain of a mAb will contain a tryptophan residue upon Lys-C cleavage, which in large part makes this detection mechanism feasible. Three fluorescence chromatograms obtained for the Lys-C glycopeptides from the NIST IgG1K are presented in Figure 7. These three chromatograms were obtained from the use of the ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm as well as two commercially available alternatives (Columns A and B). Peak capacities have been measured for each specific column using the retention windows demarcated by the most extreme glycopeptide retention times (*) and the half-height peak widths of the K16+FA2, K16+FA2G1, K16+FA2G1', K16+FA2G2, and K16+FA2G2Ga1 peaks. This analysis shows that these columns exhibit strikingly different resolving power. With an effective peak capacity of 72.8, the Glycoprotein BEH Amide column shows a superior peak capacity and performance increases over the alternative amide column technologies of 40 and 96%.

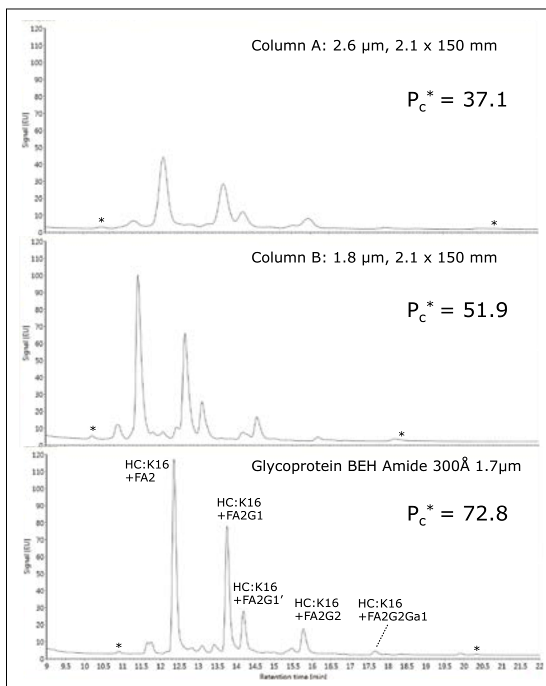


Figure 7. Lys-C glycopeptide mapping of an IgG1K using fluorescence detection and various 2.1 x 150 mm columns packed with amide bonded stationary phase: a Competitor Column A: 150Å 2.6 µm, 2.1 x 150 mm (Top), a Competitor Column B: 1.8 µm, 2.1 x 150 mm (Middle), and on an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm column (Bottom). Peak capacities were calculated based on the half-height peak widths of the labeled glycopeptides and the retention window established by the earliest and latest eluting glycopeptide species, marked with asterisks(*). Comparative separations may not be representative in all applications.

CONCLUSIONS

Glycopeptide mapping of glycoproteins presents a highly effective technique that can be used to elucidate both domain and peptide-specific glycosylation. In this work, we have demonstrated the use of an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column to obtain HILIC separations of glycopeptides that complement the chromatographic information afforded by a reversed phase separation. In addition, our results indicate that these HILIC separations provide exemplary peak capacity in comparison to other commercially available amide column technologies. That the HILIC separation is MS-compatible means that information-rich data can be readily acquired to characterize a glycopeptide map. For instance, this work shows that it can be a relatively straightforward exercise to characterize multidomain protein glycosylation, such as the Fc and Fab domain glycosylation of cetuximab. Combined with other recently developed strategies, such as HILIC subunit mapping and GlycoWorks *Rapi*Fluor-MS released N-glycan analyses, glycopeptide mapping with the ACQUITY UPLC Glycoprotein BEH Amide Column shows significant promise for facilitating the characterization of protein glycosylation to unprecedented levels of detail.

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[APPLICATION NOTE]

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Mapping IgG Subunit Glycoforms Using HILIC and a Wide-Pore Amide Stationary Phase

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved HILIC separations of IgG subunit glycoforms.
- MS-compatible HILIC to enable detailed investigations of sample constituents.
- Orthogonal selectivity to conventional reversed-phase (RP) separations for enhanced characterization of hydrophilic protein modifications.
- Domain-specific glycan information that complements profiling glycosylation by *Rapi*Fluor-MS released N-glycan analyses.
- Glycoprotein BEH amide, 300Å, 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility.

WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide, 300Å Column

Glycoprotein Performance Test Standard

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY UPLC H-Class Bio System

Xevo® G2 QTof Mass Spectrometer

SYNAPT® G2-S HDMS

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, Glycans, Glycosylated Proteins, Glycosylation, HILIC, IdeS

INTRODUCTION

Without question, the most successfully exploited protein modality for therapeutic applications has been monoclonal antibodies (mAbs), which currently account for nearly half of the biopharmaceutical market.[1] An intriguing characteristic of mAbs, in particular IgG-based mAbs, is that they are formed by the linking of two identical light chains and two identical heavy chains through disulfide bonding and non-covalent interactions. Moreover, the resulting mAb structure exhibits functionally significant subunits, for instance one crystallizable fragment (Fc domain) and two equivalent antigen binding fragments (Fab domains). In what is commonly referred to as a middle-up or middle-down analysis,[2-5] native mAbs can be proteolyzed into these and other related subunits enzymatically, as a means to perform cell-based studies and to facilitate characterization. One increasingly popular way to produce subunit digests of mAbs is via the IdeS protease (Immunoglobulin Degrading Enzyme of S. *pyogenes*).[2,6] IdeS cleaves with high fidelity at a conserved sequence motif in the hinge region of humanized mAbs to cleanly produce, upon reduction, three 25 kDa mAb fragments that are amenable to mass spectrometry and useful for localizing different attributes of therapeutic mAbs (Figure 1).[3] IdeS digestion combined with reversed-phase (RP) chromatography has, in fact, been proposed as a simple identity test for mAbs and fusion proteins, because IdeS produced subunits from different drug products will exhibit diagnostic RP retention times.[3] Additionally, RP techniques have been shown to be useful in assaying and obtaining domain specific information about oxidation, since RP retention can be dramatically affected by the oxidation of protein residues, such as methionine.[3]

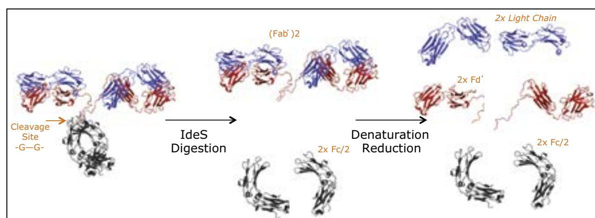


Figure 1. IdeS digestion and reduction scheme for preparing IgG LC, Fd', and Fc/2 subunits.

EXPERIMENTAL

Sample description

IdeS digestion and reduction of mAbs:

Formulated trastuzumab was diluted 7 fold into 20 mM phosphate (pH 7.1) and incubated at a concentration of 3 mg/mL with IdeS (Promega, Madison, WI) for 30 minutes at 37 °C at a 50:1 w/w ratio of trastuzumab to IdeS. The resulting IdeS-digested antibody was denatured and reduced by the addition of 1M TCEP (tris(2-carboxyethyl)phosphine) and solid GuHCl (guanidine hydrochloride). The final buffer composition for the denaturation/reduction step was approximately 6 M GuHCl, 80 mM TCEP, and 10 mM phosphate (pH 7.1). IdeS-digested trastuzumab (1.5 mg/mL) was incubated in this buffer at 37 °C for 1 hour. An IdeS digested, reduced sample of an IgG1K mAb obtained from NIST as candidate reference material #8670 (lot #3F1b) was prepared in the same manner.

Cetuximab IdeS/carboxypeptidase B digestion and reduction:

Prior to digestion with IdeS,[10] cetuximab was treated with carboxypeptidase B to complete the partial removal of the lysine-C-terminal residues that is typical of the antibody.[4] Formulated cetuximab was mixed with carboxypeptidase B (223 μ/mg, Worthington, Lakewood, NJ) at a ratio of 100:1 (w/w), diluted into 20 mM phosphate (pH 7.1), and incubated at a concentration of 1.8 mg/mL for 2 hours at 37 °C. The carboxypeptidase B treated cetuximab was then added to 100 units of IdeS and incubated for 30 minutes at 37 °C. The resulting IdeS digest was denatured and reduced by the addition of 1 M TCEP and solid GuHCl. The final buffer composition for the denaturation/reduction step was approximately 6 M GuHCl, 80 mM TCEP, and 10 mM phosphate (pH 7.1). IdeS-digested cetuximab (0.9 mg/mL) was incubated in this buffer at 37 °C for 1 hour.

Preparation of *Rapi*Fluor-MS Labeled N-Glycans from Cetuximab:

*Rapi*Fluor-MS labeled N-glycans were prepared from cetuximab using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606) according to the guidelines provided in its Care and Use Manual (715004793).

Method conditions

(unless otherwise noted)

Column conditioning

ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 μm columns (as well as other amide columns intended for glycoprotein separations) should be conditioned via two sequential injections and separations of 40 μg Glycoprotein Performance Test Standard (p/n 186008010; 10 μL injections of 4 mg/mL in 0.1% trifluoroacetic acid [TFA], 80% acetonitrile [ACN]) or with equivalent loads of a sample for which the column has been acquired. The separation outlined by the following method can be employed for conditioning with the Glycoprotein Performance Test Standard.

Column conditioning gradient:

Column dimensions: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time (min)	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	33.0	67.0	6
21.0	40.0	60.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

LC conditions for IgG subunit separations

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 5 °C

Analytical column temp.: 45 °C (trastuzumab and NIST IgG1K subunit HILIC separations)

60 °C (cetuximab subunit HILIC separations)

80 °C (trastuzumab reversed phase (RP) subunit separations)

Flow rate: 0.2 mL/min

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

UV detection: 214 nm, 10 Hz

Injection volume: ≤1.2 µL (aqueous diluents). Note: It might be necessary to avoid high organic diluents for some samples due to the propensity for proteins to precipitate under ambient conditions. A 2.1 mm I.D. column can accommodate up to a 1.2 µL aqueous injection before chromatographic performance is negatively affected.

Waters columns: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard);

ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742);

ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 186004497)

Other columns: Agilent® AdvanceBio Glycan Mapping, 1.8 µm, 2.1 x 150 mm;

Thermo Scientific® Accucore™ Amide 150 HILIC, 150Å 2.6 µm, 2.1 x 150 mm

Vials: Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL volume (p/n 186002640)

Gradient used for reversed-phase (RP) separations of trastuzumab subunits (Figure 2A):

Time (min)	%A	%B	Curve
0.0	95.0	5.0	6
1.0	66.7	33.3	6
21.0	59.7	40.3	6
22.0	20.0	80.0	6
24.0	20.0	80.0	6
25.0	95.0	5.0	6
35.0	95.0	5.0	6

Gradient used for HILIC separations of IgG subunits (Figures 2–7):

Time (min)	%A	%B	Curve
0.0	20.0	80.0	6
1	30.0	70.0	6
21	37.0	63.0	6
22	100.0	0.0	6
24	100.0	0.0	6
25	20.0	80.0	6
35	20.0	80.0	6

MS conditions for IgG subunit separations

MS system: Xevo G2 QTof or SYNAPT G2-S HDMS

Ionization mode: ESI+

Analyzer mode: Resolution (~20 K)

Capillary voltage: 3.0 kV

Cone voltage: 45 V

Source temp.: 150 °C

Desolvation temp.: 350 °C

Desolvation gas flow: 800 L/Hr

Calibration: NaI, 2 µg/µL from 500–5000 *m/z*

Acquisition: 500–4000 *m/z*, 0.5 sec scan rate

Data management: MassLynx® Software (v4.1)/UNIFI V1.7

LC conditions for *Rapi*Fluor-MS Released N-Glycan HILIC separations:

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 60 °C

Fluorescence detection: Ex 265/Em 425 nm (*Rapi*Fluor-MS) (5 Hz scan rate [50 mm column], Gain =1)

Injection volume: 10 µL (DMF/ACN diluted sample)

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Columns: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186004740)

Vials: Polypropylene 12 x 32mm, 300 µL, Screw Neck Vial, (p/n 186002640)

Gradient used for *Rapi*Fluor-MS N-Glycan HILIC Separations (Figure 7B):

Time (min)	Flow Rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

MS conditions for *Rapi*Fluor-MS N-Glycan HILIC separations

MS system:	SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	TOF MS, resolution mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–2500 *m/z*
Lockspray (ASM B-side):	100 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water every 90 seconds
Acquisition:	500–2500 *m/z*, 1 Hz scan rate
Data management:	MassLynx Software (v4.1)

It should, however, be kept in mind that many IgG modifications more strongly elicit changes in the hydrophilicity of a mAb along with its capacity for hydrogen bonding. A very obvious example of this type of modification is glycosylation. Glycans released from a mAb are very often profiled by hydrophilic interaction chromatography (HILIC), in which case an amide bonded stationary phase has historically been used, because it affords high retentivity as a consequence of its hydrophilicity and propensity for hydrogen bonding.[7] Here, we propose that HILIC with an amide bonded stationary phase also be considered for IgG subunit separations. For such an application, a stationary phase with a wide average pore diameter is critical, so that large subunit structures will have access to the majority of the porous network and be less prone to restricted diffusion while eluting through a column.[8-9] Through the development of a sub-2-µm wide-pore amide stationary phase, we have facilitated a novel and complementary workflow to RP based subunit analyses. In this application note, we demonstrate the use of a glycoprotein BEH amide, 300Å, 1.7 µm column to develop LC-MS and LC-UV techniques that can be used to rapidly profile domain specific information about the N-linked glycosylation of IgG molecules.

RESULTS AND DISCUSSION

Orthogonal, complementary IgG subunit separations

To demonstrate a conventional approach to IgG subunit mapping, we first analyzed a reduced\IdeS digest of an IgG1 mAb using a RP chromatographic separation with a wide-pore C4 bonded stationary phase (Protein BEH C_4, 300Å, 1.7 µm). The IgG1 mAb selected for this work was trastuzumab, given its prominence as a first generation mAb drug product and a potential target for biosimilar development.[11] Figure 2A shows a UPLC chromatogram that is typical for reduced, IdeS-digested trastuzumab, wherein three peaks are near equally spaced with an elution order corresponding to the Fc/2, LC and Fd' subunits, respectively. The conditions to produce this high resolution separation entail the use of TFA for ion-pairing. Interestingly, the same mobile phases have proven to be optimal for protein HILIC, as they reduce the hydrophilicity of protein residues by masking them via a hydrophobic ion pair. This, in turn, leads to improved selectivity for hydrophilic modifications.[12] That is, an orthogonal method to the RP separation can be achieved via HILIC by simply reversing a gradient and using a newly developed wide-pore amide bonded stationary phase (glycoprotein BEH Amide, 300Å, 1.7 µm).

An example of a chromatogram obtained from a column packed with this wide-pore amide material is shown in Figure 2B. Here, the same reduced, IdeS digested trastuzumab is separated into approximately 10 peaks. The first two eluting peaks correspond to the Fd' and LC subunits, while the remaining, more strongly retained peaks correspond to the glycoforms of the Fc/2 subunit. By focusing on the more strongly retained peaks, an analyst can elucidate information about the heterogeneity of glycosylation (Figure 3A). Given that this is a method with volatile mobile phases, the glycoform peaks can be readily interrogated by ESI-MS. Deconvoluted mass spectra and molecular weights corresponding to species in the glycoform profile are presented in Figures 3B and 3C. In Figure 3, chromatographic peaks are labeled with the same color as their corresponding mass spectra. Notice that this HILIC separation facilitates producing deconvoluted mass spectra for individual glycoforms with limited interference between similar molecular weight species, for instance the Fc/2+A2G1 versus the Fc/2+FA2 species (orange versus blue spectrum). In a first pass analysis, all glycan species from trastuzumab that are known to be present at a relative abundance greater than 2% are readily detected.[13] It should be noted that lower abundance species, such as Fc/2+M5 (Man5), are also detected and can be observed by extracted ion chromatograms (XICs). This indicates there is a possibility to perform selected reaction monitoring (SRM) MS analyses when and if there is a need to monitor particular low abundance structures. While it is not resolved under these conditions, the M5 Fc/2 glycoform is resolved in a different example separation (see below, Figure 7A).

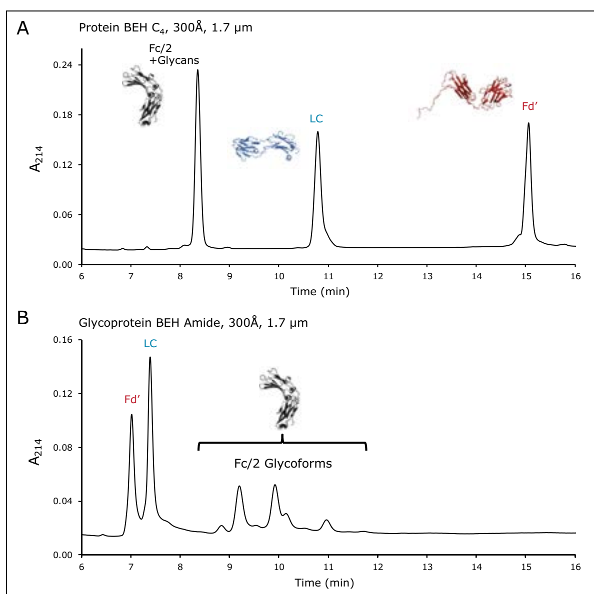
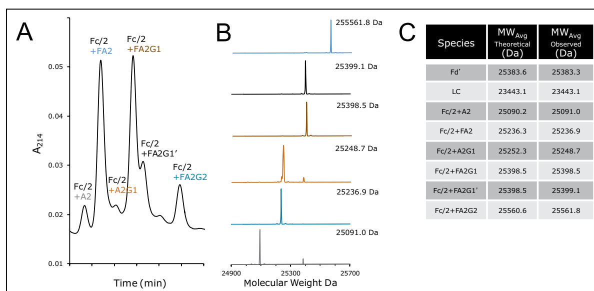


Figure 2. Trastuzumab subunit separations. (A) 1 µg of reduced, IdeS digested separated using an ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm Column (0.7 µL aqueous injection). (B) 1 µg of reduced, IdeS digested separated using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column (0.7 µL aqueous injection).



Species	MW Avg Theoretical (Da)	MW Avg Observed (Da)
Fd'	25383.6	25383.3
LC	23443.1	23443.1
Fc/2+A2	25090.2	25091.0
Fc/2+FA2	25236.3	25236.9
Fc/2+A2G1	25252.3	25248.7
Fc/2+FA2G1	25398.5	25398.5
Fc/2+FA2G1'	25398.5	25399.1
Fc/2+FA2G2	25560.6	25561.8

Figure 3. Profiling trastuzumab Fc/2 subunit glycoforms. (A) Retention window from Figure 2B corresponding to the glycoform separation space. (B) Deconvoluted ESI mass spectra for the HILIC chromatographic peaks. Chromatographic peaks are labeled with the same color as their corresponding mass spectra. (C) Molecular weights for the observed trastuzumab subunits.

Batch-to-batch analysis of trastuzumab Fc/2 glycosylation by HILIC-UV profiling

Clearly, data generated by subunit-level HILIC-MS are very information-rich. Optical detection based subunit HILIC separations can be equally informative. To this end, we have applied a HILIC-UV method to perform batch-to-batch analysis of trastuzumab Fc/2 glycosylation, as exemplified in Figure 4. Two example HILIC chromatograms for Fc/2 glycoforms obtained from two different lots of trastuzumab are shown in Figure 4A. Previous testing on these lots has demonstrated differences in glycosylation at the released glycan level.[14] Here, by integration of peaks across the profile, we have found that the two lots of trastuzumab indeed differ with respect to their Fc domain glycosylation profiles, in ways consistent with the mentioned released glycan assays. In particular, these lots of trastuzumab differ with respect to their extents of terminal galactosylation, as estimated from the abundances of FA2, FA2G1, and FA2G2 Fc/2 subunits (Figure 4B). This is an informative observation, since the extent of galactosylation can affect complement-dependent cytotoxicity (CDC).[15]

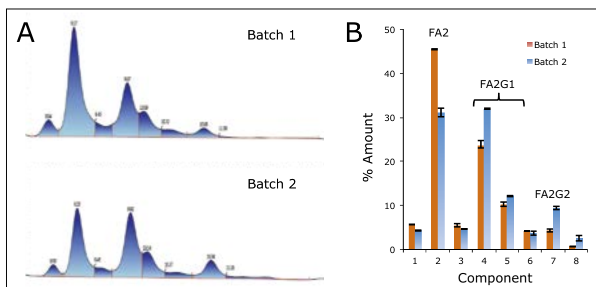


Figure 4. Batch-to-batch profiling of trastuzumab Fc/2 subunit glycoforms. (A) HILIC chromatograms of trastuzumab Fc/2 subunit glycoforms from two different lots of drug product. (B) Relative abundances of the major sample components. Analyses were performed in triplicate using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column.

Lifetime testing of glycoprotein BEH amide 300Å, 1.7µm columns for profiling IgG subunit glycoforms

The ability of a BEH amide, 300Å, 1.7 µm column to robustly deliver the above mentioned separations over time was tested by performing a series of experiments involving a single column being subjected to 300 sequential injections of a reduced, IdeS digested trastuzumab sample. This was a potentially challenging use scenario given that the reduced, IdeS digested mAb sample contains both high concentrations of guanidine denaturant and TCEP reducing agent. Total ion chromatograms corresponding to the 20th, 180th, and 300th injections of this experiment are displayed in Figure 5A. In these analyses, particular attention was paid to the half-height resolution of the Fc/2+A2 and Fc/2+FA2 species, which was assessed every 20th separation using extracted ion chromatograms (XICs).

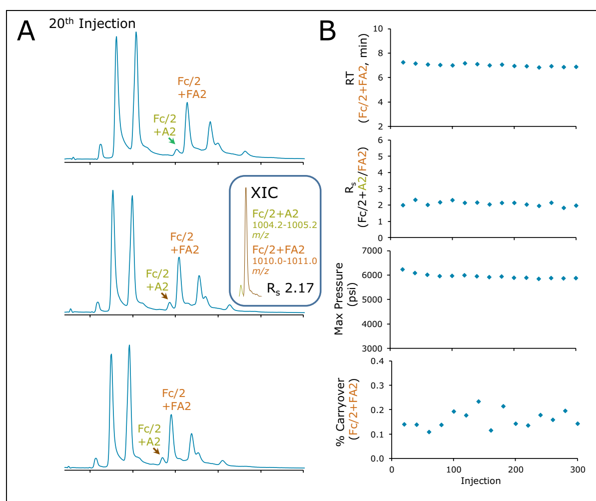


Figure 5. Lifetime testing of an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column for sequential injections of reduced, IdeS digested trastuzumab. (A) Total ion chromatograms (TICs) from the 20th, 180th, and 300th injections. Example extracted ion chromatograms (XICs) for Fc/2+A2 and Fc/2+FA2 that were used to measure resolution. (B) Chromatographic parameters observed across the 300 injection lifetime test. Each panel shows results for each 20th injection, including retention time (RT) of the FA2 glycoform, R_s between A2 and FA2 glycoforms, maximum pressure across the run, and % carryover as measured by a repeat gradient and XICs.

In this testing, several additional chromatographic parameters were also monitored, including the retention time of the Fc/2+FA2 species, the maximum system pressure observed during the chromatographic run, and the percent (%) carryover of the most abundant glycoform, the Fc/2+FA2 species (Figure 5B). Plots of these parameters underscore the consistency of the subunit separation across the lifetime of the column. With noteworthy consistency, the column produced relatively stable retention times, a consistent resolution of the A2 and FA2 glycoforms ($R_s \approx 2$), a maximum system pressure consistently at only ~6 Kpsi, and a remarkably low carryover between 0.1 and 0.2%. This latter aspect of the HILIC separations is particularly noteworthy since it indicates that carryover with these methods is almost an order of magnitude lower than analogous C4 based RP methods.

Benchmarking the capabilities of the glycoprotein BEH amide, 300Å, 1.7 µm column

We have benchmarked the performance of this new wide-pore column technology against not only its standard pore diameter analog but also its two most closely related, commercially-available alternatives. Figure 6 presents chromatograms obtained for a reduced, IdeS digested sample of an IgG1K mAb acquired from NIST using these different column technologies. In a visual comparison, it is clear that the glycoprotein BEH amide 300Å column significantly outperforms the other three columns. To quantify this assessment, peak-to-valley ratios were calculated for the separation of the FA2 glycoform away from the FA2G1 glycoform. The glycoprotein BEH amide 300Å column was found to demonstrate improvements of 48%, 152%, and 261% over the 130Å glycan BEH amide, the Accucore amide, and AdvanceBio glycan mapping columns. This mAb sample also has a particularly interesting attribute in that it has a reasonably high relative abundance of an immunogenic alpha-1,3-galactose containing glycan (an FA2G2Ga1 structure).[16-17] As shown in Figure 6, this Fc/2+FA2G2Ga1 species can be readily visualized with the wide-pore amide column. This represents a sizeable improvement in the peak capacity of large molecule HILIC separations for this emerging application.

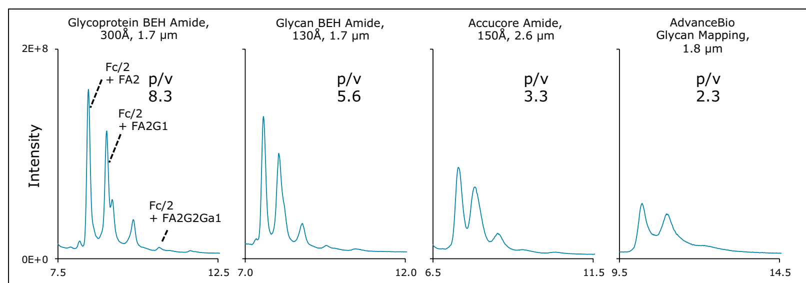
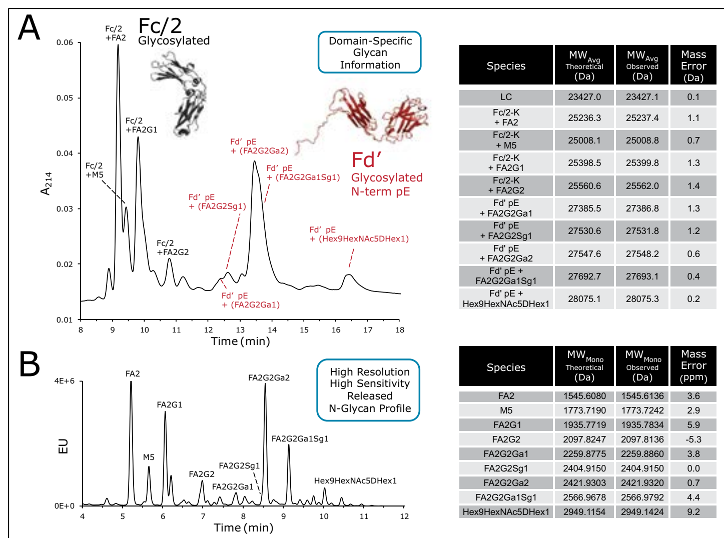


Figure 6. Subunit glycoform profiles of an IgG1K obtained with various 2.1 x 150 mm columns: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column, ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Column, Thermo Scientific Accucore Amide, 150Å, 2.6 µm column, and Agilent AdvanceBio Glycan Mapping, 1.8 µm column. Peak-to-valley (p/v) ratios for the Fc/2+FA2 versus FA2G1 glycoforms are provided. An alpha gal containing Fc/2+FA2G2Ga1 is readily visualized with the glycoprotein BEH amide, 300Å, 1.7 µm column.

Complementing *Rapi*Fluor-MS N-glycan analyses with domain specific information about mAb glycosylation

One of the key advantages to profiling IgG subunits by HILIC is being able to elucidate domain specific information about glycosylation. In an IgG structure, there exists one conserved N-glycosylation site at Asn297 of the heavy chain. As a consequence, most IgGs will be modified with two glycans in the CH2 domains (constant heavy chain 2 domains) of the Fc subunit. However, it is estimated that 20% of human IgGs are also modified in their CH1 domains, which reside in the Fab subunits, and more specifically the IdeS generated Fd' subunit.[18-19] For example, it is known that cetuximab, a chimeric mAb expressed from a murine cell line, is glycosylated in both its CH1 and CH2 domains.[20] Characterization of this mAb has thus proven to be an interesting case study for the application of our newly developed techniques. HILIC separations obtained for a reduced, IdeS digested sample of carboxypeptidase B treated cetuximab showed only one weakly retained subunit species, which could be easily assigned to the LC subunit by online ESI-MS (data not shown). Furthermore, and as shown in Figure 7A, the glycoform retention window for cetuximab was populated with twice as many peaks as had been observed for trastuzumab and its glycosylated Fc/2 subunit. Deconvoluted ESI-MS data from these HILIC-MS separations confirmed that the first grouping of peaks (labeled in gray) corresponded to Fc/2 glycoforms and typical mAb glycan species, such as FA2, FA2G1, M5, and FA2G2. Meanwhile, the second grouping of peaks were found to be distinctively related to glycoforms of the Fd' subunit given their unique masses. Curiously, each of the identified Fd' glycoforms (labeled in red) are immunogenic in nature, containing either non-human alpha-1,3-galactose or non-human N-glycolyl-neuraminic acid epitopes.[21]

The identification of these glycan species has been confirmed through released N-glycan analyses. Using the newly developed GlycoWorks *Rapi*Fluor-MS N-Glycan Kit,[22] cetuximab N-glycans were rapidly prepared and labeled with the novel fluorescence and MS-active labeling reagent, *Rapi*Fluor-MS. The resulting labeled N-glycans were subsequently separated using a glycan BEH amide, 130Å, 1.7 µm column and detected by fluorescence and positive ion mode ESI-MS, as portrayed in Figure 7B. The sensitivity gains afforded by the *Rapi*Fluor-MS label facilitated making confident assignments of the released N-glycan structures. The species that have been assigned as a result of both this released glycan analysis as well as the subunit HILIC-UV-MS method are supported by previous reports on cetuximab glycosylation.[6,20]



Species	MW Avg Theoretical (Da)	MW Avg Observed (Da)	Mass Error (Da)
LC	23427.0	23427.1	0.1
Fc/2-K + FA2	25236.3	25237.4	1.1
Fc/2-K + M5	25008.1	25008.8	0.7
Fc/2-K + FA2G1	25398.5	25399.8	1.3
Fc/2-K + FA2G2	25560.6	25562.0	1.4
Fd' pE + FA2G2Ga1	27385.5	27386.8	1.3
Fd' pE + FA2G2Sg1	27530.6	27531.8	1.2
Fd' pE + FA2G2Ga2	27547.6	27548.2	0.6
Fd' pE + FA2G2Ga1Sg1	27692.7	27693.1	0.4
Fd' pE + Hex9HexNAc5DHex1	28075.1	28075.3	0.2

Species	MW Mono Theoretical (Da)	MW Mono Observed (Da)	Mass Error (ppm)
FA2	1545.6080	1545.6136	3.6
M5	1773.7190	1773.7242	2.9
FA2G1	1935.7719	1935.7834	5.9
FA2G2	2097.8247	2097.8136	-5.3
FA2G2Ga1	2259.8775	2259.8860	3.8
FA2G2Sg1	2404.9150	2404.9150	0.0
FA2G2Ga2	2421.9303	2421.9320	0.7
FA2G2Ga1Sg1	2566.9678	2566.9792	4.4
Hex9HexNAc5DHex1	2949.1154	2949.1424	9.2

Figure 7. HILIC Profiling of cetuximab glycosylation. (A) HILIC-UV chromatogram of reduced, IdeS/carboxy peptidase B-digested cetuximab obtained using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. Species corresponding to Fc/2 and Fd' subunits are labeled in gray and red, respectively. Subunit glycan assignments based on deconvoluted mass spectra are provided. (B) HILIC-fluorescence chromatograms of RapiFluor-MS labeled N-glycans from cetuximab obtained using an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm Column. Mass spectral data supporting the assignments of the RapiFluor-MS labeled N-glycans are provided.

With the combination of released glycan and subunit-derived glycan information, cetuximab glycosylation has been characterized with significant detail. With the *Rapi*Fluor-MS released glycan analysis, a very high resolution separation has been achieved with an LC-MS compatible method in which glycans can even be subjected to detailed MS/MS analyses. With an equally MS-compatible subunit HILIC separation, domain-specific glycan information has been readily obtained with minimal sample preparation. Each method has therefore provided complementary information on the glycosylation of the mAb. Nevertheless, the widepore amide HILIC method stands out as a useful technique for rapidly screening mAbs for multidomain glycosylation.

CONCLUSIONS

Subunit analyses of mAbs represent a useful strategy for rapidly investigating domain-specific modifications. The combination of high fidelity IdeS proteolysis with high resolution LC-UV-MS has presented a new approach to mAb identity testing and assaying oxidation.[3] The current subunit mapping strategies have exclusively relied upon reverse phase chromatography. However, since N-linked glycosylation of IgG proteins elicit dramatic changes in hydrophilicity and hydrogen bonding characteristics, a separation by hydrophilic interaction chromatography (HILIC) can be effectively used for this application or as a complementary method to reversed-phase separations since the same mobile phases can be employed. For this reason, we have proposed the use of HILIC with an amide bonded stationary phase that has been optimized for large molecule separations, the wide-pore glycoprotein BEH amide, 300Å, 1.7 µm stationary phase. Along with new developments in released N-glycan analysis afforded by *Rapi*Fluor-MS,[22] the glycoprotein BEH amide, 300Å, 1.7 µm column enables new possibilities for routine monitoring and detailed characterization of mAb glycosylation, including elucidation of domain-specific glycan information.

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Structural Comparison of Infliximab and a Biosimilar via Subunit Analysis Using the Waters Biopharmaceutical Platform with UNIFI

Henry Shion and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

A streamlined workflow within an integrated UPLC®-MS/MS system solution that features automated data acquisition, processing, and reporting, and that is deployable to both regulated and non-regulated laboratories, enables efficient structural analysis and comparative analysis of multiple batches of an innovator product and its biosimilar candidate within a comparability study.

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ACQUITY UPLC® H-Class Bio System

Xevo® G2-S QTof

ACQUITY UPLC Tunable UV Detector

UNIFI Scientific Information System

ACQUITY UPLC Protein BEH C_4 Column

KEY WORDS

Biosimilar, intact mass analysis, intact mass subunit analysis, light chain, heavy chain, glycosylation, glycoprofile, infliximab, mAb, biotherapeutic characterization

INTRODUCTION

The expiration of patents and other intellectual property rights for originator biologics over the next decade opens up ample opportunities for biosimilars to enter the market and push industry competition to a high level.[1-4]

Compared to small molecule drugs, biopharmaceuticals have much more complex structures and are more expensive to develop. The complexity of the biopharmaceutical molecular entity puts greater challenges on organizations seeking to manufacture safe and effective biosimilar products for patients. Regulatory bodies such as the U.S. FDA and EMA[5-8] require a demonstration of comprehensive characterization for the drug substance: Confirming primary sequence and identifying post-translational modifications (PTMs), establishing biophysical and functional comparability for the innovator and candidate biosimilar, and performing studies that establish expected variation within an innovator biotherapeutic.

Infliximab (Remicade) is a monoclonal antibody (mAb) used to treat autoimmune diseases; it was first approved by the FDA for the treatment of Crohn's disease in 1998, and in 2013 two biosimilars have been submitted for approval in Europe.

In this application note, we characterize infliximab and a biosimilar candidate, produced in a different cell line, using Waters® Biopharmaceutical Platform Solution with UNIFI Scientific Information System. The objective is to screen multiple lots of both the innovator and biosimilar products at the subunit level (light chain (LC) and heavy chain (HC)) to establish comparability at this higher level of structure. Lot-to-lot and batch-to-batch comparisons will show product variation, illustrating the range of quality attributes to be considered in a candidate biosimilar.

EXPERIMENTAL

Biopharmaceutical Platform Solution with UNIFI

- ACQUITY UPLC H-Class Bio System
- Xevo G2-S QTof
- ACQUITY UPLC Tunable UV Detector
- UNIFI Scientific Information System

Intact protein LC-MS conditions

Column: ACQUITY UPLC Protein BEH C_4 Column, 300Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186004495)

Column temp.: 80 °C

Mobile phase A: Water

Mobile phase B: Acetonitrile

Mobile phase C: Not used

Mobile phase D: 0.5% TFA (in water)

Detection: UV 280 nm

LC gradient table:

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.20	65.2	29.8	0	5.0	Initial
12.0	0.20	63.5	31.5	0	5.0	6
14.0	0.20	63.5	31.5	0	5.0	6
14.1	0.20	10.0	85.0	0	5.0	6
15.1	0.20	10.0	85.0	0	5.0	6
15.2	0.20	65.2	29.8	0	5.0	6
18.0	0.20	65.2	29.8	0	5.0	6

Total run time: 20.0 min

MS conditions

Capillary: 3.0 kV

Sampling cone: 80 V

Extraction cone: 4 V

Source temp.: 125 °C

Desolvation temp.: 350 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 800 L/Hr

Data acquisition and processing

MaxEnt™ 1 for MS spectra deconvolution

UNIFI Scientific Information System

Sample preparation

Three batches of innovator infliximab were acquired from Jenssen Biotech, Inc. (Horsham, PA, USA). The batches were produced by the SP2/0 mouse cell line. Three batches of candidate biosimilar infliximab produced by an alternative mammalian cell line (Chinese hamster ovary (CHO) were obtained from a third-party collaborator. All of the samples were stored at -80 °C before analysis.

A reduction buffer solution containing 25 mM NaCl, 25 mM Tris, 1 mM EDTA (pH 8.0) was made to prepare mAb subunits. For each of the six batches, 10 µL of formulated mAb solution (21 mg/mL, the commonly used concentration level for patient injection) was mixed with 180 µL of reduction buffer in a 1.5 mL Eppendorf tube for a protein concentration of 1.0 mg/mL. A concentrated dithiothreitol (DTT) solution (100 mM in H_2O) was then added to each solution to obtain a final DTT concentration of 1.0 mM. The samples were incubated at 37 °C for 20 minutes. The samples were briefly centrifuged, then 105 µL of each sample was mixed with an equal volume of aqueous solution containing 3% acetonitrile and 0.1% formic acid. The final concentration of the mAb was about 0.5 mg/mL. Triplicate injections of each sample were made onto an ACQUITY UPLC Protein BEH C_4 Column, 300Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186004495) LC-MS analysis of the mAb subunit.

RESULTS AND DISCUSSION

Subunit characterization for Infliximab from two cell lines

Figure 1 shows the reversed-phase LC-MS chromatograms from the analysis of reduced infliximab from both the innovator and biosimilar products. There are two major components to each chromatogram, a peak at ~3.5 minutes and a complex set of peaks at ~10 minutes. The chromatographic peaks eluting around 3.5 minutes have ESI-MS measurements of 23434.0 Da, respectively, in full agreement with the calculated mass of the light chain of infliximab (23434.0 Da). The complex peak eluting at ~10 minutes is comprised of several species with MW in the 51,000 Da range, corresponding to the glycosylated heavy chain.

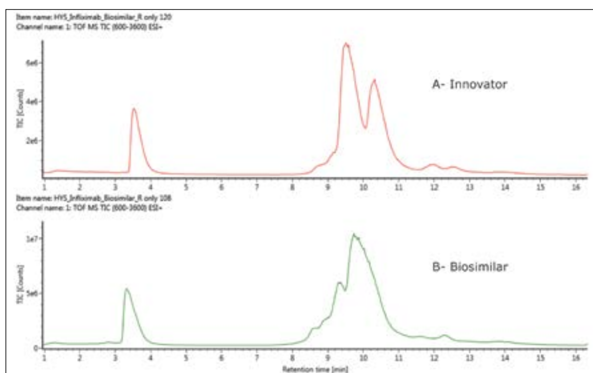

Figure 1. Reversed-phase (C_4) chromatograms of the innovator infliximab (top) and a biosimilar infliximab (bottom). The signal trace is the Total Ion Current (TIC) from the mass spectrometer.

Figure 2 shows the comparison of the light chain spectra in a mirror plot using UNIFI Scientific Information System's software, with the combined raw MS spectra shown on the left panel (as demonstrated by multiple charged spectrum envelopes) and the MaxEnt 1 deconvoluted spectra displayed on the right.

The results indicate that there is only one isoform and no noticeable difference in the light chains between the innovator and biosimilar samples. This observation is consistent with other IgG1 biosimilar studies[9] that show little or no post-translational modifications of LC subunits.

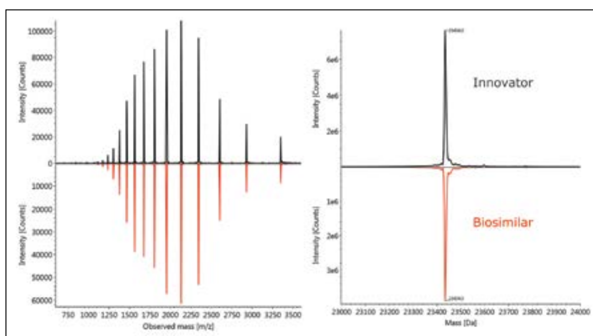

Figure 2. The combined raw MS spectra and deconvoluted spectra in mirror image plots. The MS spectra of the light chain (eluting around 3.5 min in Figure 1) from the innovator sample (top) and the biosimilar sample (bottom) are displayed.

The chromatographic profile of the heavy chains of infliximab was more complicated than that of the light chain. A cluster of peaks is observed around 10 minutes in Figure 1, corresponding to different isoforms of the heavy chains, and they exhibit significantly different chromatographic behavior from that of the light chain. Similarly, as shown by Figure 1 and 3, the heavy chains of infliximab from the two cell lines show quite distinct chromatographic and spectral differences.

The two major chromatographic peaks (at 9.5 min and 10.5 min) from the analysis of reduced innovator infliximab (Figure 1A) come from the heavy chains and appear to have multiple isoforms (Figure 3C). Mass spectrometry analysis of these peaks (Figure 3) shows that variation in both the polypeptide sequence (+/- lysine) and glycosylation contribute to the heterogeneity of the innovator HC.

This is in contrast to the biosimilar sample, which displays a more homogeneous peak at 9.8 minutes (Figure 1B) and fewer mass variants (Figure 3D).

Several major glycoforms (e.g. G0, G0F, G1F, G2F, and Man5) were identified for the innovator heavy chain as shown in Figure 4, demonstrating a high degree of heterogeneity of the innovator infliximab. The biosimilar has three major glycoforms (G0, G0F, and G1F) and no apparent amino acid variations. All of the MS peaks in the deconvoluted spectrum can be automatically identified in UNIFI Scientific Information System's software based on the mAb's reported sequence and the suspected PTM, and annotated, as displayed in Figure 4.

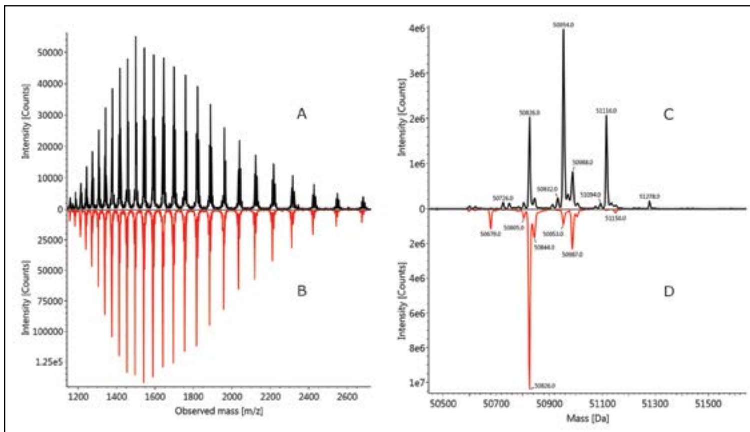

Figure 3. The combined raw MS spectra mirror image comparison between the heavy chains (eluted around 10 min in Figure 1) for the innovator and the biosimilar.

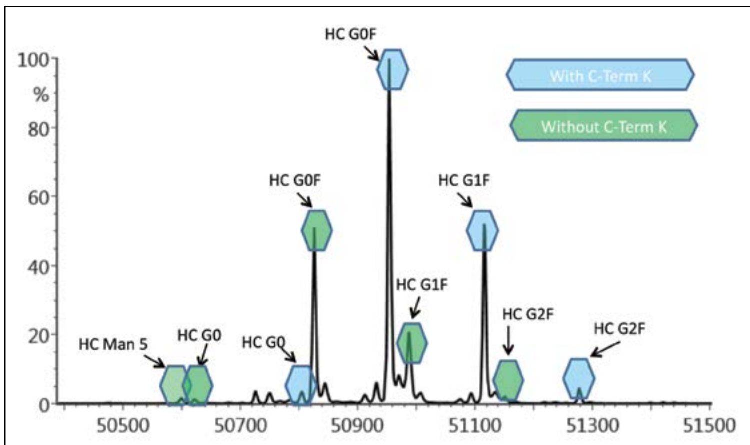

Figure 4. The innovator heavy chain deconvoluted spectra reveals the presence of glycoforms G0, G0F, G1F, G2F, and Man5 as well as lysine variations. Incomplete removal of lysine from the C-terminus of the HC is a known variant for IgG1.

Assessment of batch-to-batch variability

The analysis of reduced IgG is a straightforward, high-sensitivity method that provides valuable information on the identity and amount of related variants of mAb structure. Analysis of the reduced infliximab indicates that its structural heterogeneity resides within the heavy chain of the antibody, and includes variation in both glycosylation and amino acid sequence. The incomplete removal of C-terminal lysine residues is a known structural variant, so it can be surmised that this PTM is occurring in the innovator infliximab.

As demonstrated by the spectra of the HC (Figure 4), the biantennary oligosaccharides G0F, G1F, and G2F, along with smaller amounts of the high mannose forms, are the major glycoforms of infliximab. Since there is only one N-glycosylation site on the HC, the intensity of peaks for the various oligosaccharide structures can be used to quantify the relative abundance of the various glycoforms. The MaxEnt 1 algorithm used for generating the deconvoluted spectra preserves the intensity information from the raw spectra, for quantitative assessment of structural variation.

This measurement establishes a foundation upon which structural comparison for multiple batches of infliximab can be performed, thus making the analysis at the subunit level an attractive approach to establishing development requirements for biosimilars.

On the basis of the analysis of reduced infliximab subunits, we compared the structure differences among multiple batches of infliximab from the two cell lines. Regulatory guidelines for biosimilar development recommend that any analytical characterization first establish the structural variation range of the reference product. As such, analysis of multiple lots of reference products (infliximab from SP2/0 cells) as well as biosimilar products (inflixmab from CHO cells) is necessary to establish the range of values for critical structural features. In the meantime, replicate analysis is also performed for each sample to demonstrate the reproducibility of the LC-MS method itself.

The analysis of multiple samples in triplicate helps establish a vigorous analytical procedure to provide sound analytical support for biosimilar development. However, this approach generates a high volume of data that requires efficient informatics tools to process data and produce meaningful results. The UNIFI Scientific Information System automatically acquires and processes the data and generates reports on the results, demonstrating the great power and flexibility available for such data analysis tasks.

Next, we demonstrate how UNIFI Scientific Information System's software can be utilized to streamline the structural comparison of reduced infliximab from two cell lines.

Structural comparison

Figure 5A displays the MS response summary plot for glycoform G0 in percentage. This UNIFI Scientific Information System plot offers a simple and direct view to demonstrate the variation in relative abundance of the G0 glycoform across the injections of innovator and biosimilar batches. This functionality removes the scientific and compliance burden of summarizing reports of such data in Excel or other data analysis tools that are not core features of the instrument's software. By including both automated processing statistical reporting within UNIFI, the software also prevents human transcription errors that may require significant time and effort to identify and correct. Similar plots can be readily generated within UNIFI Scientific Information System for other glycoforms identified in the analysis, such as G1F and Man5, as shown in Figure 5B and 5C.

The triplicate analysis for each sample shows a highly reproducible measurement. There is some minor batch-to-batch variability, notably in the abundance of G1F in the innovator (5B) as well as the Man5 content in the biosimilar (5C). On the other hand, it appears that the biosimilar, produced in CHO cells, has approximately 10 times more non-fucosylated G0 glycoform compared to that of the innovator (SP2/0 cell line) product. It is also observed that there are about twice as many G1F glycoforms (by percentage) in the biosimilar batches than in the innovator, and there is about 30% more Man5 glycoform in the innovator batches than in the biosimilar sample batches.

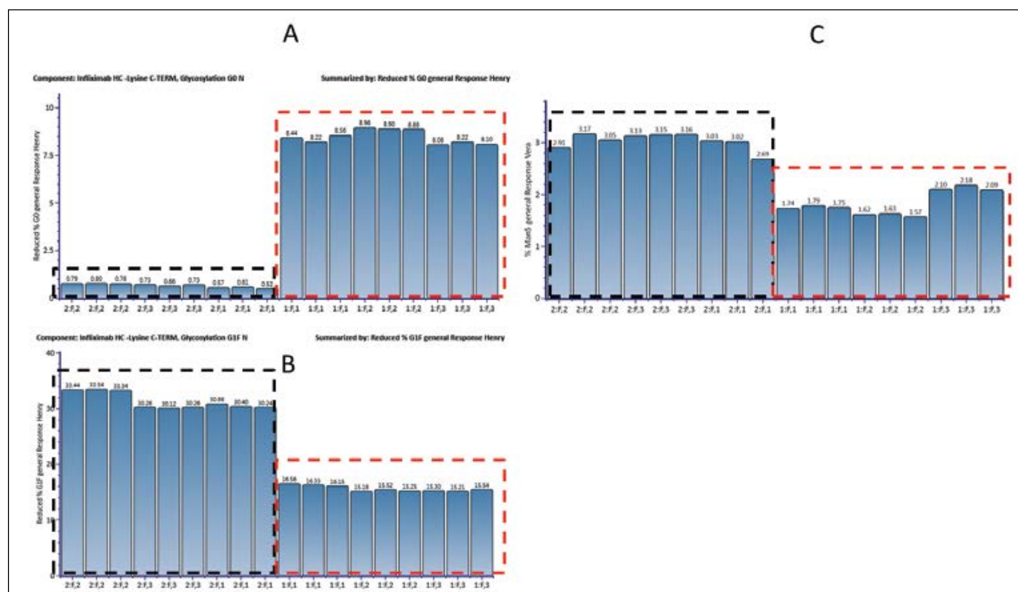

Figure 5. Relative abundance of the G0 (A), G1F (B), and Man5 (C) glycoforms in infliximab HC, from all the injections of the innovator (left, blue) and biosimilar (right, Red) batches.

As this example shows, the glycoforms of infliximab from two cell lines can be readily analyzed and information on the glycosylation variation can be quickly obtained via UNIFI Scientific Information System software's automated workflow covering data acquisition, processing, and reporting. Additionally, the workflow can be deployed in both non-regulated and regulated environments, so a common analytical platform can be employed and consistent information acquired across the entire development process.

Another major source of HC heterogeneity is lysine variants. Depending on the cell line and other production conditions, a lysine residue may remain on the C-terminus of the polypeptide chain. Figure 6 displays the percentage of clipped-lysine variants, automatically calculated in UNIFI Scientific Information System software, for both the innovator and biosimilar batches. As can be seen, the percentage was much smaller for the biosimilar sample batches (from CHO cell line) as compared to that observed in the innovator (SP2/0 cell line) batches. This experimental result confirms that there was a much lower level of C-terminal lysine in antibodies derived from the CHO cell line, and the lysine content is more consistent from batch-to-batch. The innovator infliximab has a lower overall abundance for variants with the complete removal of lysine, and the amount does vary from batch to batch.

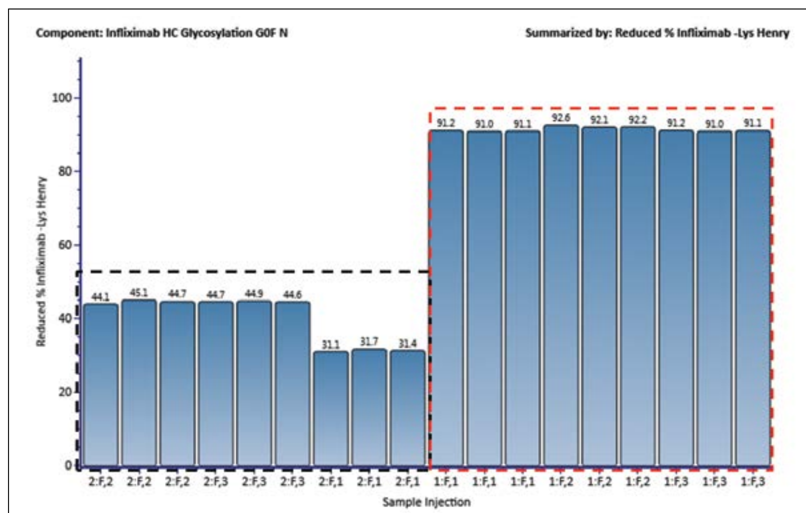

Figure 6. The percentage of clipped-lysine (0K) variants, automatically calculated in UNIFI Scientific Information System, is shown for the innovator (left, blue) and biosimilar (right, red) batches.

CONCLUSIONS

In this work, the extent of comparability was established between multiple batches of innovator and candidate biosimilar infliximab, using an integrated analytical platform with capabilities for automated data processing and reporting. The Biopharmaceutical Platform Solution with UNIFI Scientific Information System was applied to study these samples at the level of reduced heavy and light chain subunits, and to report on several biotherapeutic structural differences between these preparations.

Overall, the innovator molecule exhibited more heterogeneity with respect to PTM's (glycosylation and C-terminal lysine) compared to the candidate biosimilar. Potentially significant differences were found between the innovator and the biosimilar samples, particularly in regard to the presence of fucosylated glycans. We found that the biosimilar had a much higher abundance of the non-fucosylated glycoform G0, and less of the fucosylated G1F, in comparison to the innovator. Some batch-to-batch variability was observed among both the innovator batches and the biosimilar batches.

The power to universally deploy high resolution analytics to address these important questions, combined with the ability to quickly communicate these results, enables organizations to make rapid and confident decisions in the race to market with safe and effective innovator and biosimilar therapeutics.

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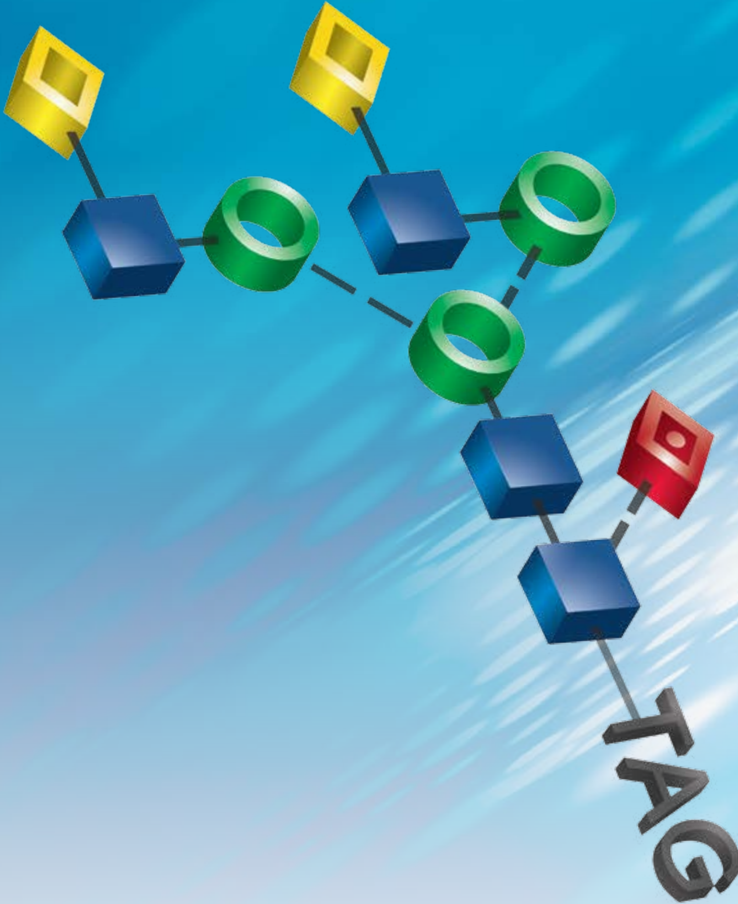
RELEASED N-GLYCANS



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Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent

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[1] Waters Corporation, Milford, MA, USA
[2] New England BioLabs, Ipswich, MA, USA

APPLICATION BENEFITS

- Preparation of labeled *N*-glycans (from glycoprotein to analysis ready sample) in 30 minutes
- Complete deglycosylation to produce unbiased results
- Simple, streamlined protocol provided with the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit
- Unprecedented sensitivity for labeled *N*-glycans with at least 2 and 100 fold increases to fluorescence and MS detection, respectively
- Accurate profiling based on robust SPE for neutral to tetrasialylated *N*-glycans

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS™ *N*-Glycan Kit

GlycoWorks HILIC µElution Plate

ACQUITY UPLC® Glycan BEH Amide 130Å Column

ACQUITY UPLC H-Class Bio System

ACQUITY® QDa® Mass Detector

Xevo® G2-XS QTof MS

SYNAPT® G2-S HDMS

KEY WORDS

GlycoWorks, *Rapi*Fluor-MS, *Rapi*Gest™ SF, Rapid Tagging, PNGase F, Deglycosylation, ACQUITY UPLC H-Class Bio System, BEH Amide 130Å, Glycans, Glycoproteins, Glycosylation, HILIC, Fluorescence

INTRODUCTION

The *N*-glycan profile of a biopharmaceutical is commonly defined as a critical quality attribute, since it can be a measure of efficacy, safety, and manufacturing conditions.[1-2] Therefore, it is important that approaches for the glycan analysis of clinical and commercial biotherapeutic formulations exhibit high sensitivity and facilitate detailed characterization. Additionally, it would be highly advantageous if such an analysis could also be performed with rapid turnaround times and high throughput capacity to expedite product development. Most analytical strategies for evaluating *N*-glycans from glycoproteins involve deglycosylation via PNGase F and the labeling of the resulting *N*-glycans with a chemical moiety that imparts a detectable attribute. In one, highly effective approach, labeled glycans are separated by hydrophilic interaction chromatography (HILIC) and detected by fluorescence (FLR) and sometimes mass spectrometry (MS).[3-10]

Unfortunately, conventional approaches to the preparation of *N*-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromises in sensitivity.[11] For instance, a conventional deglycosylation procedure requires that a glycoprotein sample be incubated for about 1 hour, while many analysts generically employ an overnight (16 hour) incubation. Combined with this process is a lengthy, 2 to 3 hour labeling step that relies on reductive amination of reducing, aldehyde termini that form on *N*-glycans only after they hydrolyze from their glycosylamine forms. And in the case of one of the most frequently employed labeling compounds, 2-aminobenzamide (2-AB), the resulting glycans can be readily detected by fluorescence but are rather challenging to detect by electrospray ionization mass spectrometry (ESI-MS).

Variations to conventional approaches for *N*-glycan sample preparation have been explored, but have not, as of yet, presented a solution that combines the desired attributes of simplicity, high MS sensitivity, and high throughput. Alternative labeling reagents, for example procainamide, that have functional groups to enhance electrospray ionization efficiency have been used,[12] but this does not address the cumbersome, time consuming nature of relying on a reductive amination labeling step. Rapid tagging procedures that yield labeled glycans in a matter of minutes have consequently been investigated. In fact, two rapid tagging glycan labels were recently introduced, including a rapid tagging analog

EXPERIMENTAL

Method conditions (unless otherwise noted):

LC conditions

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 5 °C

Analytical column temp.: 60 °C

Flow rate: 0.4 mL/min

Fluorescence detection: Ex 265/Em 425 nm (*Rapi*Fluor-MS)

Ex 278/Em 344 nm (Instant AB)

Ex 330/Em 420 nm (2-AB)

(2 Hz scan rate [150 mm column]/5 Hz scan rate [50 mm column], Gain =1)

Injection volume: ≤1 μL (aqueous diluents with 2.1 mm I.D. columns)

≤30 μL (DMF/ACN diluted samples with 2.1 mm I.D. columns)

Columns: ACQUITY UPLC Glycan BEH Amide 130Å, 1.7 μm, 2.1 x 50 mm (p/n 186004740)

ACQUITY UPLC Glycan BEH Amide 130Å, 1.7 μm, 2.1 x 150 mm (p/n 186004742)

Sample collection/ vials: Sample Collection Module (p/n 186007988)

Polypropylene 12 x 32 mm Screw Neck Vial, 300 μL volume (p/n 186002640)

Gradient used with 2.1 x 50 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

Gradient used with 2.1 x 150 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

MS conditions

MS system:	SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	TOF MS, resolution mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–2500 *m/z*
Lockspray (ASM B-side):	100 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds
Acquisition:	500–2500 *m/z*, 1 Hz scan rate
Data management:	MassLynx Software (V4.1)

of aminobenzamide (AB).[13] In a rapid reaction, the precursor glycosylamines of reducing, aldehyde terminated glycans are modified via a urea linked aminobenzamide. Although such a rapid tagging reagent accelerates the labeling procedure, it does not provide the enhanced ionization efficiencies needed in modern *N*-glycan analyses.

To address the above shortcomings, we have developed a sample preparation solution that enables unprecedented FLR and MS sensitivity for glycan detection while also improving the throughput of *N*-glycan sample preparation. A novel labeling reagent has been synthesized that rapidly reacts with glycosylamines upon their release from glycoproteins. Within a 5 minute reaction, *N*-glycans are labeled with *Rapi*Fluor-MS, a reagent comprised of an *N*-hydroxysuccinimide (NHS) carbamate rapid tagging group, an efficient quinoline fluorophore, and a highly basic tertiary amine for enhancing ionization. To further accelerate the preparation of *N*-glycans, rapid tagging has been directly integrated with a Rapid PNGase F deglycosylation procedure involving *Rapi*Gest SF surfactant and a HILIC µElution SPE clean-up step that provides highly quantitative recovery of the released and labeled glycans with the added benefit of not requiring a solvent dry-down step prior to the LC-FLR-MS analysis of samples.

SAMPLE DESCRIPTION

N-glycans from Intact mAb Mass Check Standard (p/n 186006552), bovine fetuin (Sigma F3004), and pooled human IgG (Sigma I4506) were prepared according to the guidelines provided in the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit Care and Use Manual (715004793).

To compare the response factors of Instant AB™ and *Rapi*Fluor-MS labeled glycans, labeling reactions were performed with equivalent molar excesses of reagent, and crude reaction mixtures were directly analyzed by HILIC-FLR-MS in order to avoid potential biases from SPE clean-up procedures. Response factors were determined as ratios of the FA2 *N*-glycan (Oxford notation) chromatographic peak area to the mass of glycoprotein from which the glycan originated.

To compare the response factors of 2-AB labeled versus *Rapi*Fluor-MS labeled glycans, equivalent quantities of labeled *N*-glycans from pooled human IgG were analyzed by HILIC-FLR-MS. Column loads were calibrated using external quantitative standards of 2-AB labeled triacetyl chitotriose and *Rapi*Fluor-MS derivatized propylamine (obtained in high purity; confirmed by HPLC and 1H NMR). Response factors were determined as ratios of the FA2 chromatographic peak area to the mole quantity of glycan.

The procedure for extracting labeled *Rapi*Fluor-MS glycans after derivatization was evaluated using a test mixture containing *N*-glycans released and labeled from a 1:1 mixture (by weight) of pooled human IgG and bovine fetuin. The test mixture was prepared and then reconstituted in a solution equivalent in

composition to the solution glycans are subjected to when following the protocol of the *Rapi*Fluor-MS *N*-Glycan Kit. All other sample preparation techniques are described in the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit Care and Use Manual (715004793).

RESULTS AND DISCUSSION

Rational design of a new *N*-Glycan labeling reagent

A new labeling reagent for facilitating *N*-glycan analysis has been synthesized based on rational design considerations (Figure 1) that would afford rapid labeling kinetics, high fluorescence quantum yield, and significantly enhanced MS detectability. Conventional *N*-glycan sample preparation is dependent on reductive amination of aldehyde terminated saccharides, a process that requires glycans to undergo multiple chemical conversions and a lengthy high temperature incubation step.[11] Moreover, glycans must be reductively aminated in anhydrous conditions in order to minimize desialylation. Sample preparations are therefore burdened with transitioning a sample from aqueous to anhydrous conditions. For these reasons, the newly designed labeling reagent foregoes reductive amination and instead takes advantage of an aqueous rapid tagging reaction. To this end, Waters has drawn upon its experience with rapid fluorescence labeling of amino acids to develop a new reagent that meets the needs of modern, *N*-glycan analysis. More than 20 years ago, Waters introduced a rapid tagging labeling reagent, known as AccQ•Fluor™, that is now widely use to accurately profile the amino acid composition of protein samples via fluorescence detection.[14-15]

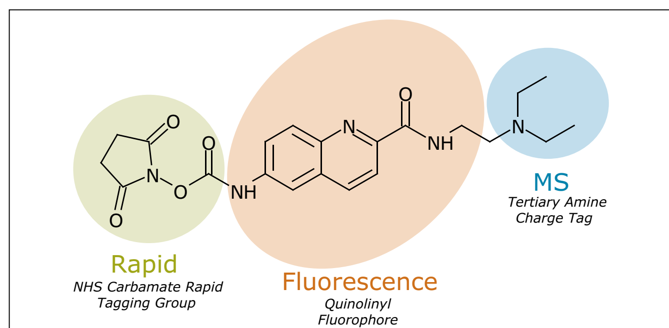

Figure 1. RapiFluor-MS Molecular Structure. Features of the chemical structure that enable rapid tagging, efficient fluorescence, and enhanced ionization efficiency are highlighted.

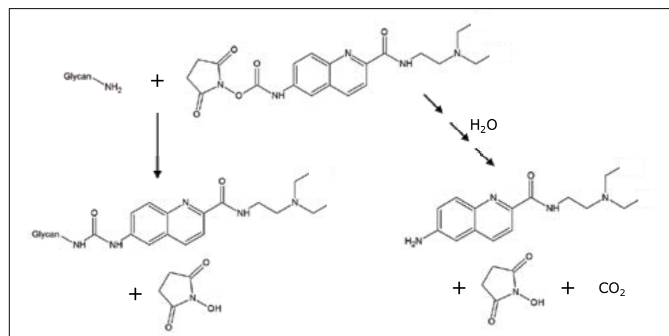

Figure 2. Reaction Schematic for RapiFluor-MS Derivatization of an N-glycosylamine. The pathway on the left shows the derivatization of a glycosylamine, which produces an N-glycan with a urea (NH-CO-NH) linked RapiFluor-MS label. Hydrolysis of RapiFluor-MS is shown in the pathway on the right.

AccQ•Fluor possesses two important chemical characteristics: an NHS-carbamate rapid tagging reactive group and a highly efficient quinolinyl fluorophore. These features of AccQ•Fluor form the basis of the new glycan labeling reagent. The NHS-carbamate reactive group of this reagent enables glycosylamine bearing *N*-glycans to be rapidly labeled following their enzymatic release from glycoproteins. Within a 5 minute reaction, *N*-glycans are labeled with the new reagent under ambient, aqueous conditions to yield a highly stable urea linkage (Figure 2). In addition to rapid tagging capabilities, the new labeling reagent also supports high sensitivity for both MS and fluorescence detection. A quinoline fluorophore serves as the central functionality of the new reagent that, as with AccQ•Fluor, facilitates high sensitivity fluorescence detection. In addition to AccQ•Fluor, however, the new reagent has been synthesized with a tertiary amine side chain as a means to enhance MS signal upon positive ion mode electrospray ionization (ESI+). In summary, the resulting *N*-glycan labeling reagent is built upon our expertise in chemical reagents and three important chemical attributes, a rapid tagging reactive group, an efficient fluorophore, and a highly basic MS active group. To describe these noteworthy attributes, the new labeling reagent has accordingly been named *Rapi*Fluor-MS.

***Rapi*Fluor-MS enables high sensitivity detection**

*Rapi*Fluor-MS *N*-glycan labeling has been extensively studied. In particular, the response factors of *Rapi*Fluor-MS labeled glycans have been benchmarked against those observed for glycans labeled with alternative reagents. The most closely related, commercially available alternative to *Rapi*Fluor-MS is an NHS carbamate analog of aminobenzamide, known as Instant AB.[13] Figures 3A and 3B present HILIC fluorescence and base peak intensity (BPI) MS chromatograms for equivalent quantities of *N*-glycans released from a murine monoclonal antibody (Intact mAb Mass Check Standard, p/n 186006552) and labeled with *Rapi*Fluor-MS and Instant AB, respectively. Based on the observed chromatographic peak areas, response factors for fluorescence and MS detection were determined for the most abundant glycan in the IgG profile, the fucosylated, biantennary FA2 glycan (Figure 3C). Our results for the FA2 glycan indicate that *Rapi*Fluor-MS labeled glycans produce 2 times higher fluorescence signal and, more astoundingly, 780 times greater MS signal than *N*-glycans labeled with Instant AB.

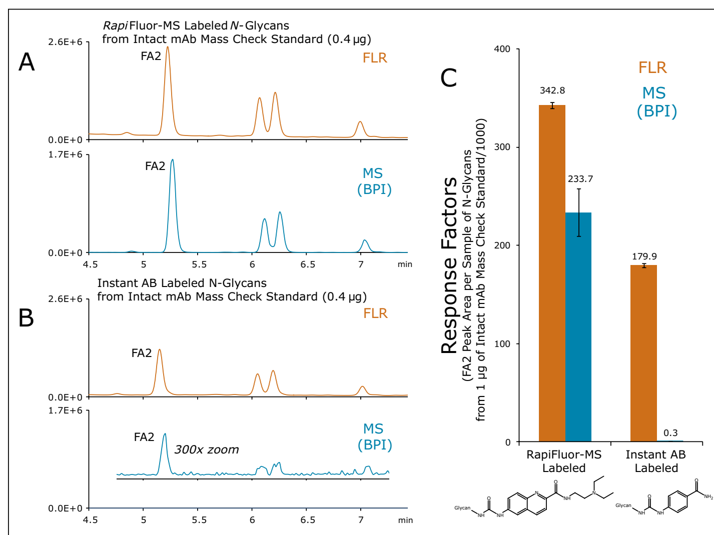

Figure 3. HILIC-FLR-MS of (A) RapiFluor-MS and (B) Instant AB Labeled N-Glycans from Intact mAb Mass Check Standard. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. Labeled glycans (from 0.4 µg of glycoprotein, 1 µL aqueous inection) were separated using a ACQUITY UPLC BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm Column. (C) Response factors for RapiFluor-MS and Instant AB labeled glycans (measured as the FA2 peak area per sample of N-glycans resulting from 1 µg of Intact mAb Mass Check Standard). Fluorescence (FLR) and MS (base peak intensity) response factors are shown in orange and blue, respectively. Analyses were performed in duplicate.

In a similar fashion, *Rapi*Fluor-MS labeling has also been compared to conventional 2-AB labeling. To draw such a comparison, *N*-glycans released from pooled human IgG and labeled with either *Rapi*Fluor-MS or 2-AB were analyzed by HILIC-FLR-MS at equivalent mass loads (Figures 4A and 4B, respectively). Given that rapid tagging and reductive amination are performed by significantly different procedures, external calibrations were established using quantitative standards in order to determine the amounts of FA2 glycan loaded and eluted from the HILIC column. Response factors determined using these calibrated amounts of FA2 glycan are provided in Figure 4C. Again, it is found that *Rapi*Fluor-MS labeled glycans are detected with significantly higher signal (14 times higher fluorescence and 160 times greater MS signal versus 2-AB labeled glycans.)

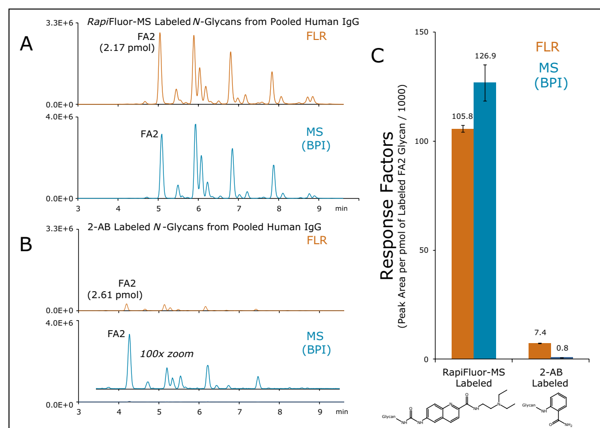

Figure 4. HILIC-FLR-MS of (A) RapiFluor-MS and (B) 2-AB Labeled N-Glycans from Pooled Human IgG. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. Labeled glycans (~14 pmol total glycan, 1 µL aqueous injection) were separated using a ACQUITY UPLC Glycan BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm Column. The quantities of FA2 glycan were calibrated via two-point external calibrations with quantitative standards. (C) Response factors for RapiFluor-MS and 2-AB labeled glycans (measured as the FA2 peak area per picomole of FA2 determined by the external calibration). Fluorescence (FLR) and MS (BPI) response factors are shown in orange and blue, respectively. Analyses were performed in duplicate.

To summarize the above observations, we have plotted the response factors of Instant AB and 2-AB as percentages of the response factors of *Rapi*Fluor-MS (Figure 5). The gains in fluorescence and MS sensitivity are apparent in this plot, since it portrays response factors for Instant AB and 2-AB normalized to those for *Rapi*Fluor-MS. In this plot, the relative performance of reductive amination with another alternative labeling reagent, procainamide, is also provided. Procainamide is a chemical analog to aminobenzamide that has recently been exploited to enhance the ionization of reductively aminated glycans when they are analyzed by HILIC-ESI(+)-MS. Previous studies have shown that procainamide labeled glycans yield comparable fluorescence signal and up to 50 times greater MS signal when compared to 2-AB labeled glycans.[12,16] Compared to procainamide, *Rapi*Fluor-MS will therefore provide sizeable gains in MS sensitivity. That is to say, the novel *Rapi*Fluor-MS labeling reagent not only supports rapid tagging of glycans but it also provides analysts with unmatched sensitivity for fluorescence and MS based detection.

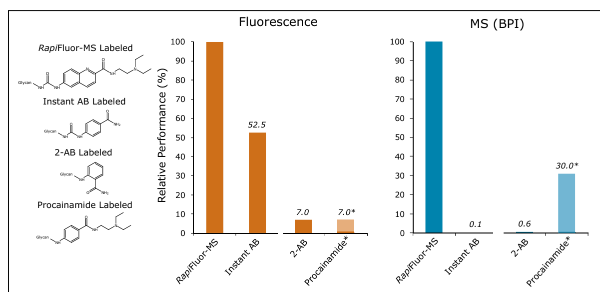

Figure 5. Relative Performance of Glycan Labels. Response factors normalized to the fluorescence and MS response factors of RapiFluor-MS labeled N-glycans. (*) Comparative result extrapolated from a published comparison of N-glycans, wherein it was found that procainamide provided comparable fluorescence and up to 50 fold greater ESI-MS sensitivity when compared to 2-AB (Klapoetke et al. 2010).

Rapid deglycosylation with a novel formulation of Rapid PNGase F and *Rapi*Gest SF Surfactant

*Rapi*Fluor-MS labeling revolutionizes *N*-glycan sample preparation and can be readily adopted in the laboratory with the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit. This complete solution from Waters and New England BioLabs was purposefully designed to remove the bottlenecks from all aspects of *N*-glycan sample preparation. The optimized *N*-glycan sample preparation workflow requires a minimum of three steps, including deglycosylation (to release glycans from a glycoprotein), labeling (to impart a detectable chemical entity to glycans), and a clean-up step (to remove potential interferences from the sample) (Figure 6). Conventional approaches to *N*-glycan sample preparation can be very time consuming due to not only lengthy labeling procedures but also lengthy deglycosylation steps that range from 1 to 16 hours. To ensure rapid labeling with *Rapi*Fluor-MS was not encumbered by a time-consuming deglycosylation process, Waters partnered with New England BioLabs to co-develop a Rapid PNGase F deglycosylation procedure specifically designed for integration with rapid tagging labeling reagents.

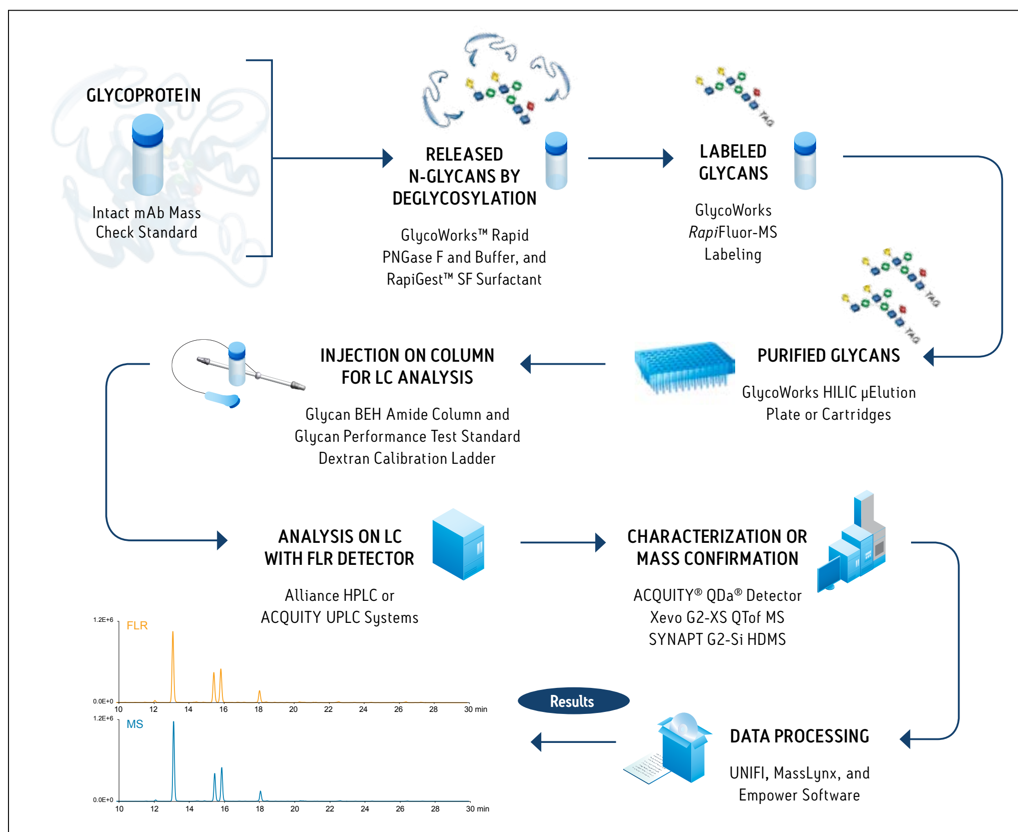

Figure 6. Workflow for the Rapid Preparation of N-glycans Using the RapiFluor-MS N-Glycan Kit.

The GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit provides a novel formulation of Rapid PNGase F and *Rapi*Gest SF Surfactant that can be used to completely deglycosylate a diverse set of glycoproteins in an approximately 10 minute procedure. This fast deglycosylation procedure is facilitated by the use of *Rapi*Gest, an anionic surfactant, that is used to ensure that *N*-glycans are accessible to Rapid PNGase F and that glycoproteins remain soluble upon heat denaturation. Most importantly, *Rapi*Gest is an enzyme-friendly reagent and can therefore be used at high concentrations without hindering the activity of Rapid PNGase F. In the developed fast deglycosylation technique, a glycoprotein is subjected to a high concentration of *Rapi*Gest (1%) and heated to ≥80°C for 2 minutes. Subsequently and without any additional sample handling, Rapid PNGase F is added to the solution and the mixture is incubated at an elevated, 50°C temperature for 5 minutes to achieve complete, unbiased deglycosylation.

The effectiveness of this rapid deglycosylation process has been evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is an effective technique for separating proteins based on their size in solution and can often be used to separate the glycosylated and de-glycosylated forms of proteins.[17-18] A diverse set of glycoproteins were deglycosylated according to the rapid deglycosylation procedure and analyzed by SDS-PAGE along with negative controls containing no PNGase F and positive controls, in which the glycoproteins were subjected to conventional multiple step deglycosylation with SDS based denaturation and PNGase F incubation for 30 minutes at 37 °C. Figure 7 shows the results of this study, where it can be seen that for each of the tested proteins there is a significant decrease in protein apparent molecular weight after they are subjected to the rapid deglycosylation procedure. Moreover, the apparent molecular weight decreases are visually comparable to those observed for proteins deglycosylated by the control method. These results demonstrate that the fast deglycosylation approach facilitated by a unique formulation of Rapid PNGase F and *Rapi*Gest SF Surfactant produces deglycosylation comparable to a conventional approach but in only a fraction of the time required.

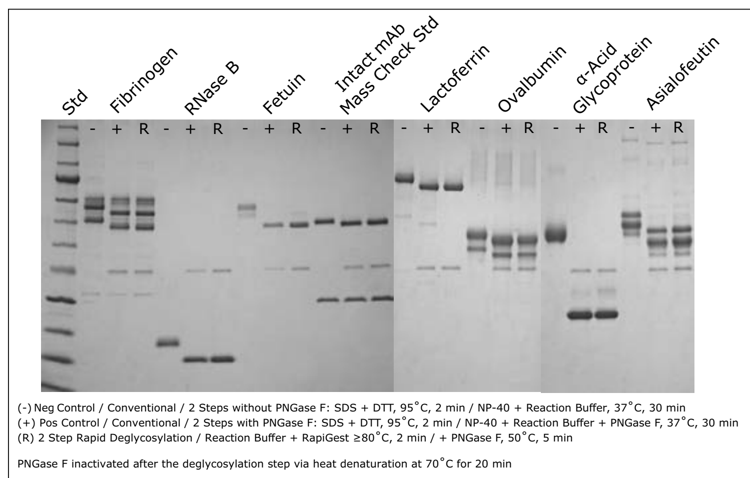

Figure 7. Gel Electrophoresis Assay for Deglycosylation of Glycoproteins. A negative control (-) shows the migration distance and apparent molecular weight of the native glycoproteins, and a positive control (+) shows the migration distance and decreased apparent molecular weight of deglycosylated proteins as obtained by conventional two step deglycosylation using SDS denaturation and a subsequent 30 minute incubation with PNGase F at 37 °C. Results demonstrating the complete deglycosylation of these glycoproteins with a fast procedure involving a two step approach with RapiGest-assisted heat denaturation and a subsequent 5 minute incubation with Glycoworks Rapid PNGase F at 50°C are also shown (R). Coomassie blue staining was used for band visualization.

Robust, quantitative HILIC SPE

As mentioned earlier, the final step in an *N*-glycan sample preparation aims to extract the labeled glycans in preparation for their analysis. An effective approach for extraction of labeled glycans from reaction byproducts has been devised using solid phase extraction (SPE). In particular, this SPE is designed to selectively extract *Rapi*Fluor-MS labeled *N*-glycans from a mixture comprised of deglycosylated protein, PNGase F, buffer/formulation components, *Rapi*Gest Surfactant, and labeling reaction byproducts, which otherwise interfere with analysis of the labeled glycans by HILIC column chromatography (Figure 8). For the *Rapi*Fluor-MS *N*-Glycans kit, a GlycoWorks µElution plate is provided that contains a silica based aminopropyl sorbent specifically selected for this application.[19-22] Due to its highly polar nature, the GlycoWorks SPE sorbent readily and selectively retains polar compounds such as glycans. In addition, this sorbent possesses a weakly basic surface that provides further selectivity advantages based on ion exchange and electrostatic repulsion. It is also worth noting that the GlycoWorks µElution Plate is designed for minimal elution volumes such that samples can be immediately analyzed without a dry down step. Moreover, the GlycoWorks µElution Plate is constructed as a 96 well format, meaning it can be used to perform high throughput experiments or used serially (with appropriate storage, see the Care and Use Manual) for low throughput needs.

In this HILIC SPE process, the sorbent is first conditioned with water and then equilibrated to high acetonitrile loading conditions. Thereafter, glycan samples that have been diluted with acetonitrile are loaded and washed free of the sample matrix using an acidic wash solvent comprised of 1% formic acid in 90% acetonitrile. This washing condition achieves optimal SPE selectivity by introducing electrostatic repulsion between the aminopropyl HILIC sorbent and reaction byproducts and by enhancing the solubility of the matrix components. After washing, the labeled, released glycans are next eluted from the HILIC sorbent. Since the GlycoWorks SPE sorbent has a weakly basic surface, and the capacity for anion exchange, just as it has the capacity for cation repulsion, it is necessary to elute the labeled glycans with an eluent of significant ionic strength. We have, as a result, developed an elution buffer comprised of a pH 7 solution of 200 mM ammonium acetate in 5% acetonitrile (p/n 186007991). Upon their elution, the *Rapi*Fluor-MS labeled glycans can be diluted with a mixture of organic solvents (acetonitrile and dimethylformamide) and directly analyzed by UPLC or HPLC HILIC column chromatography using fluorescence and/or ESI-MS detection.

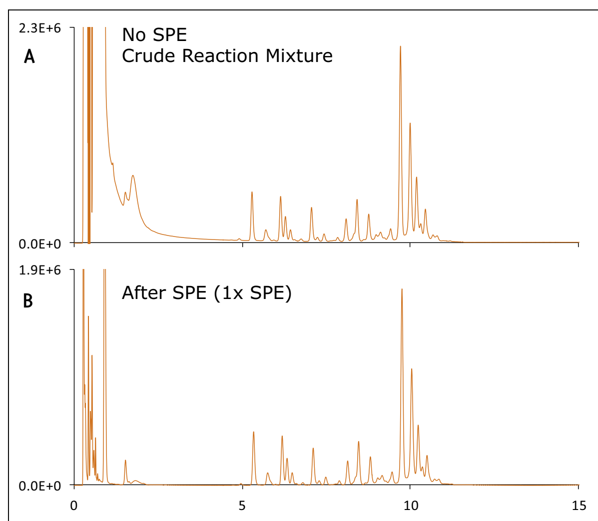

Figure 8. HILIC SPE to Remove Chromatographic Interference. (A) A test mixture comprised of RapiFluor-MS labeled glycans from pooled human IgG and bovine fetuin separated on an ACQUITY UPLC BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm Column and detected via fluorescence (labeled N-glycans from 0.4 µg glycoprotein, 1 µL injection crude reaction mixture). (B) The test mixture after extraction by HILIC SPE (labeled N-glycans from 0.4 µg glycoprotein, 10 µL injection of ACN/DMF diluted SPE eluate).

As with other aspects of the *Rapi*Fluor-MS *N*-Glycan Kit, this GlycoWorks SPE step has been extensively evaluated. A test mixture was created to assess the recovery of a diverse set of *Rapi*Fluor-MS labeled *N*-glycans, ranging from small neutral glycans to high molecular weight, tetrasialylated glycans. Such a mixture was prepared by releasing and labeling *N*-glycans from both pooled human IgG and bovine fetuin. An example analysis of this test mixture by HILIC column chromatography and fluorescence detection is shown in Figure 9A. Species representing extremes in glycan properties are labeled, including an asialo FA2 glycan and a glycan with a tetrasialylated, triantennary structure (A3S1G3S3). To evaluate the effects of the SPE process, this mixture was subjected to a second pass of GlycoWorks SPE and again analyzed by HILIC chromatography and fluorescence detection, as shown in Figure 9B. It can be observed that the sample processed twice by SPE presents a labeled glycan profile comparable to the profile observed for the sample processed only once by SPE. Indeed, this SPE step has been found to exhibit an absolute recovery of approximately 70-80% for all purified glycans and more importantly highly accurate relative yields. Figure 9C shows the relative abundances for four glycans (FA2, FA2G2S1, A3G3S3, and A3S1G3S3) as determined for samples subjected to one pass versus two passes of SPE. The largest deviation in relative abundance was observed for the tetrasialylated A3S1G3S3, in which case relative abundances of 5.7% and 6.1% were determined for samples processed by one and two passes of SPE, respectively. With these results, it is demonstrated that GlycoWorks SPE provides a mechanism to immediately analyze a sample of extracted, labeled glycans and does so without significant compromise to the accuracy of the relative abundances determined for a wide range of *N*-glycans.

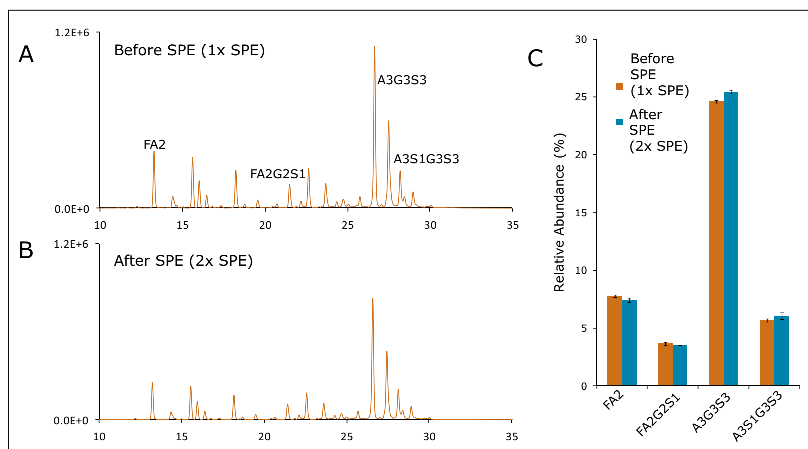

Figure 9. Extraction of RapiFluor-MS Labeled N-glycans by SPE with a GlycoWorks HILIC µElution Plate. (A) A test mixture comprised of RapiFluor-MS labeled glycans from pooled human IgG and bovine fetuin separated on an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column and detected via fluorescence (labeled N-glycans from 0.4 µg glycoprotein, 10 µL injection of ACN/DMF diluted sample). (B) The test mixture after extraction by HILIC SPE. (C) Relative abundances determined for a set of RapiFluor-MS labeled glycans before and after GlycoWorks HILIC SPE.

CONCLUSIONS

Conventional approaches to the preparation of *N*-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromises in sensitivity. With the development of the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit, we address these shortcomings by enabling unprecedented sensitivity for glycan detection while also improving the throughput of *N*-glycan sample preparation. With this approach, glycoproteins are deglycosylated in approximately 10 minutes to produce *N*-glycosylamines. These glycans are then rapidly reacted with the novel *Rapi*Fluor-MS reagent within a 5 minute reaction and are thereby labeled with a tag comprised of an efficient fluorophore and a highly basic tertiary amine that yields enhanced sensitivity for both fluorescence and MS detection. In a final step requiring no more than 15 minutes, the resulting *Rapi*Fluor-MS labeled glycans are extracted from reaction byproducts by means of µElution HILIC SPE that has been rigorously developed to provide quantitative recovery of glycans (from neutral to tetrasialylated species) and to facilitate immediate analysis of samples. Accordingly, an analyst can complete an *N*-glycan sample preparation, from glycoprotein to ready-to-analyze sample, in just 30 minutes when using the sensitivity enhancing *Rapi*Fluor-MS labeling reagent.

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Robustness of *Rapi*Fluor-MS N-Glycan Sample Preparations and Glycan BEH Amide HILIC Chromatographic Separations

Matthew A. Lauber,[1] Michael F. Morris,[1] Darryl W. Brousmiche,[1] and Stephan M. Koza[1]
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APPLICATION BENEFITS

- High yield sample preparation with quantitative recovery to ensure accurate and repeatable profiling of N-glycans
- Comparability to historical 2-AB based released glycan analysis approaches
- RapiFluor-MS Glycan Performance Test Standard for method familiarization, troubleshooting, and benchmarking
- Robust Glycan BEH Amide HILIC separations supported by GU calibration with the novel *Rapi*Fluor-MS Dextran Calibration Ladder

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

GlycoWorks HILIC µElution Plate

*Rapi*Fluor-MS Glycan Performance Test Standard

*Rapi*Fluor-MS Dextran Calibration Ladder

ACQUITY UPLC® Glycan BEH Amide, 130Å Column

XBridge® Glycan BEH Amide, 130Å Column

ACQUITY UPLC H-Class Bio System

ACQUITY® QDa® Mass Detector

Xevo® G2-XS QTof MS

SYNAPT® G2-*Si* HDMS

KEY WORDS

GlycoWorks, *Rapi*Fluor-MS, RapiGest™ SF, Rapid Tagging, PNGase F, Deglycosylation, ACQUITY UPLC H-Class Bio System, BEH Amide 130Å, Glycans, Glycoproteins, Glycosylation, HILIC, Fluorescence

INTRODUCTION

N-glycosylation of proteins is routinely characterized and monitored because of its significance to the detection of disease states[1-3] and the manufacturing of biopharmaceuticals.[4-5] Glycosylation profiles are most often assessed by means of released glycan analyses, wherein samples are often prepared by techniques that are notoriously time-consuming or lead to compromises in MS sensitivity.[6-7] With the development of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, we have addressed these shortcomings by enabling unprecedented sensitivity for glycan detection while also improving the throughput of N-glycan sample preparation.[8] Using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, glycoproteins are deglycosylated in 10 minutes to produce N-glycosylamines that are then rapidly reacted with the novel *Rapi*Fluor-MS labeling reagent (Figure 1). In a final step, the resulting labeled glycans are extracted from reaction byproducts by means of an SPE method that facilitates immediate analysis of samples. As a result, an analyst can now complete an N-glycan sample preparation, from glycoprotein to ready-to-analyze sample, in just 30 minutes and be poised to perform high sensitivity N-glycan profiling using hydrophilic interaction chromatography (HILIC) and mass spectrometric (MS) or fluorescence (FLR) detection.

Equally important as the efficiency and sensitivity gains afforded by this new sample preparation approach is its robustness and its ability to produce results consistent with historical N-glycan profiling. Within this application note, we will discuss these attributes of the *Rapi*Fluor-MS based sample preparation and the corresponding HILIC-based LC analyses.

EXPERIMENTAL

Method conditions

(unless otherwise noted)

LC conditions

LC system: ACQUITY UPLC H-Class Bio

Sample temp.: 10 °C

Analytical column temp.: 60 °C

Flow rate: 0.4 mL/min

Fluorescence detection: Ex 265/Em 425 nm (*Rapi*Fluor-MS)
Ex 330 / Em 420 nm (2-AB)
(2 Hz scan rate [150 mm column], Gain =1)
(5 Hz scan rate [50 mm column], Gain=1)

Injection volume: ≤1 µL (aqueous diluents with 2.1 mm I.D. columns)
≤30 µL (DMF/ACN diluted samples with 2.1 mm I.D. columns)

Columns: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186004740)
XBridge Glycan BEH Amide XP, 130Å, 2.5 µm, 2.1 x 150 mm (p/n 186007265)
Agilent AdvanceBio Glycan Mapping Rapid Resolution HD, 1.8 µm, 2.1 x 150 mm
Thermo Scientific Accucore™ 150 Amide HILIC, 2.6 µm, 2.1 x 150 mm

Sample collection/vials: Sample Collection Module (p/n 186007988)
Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

Gradient used with 2.1 x 50 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

Gradient used with 2.1 x 150 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

Intact mass analysis was performed by LC-MS with a Xevo G2-QTof

MS conditions

MS system: Xevo G2 QTof

Ionization mode: ESI+

Analyzer mode: TOF MS, resolution mode (~20 K)

Capillary voltage: 3.0 kV

Cone voltage: 45 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Desolvation gas flow: 800 L/Hr

Calibration: NaI, 1 µg/µL from 500–5000 m/z

Acquisition: 500–5000 m/z, 1 Hz scan rate

Data management: UNIFI® 1.7/MassLynx® Software (v4.1)

Sample description

*Rapi*Fluor-MS labeled N-glycans were prepared from glycoproteins, including Intact mAb Mass Check Standard (p/n: 186006552), using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n: 176003606) according to the guidelines provided in its Care and Use Manual (715004793).

2-AB labeled N-glycans were prepared using a Prozyme GlykoPrep® Rapid N-Glycan Preparation with 2-AB Kit according to the manufacturer's recommended protocol. In addition, 2-AB labeled N-glycans were also prepared using an approach combining the use of a Prozyme GlykoPrep Digestion Module, an in-house optimized 2-AB labeling protocol, and a GlykoPrep Cleanup Module (Prozyme, Hayward, CA).

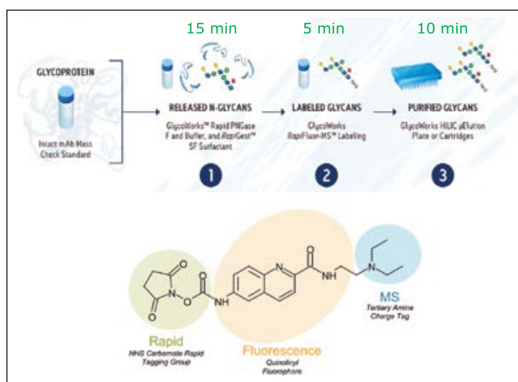


Figure 1. GlycoWorks RapiFluor-MS N-Glycan Kit sample preparation workflow and the chemical structure of the RapiFluor-MS Reagent.

*Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983) was reconstituted in 50 µL of water and injected as a 1 µL volume for chromatographic benchmarking and lifetime testing experiments. *Rapi*Fluor-MS Dextran Calibration Ladder (p/n: 186007982) was reconstituted in 100 µL of water and injected as a 1 µL volume for retention time calibrations.

Percent yields for the sample preparation workflows were determined by means of quantitative analyses. Column loads were calibrated using external quantitative standards of 2-AB labeled triacetyl chitotriose and *Rapi*Fluor-MS derivatized propylamine obtained in high purity (confirmed by HPLC and 1H NMR).

To determine percent yields, the measured quantities of FA2 glycan from Intact mAb Mass Check Standard (p/n: 186006552) were compared to theoretical yields calculated for the preparation. For example, the theoretical yield for the FA2 glycan resulting from the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit was calculated as follows:

$$1.5 \times 10^7 \text{ pg IgG} \times \frac{1 \text{ pmol}}{150{,}000 \text{ pg}} \times \frac{2 \text{ pmol glycan}}{1 \text{ pmol IgG}} \times \frac{0.45 \text{ pmol FA2}}{1 \text{ pmol total glycan pool}} \times \frac{10\ \mu\text{L injection}}{400\ \mu\text{L sample prepared}} = 2.3 \text{ pmol}$$

**This calculation is based on the assumption that the sample of Intact mAb Mass Check Standard was 15 µg, that the mAb has a molecular weight of 150 kDa, that there are only 2 N-glycans per one mAb, that the N-glycan profile of the mAb contains the FA2 glycan at a relative abundance of 45%, and that only 2.5% of the sample was analyzed.*

RESULTS AND DISCUSSION

Robust sample preparation: Deglycosylation

Each procedural step in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit has been optimized to be high yielding and to minimize the introduction of bias to an N-glycan profile. Previous work based on SDS PAGE gel shift assays has demonstrated that the rapid deglycosylation procedure developed for this kit produces complete deglycosylation of a diverse set of glycoproteins.[8] This completeness of deglycosylation is also supported by intact mass analysis using LC-MS, where the deglycosylation of a monoclonal antibody (mAb) can be readily tracked. Figure 2 presents deconvoluted ESI mass spectra for Intact mAb Mass Check Standard, a murine IgG1 mAb. The top spectrum shows the mAb before it had been subjected to rapid deglycosylation (Figure 2A). The bottom spectrum meanwhile presents the mAb after it was processed according to the approach specified in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, wherein glycoproteins are subjected to 1% (w/v) RapiGest SF Surfactant-assisted heat denaturation followed by incubation with Rapid PNGase F at 50 °C for 5 minutes (Figure 2B). The masses observed in these spectra confirm that these samples differ in terms of glycan occupancy. The control sample contains the mAb in its doubly glycosylated, native form (one glycan on each heavy chain). In contrast, the sample subjected to the proposed 2-step rapid deglycosylation procedure is homogenous with an observed molecular weight that is in agreement with the predicted molecular weight of the fully deglycosylated mAb (145.3 kDa). And although high temperatures are employed in this method for the purpose of heat denaturation, no detrimental effects on an N-glycan profile have been observed. To this point, notice that there are no differences in an N-glycan profile prepared from pooled human IgG when using an excessive 20 minute heat denaturation at 90 °C versus the rapid 3 minute procedure (Figure 3).

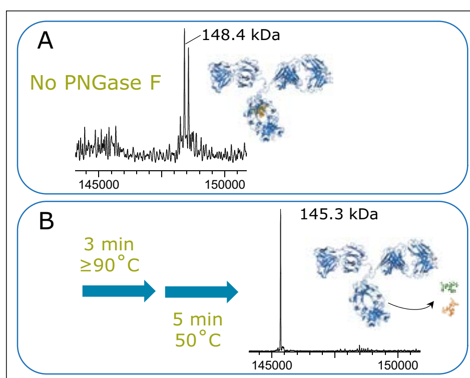


Figure 2. Intact mass analysis of Intact mAb Mass Check Standard (A) before and (B) after rapid deglycosylation with the GlycoWorks RapiFluor-MS N-Glycan Kit.

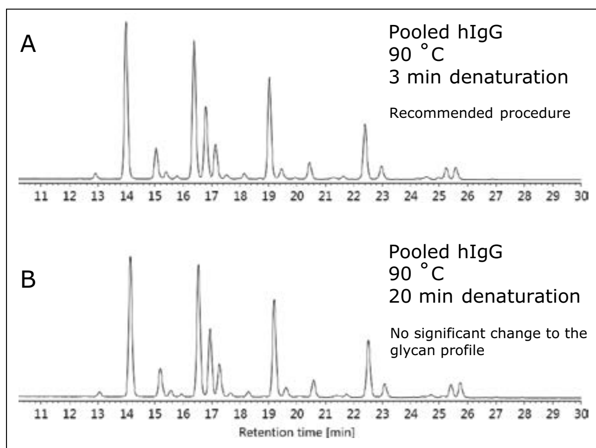
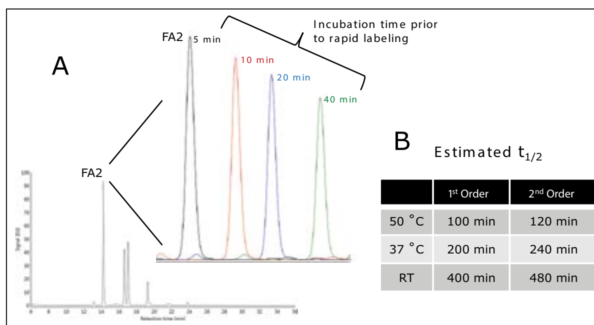


Figure 3. Testing the effects of subjecting human IgG and its N-glycans to heat denaturation. (A) The RapiFluor-MS N-glycan profile as observed using the recommended 3-minute heat denaturation versus (B) the RapiFluor-MS N-glycan profile as observed using a 20-minute heat denaturation.

Robust sample preparation: Rapid labeling

The efficiency of the sample preparation carries over from deglycosylation to *Rapi*Fluor-MS labeling. A primary concern in this step is the relative stability of the PNGase F released N-glycosylamines, which are required for *Rapi*Fluor-MS labeling, in the pH 7.9 GlycoWorks Rapid Buffer. A time-course study involving varying delays between deglycosylation and *Rapi*Fluor-MS labeling steps has shown that the N-glycosylamines have a relatively long half-life of approximately 2 hours at 50 °C (Figure 4). That is, with our 5 minute deglycosylation step, there should be little concern over sample loss (< 3% loss) due to hydrolysis of the glycosylamine. In addition, sample losses from the labeling reaction are minimal. Many experimental parameters were explored during the development of the rapid labeling reaction specified in the *Rapi*Fluor-MS N-Glycan Kit, including pH, temperature, ionic strength, time, buffer components, and reagent molar excess. Figure 5 shows an example of optimizing the reagent molar excess as needed to maximize labeling yield. Fluorescence chromatograms for labeled, released N-glycans from Intact mAb Mass Check Standard are stacked on the left (Figure 5A). Note that with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit proteins are purposely not depleted from the sample after deglycosylation to save time and to give better control over the labeling. The *Rapi*Fluor-MS Reagent is therefore used in a molar excess over all of the nucleophiles from the glycoprotein, which for an IgG corresponds to approximately seventy five protein amines and two N-glycosylamines. Each of the corresponding samples was obtained from labeling a fixed glycoprotein concentration of 0.36 mg/mL with *Rapi*Fluor-MS Reagent at concentrations varying from 18 to 108 mM. As shown in Figure 5B, plotting of the fluorescence peak areas for the resulting N-glycan profile indicates that labeling is maximized near a *Rapi*Fluor-MS Reagent concentration of 36 mM, the conditions designed into the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit. Moreover, molar excess conditions both higher and lower than the 36 mM reagent condition produced comparable fluorescence profiles, underscoring the robustness of *Rapi*Fluor-MS labeling.



	1st Order	2nd Order
50 °C	100 min	120 min
37 °C	200 min	240 min
RT	400 min	480 min

Figure 4. Estimating the half-life of N-glycosylamine hydrolysis through a time-course on deglycosylation incubation. (A) Fluorescence traces for RapiFluor-MS labeled FA2 from Intact mAb Mass Check Standard observed after implementing varying incubation times for deglycosylation (50°C incubations). (B) Approximation of the N-glycosylamine half-life assuming 1st or 2nd order reaction kinetics.

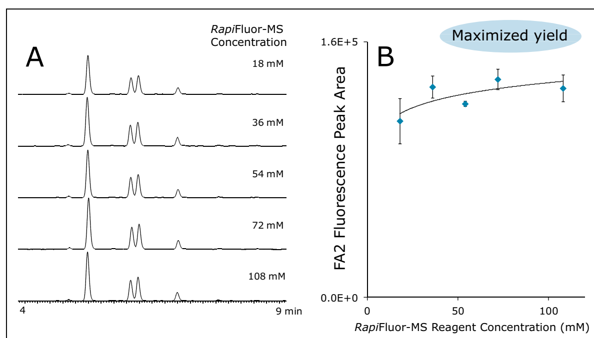


Figure 5. Optimization of labeling reagent molar excess for the GlycoWorks RapiFluor-MS N-Glycan Kit. (A) Fluorescence chromatograms for labeled glycans obtained by titration of 0.36 mg/mL deglycosylated Intact mAb Mass Check Standard with varying concentrations of RapiFluor-MS Reagent. Separations were performed with labeled glycans from 0.4 µg of glycoprotein and an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm Column. (B) Fluorescence peak area as a function of RapiFluor-MS Reagent concentration.

Robust sample preparation: μElution HILIC SPE

The last step in the sample preparation involves extraction of the *Rapi*Fluor-MS labeled glycans from reaction byproducts using HILIC SPE. This technique has been routinely used for preparations of 2-AB labeled N-glycans and has now been optimized for *Rapi*Fluor-MS labeled species.[8-9] Previous studies have shown that *Rapi*Fluor-MS labeled glycans are obtained through this SPE processing at relatively high yields of approximately 74%.[8] Nearly all of the observed sample losses in this step are non-specific. Figure 6 plots fluorescence peak areas for preparations of N-glycans from Intact mAb Mass Checked Standard, in which the final SPE elution volume was either 30, 90 or 180 μL. This plot shows that SPE recovery is a function of elution volume and that highest recoveries are achieved when employing large elution volumes. To facilitate direct analyses, however, a compromise is made such that a 90 μL elution volume is used in order to obtain a relatively concentrated glycan eluate. Regardless of the elution volume and absolute yield of glycans from the SPE sorbent, the most important characteristic of this clean-up is that the observed sample losses have been determined to be non-specific with no significant bias being introduced to a glycan profile for a wide range of glycans with diverse chemical properties, including small, neutral glycans up to large, tetrasialylated glycans (see Reference 8 for more details about GlycoWorks HILIC SPE).

Yield of *Rapi*Fluor-MS labeled N-glycans

In another measurement of robustness, it is worth looking at the yield of N-glycans through the entire workflow. This was evaluated in order to measure the collective efficiency of combining fast deglycosylation, rapid labeling, and HILIC SPE extraction of *Rapi*Fluor-MS labeled glycans (Figure 7). *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard were prepared, analyzed by HILIC-FLR, and quantified by means of an external calibration. Based on a calculated theoretical yield (see experimental) and duplicate analyses, it was determined that the percent yield through the entire *Rapi*Fluor-MS N-Glycan Kit sample preparation was approximately 73%. To provide perspective, we evaluated the yield of 2-AB labeled N-glycans from an alternative sample preparation workflow involving the use of a GlykoPrep Rapid N-Glycan Preparation with 2-AB Kit.

Quantitative analyses showed that 2-AB labeled N-glycans are prepared using this kit with a relatively low yield of approximately 35%, though it has been found that the yield of this kit can be dramatically improved by optimization and lengthening of the labeling step. Comparatively speaking, though, these results show that not only does the *Rapi*Fluor-MS approach quicken a historically time-consuming sample preparation, it also exhibits reasonably high yields.

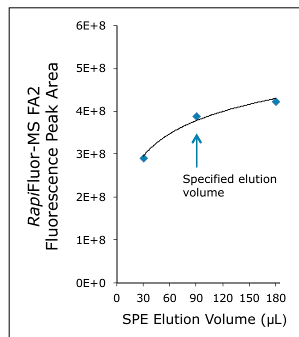


Figure 6. Fluorescence peak area as a function of SPE elution volume. The specified elution volume in the GlycoWorks RapiFluor-MS N-Glycan Kit is 90 μL.

Step	Yield	Testing to confirm minimal bias
Deglycosylation	Complete	▪ Intact mass analysis/subunit LC-MS ▪ Gel shift assays
Labeling	>95%	▪ Released glycan profile vs. subunit derived glycan information
SPE	~74%	▪ Recovery measurements ▪ Glycan profile before vs. after SPE
GlycoWorks RapiFluor-MS N-Glycan Kit	~73% Yield	
GlykoPrep® Rapid N-Glycan Preparation with 2-AB	~35% Yield	

Figure 7. Percent yield for the preparation of RapiFluor-MS labeled N-glycans with the GlycoWorks RapiFluor-MS N-Glycan Kit. Testing that has been performed to confirm minimal sample loss and quantitative recovery is listed for each procedural step. The percent yield that has been measured for the preparation of 2-AB labeled N-glycans with a GlykoPrep Rapid N-Glycan Preparation with 2-AB kit is also provided. These results may not be representative of all applications.

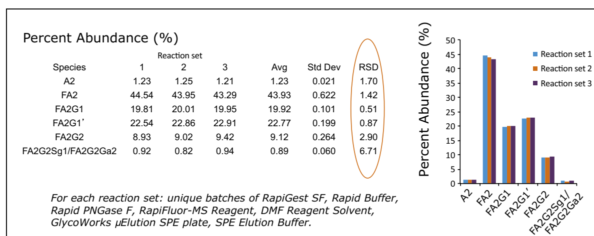
Minimal impact to glycan profiling with reagent batch variation

Lastly, sample preparations with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit have proven to be robust with respect to reagent manufacturing. A robustness study was performed to test the impact of changing the batches of each reagent that plays a critical role in the preparation of *Rapi*Fluor-MS labeled N-glycans, namely *Rapi*Gest SF, GlycoWorks Rapid Buffer, GlycoWorks Rapid PNGase F, *Rapi*Fluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE Plate, and the SPE Elution Buffer. Three sets of these reagents, each varying by batch, were tested in their application to profiling the N-glycans from Intact mAb Mass Check Standard. Average relative abundances observed for the glycan species in this standard with the three different reaction sets are presented in Figure 8. Relative abundances of N-glycans were observed to be largely comparable across the different preparations with an average RSD for the labeled N-glycan species being 2.3%.

Comparability to 2-AB N-glycan profiling

Another critical aspect to the *Rapi*Fluor-MS N-glycan sample preparation is that it can be used in place of legacy 2-AB methods without requiring significant adaptations to existing analytical techniques. With the speed of the sample preparation and the enhanced method sensitivity afforded by the *Rapi*Fluor-MS tag,[8] the task of analyzing N-glycosylation is in fact made significantly easier.

Just like 2-AB labeled glycans, *Rapi*Fluor-MS labeled glycans are ideally suited for HILIC separations with an amide bonded stationary phase, such as that found in the Waters Glycan BEH Amide Columns. Figure 9 shows example separations for 2-AB and *Rapi*Fluor-MS labeled glycans obtained from Intact mAb Mass Check Standard. The 2-AB labeled glycans, in this case, were prepared using the previously mentioned GlykoPrep Kit and an approximately 3.5 hour protocol, whereas the *Rapi*Fluor-MS labeled glycans were prepared in less than 30 minutes using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit.



Percent Abundance (%)

Species	Reaction set 1	2	3	Avg	Std Dev	RSD
A2	1.23	1.25	1.21	1.23	0.021	1.70
FA2	44.54	43.95	43.29	43.93	0.622	1.42
FA2G1	19.81	20.01	19.95	19.92	0.101	0.51
FA2G1'	22.54	22.86	22.91	22.77	0.199	0.87
FA2G2	8.93	9.02	9.42	9.12	0.264	2.90
FA2G2Sg1/FA2G2Ga2	0.92	0.82	0.94	0.89	0.060	6.71

For each reaction set: unique batches of RapiGest SF, Rapid Buffer, Rapid PNGase F, RapiFluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE plate, SPE Elution Buffer.

Figure 8. Characterization of batch-to-batch variation in the RapiFluor-MS sample preparation. Percent abundances were measured for the preparation of RapiFluor-MS labeled N-glycans from Intact mAb Mass Check Standard using three different sets of materials. Each reaction set was represented by unique batches of RapiGest SF, Rapid Buffer, Rapid PNGase F, RapiFluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE plate, SPE Elution Buffer. Testing was performed in triplicate. FA2G1' denotes the structural isomer of FA2G1.

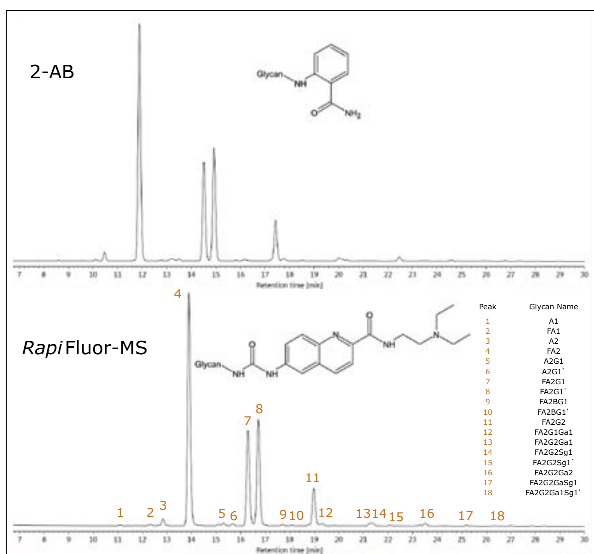
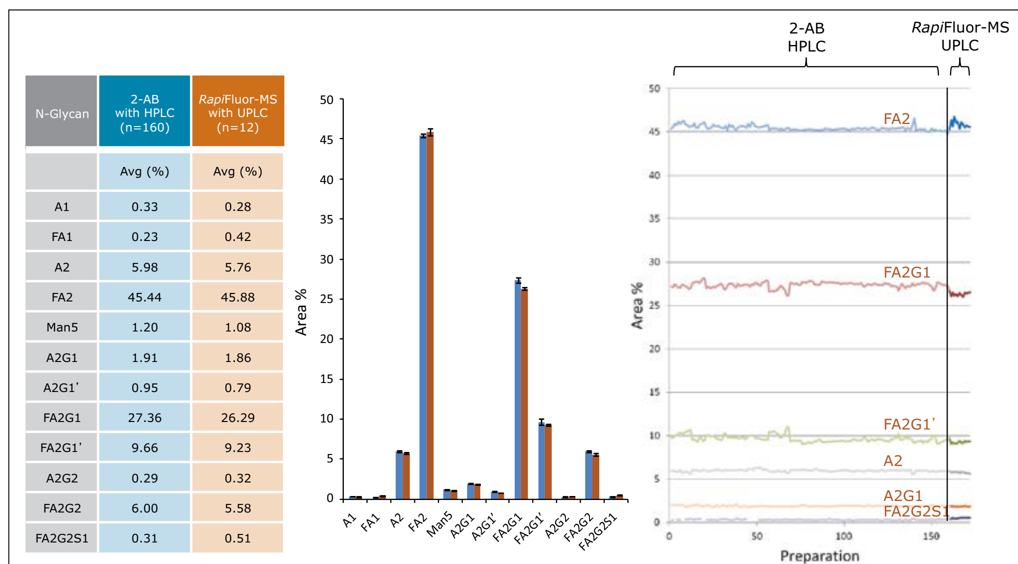


Figure 9. Similarity between 2-AB and RapiFluor-MS N-glycan HILIC profiles for a typical mAb. Fluorescence chromatograms for labeled glycans from Intact mAb Mass Check Standard using an ACQUITY UPLC BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. Peak identifications for the RapiFluor-MS labeled N-glycans are provided. 2-AB labeled N-glycans were prepared using a GlykoPrep Rapid N-Glycan Preparation with 2-AB kit. (') denotes a structural isomer.

So that chromatograms exhibiting equivalent signal-to-noise could be compared, the *Rapi*Fluor-MS sample was analyzed in this study at a significantly lower mass load than the 2-AB labeled sample. Despite being prepared by different approaches, it can be seen that the labeled N-glycans are resolved by the HILIC separation into very similar profiles. For a typical mAb profile, *Rapi*Fluor-MS and 2-AB labeling both yield HILIC glycan separations with similar selectivity. However, as a result of its additional hydrogen bonding donors/acceptors, the *Rapi*Fluor-MS label introduces a slight shift of the mAb N-glycan profile to higher retention times. This change in the absolute retention window of an N-glycan profile is predictable and can therefore be easily accounted for when transitioning from 2-AB to *Rapi*Fluor-MS based methods.

Consistency in results observed for the *Rapi*Fluor-MS-based approach compared to historical 2-AB techniques was also evaluated. N-glycan profiling of the same monoclonal IgG1 reference sample has been studied between these different methodologies. Figure 10 displays N-glycan information obtained for this mAb sample throughout 160 different profiling experiments involving 2-AB labeling and HPLC chromatography. Likewise, Figure 10 provides data from 12 recent experiments using *Rapi*Fluor-MS labeling and UPLC® chromatography. Comparable relative abundances are observed for this sample in a direct comparison (Figure 10, center panel) and a control chart demonstrates the ability to transition between these two methods (Figure 10, right panel). This consistency in N-glycan profiling makes it possible to replace time-consuming 2-AB/HPLC methods with *Rapi*Fluor-MS/UPLC techniques.

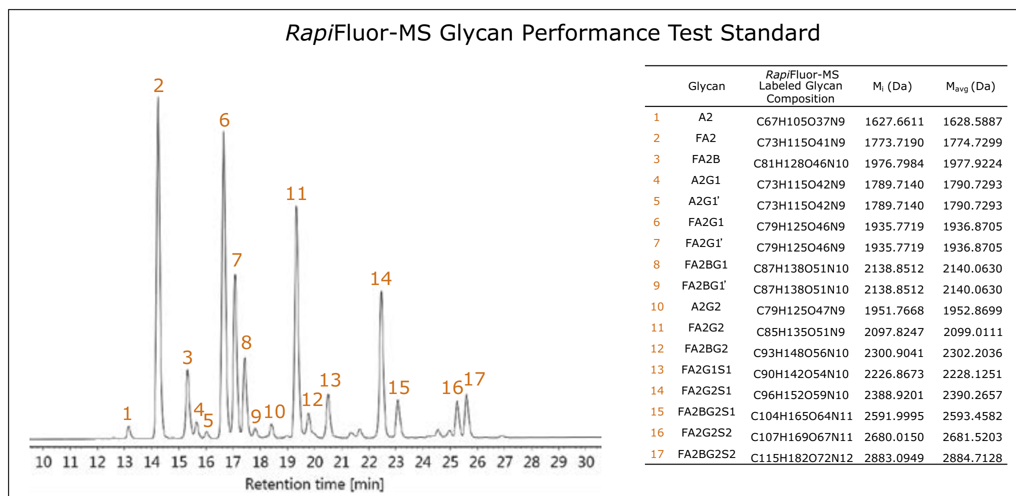


N-Glycan	2-AB with HPLC (n=160)	*Rapi*Fluor-MS with UPLC (n=12)
	Avg (%)	Avg (%)
A1	0.33	0.28
FA1	0.23	0.42
A2	5.98	5.76
FA2	45.44	45.88
Man5	1.20	1.08
A2G1	1.91	1.86
A2G1'	0.95	0.79
FA2G1	27.36	26.29
FA2G1'	9.66	9.23
A2G2	0.29	0.32
FA2G2	6.00	5.58
FA2G2S1	0.31	0.51

Figure 10. Consistency between UPLC-based RapiFluor-MS N-glycan profiling and HPLC-based 2-AB N-glycan profiling of a humanized monoclonal IgG1. Comparison of relative abundances for N-glycans detected using a method combining the GlycoWorks RapiFluor-MS N-Glycan Kit with a UPLC separation (n=12) versus a historical 2-AB sample preparation combined with an HPLC separation (n=160). Trending data for the N-glycans from the human monoclonal IgG1 (light colored lines = 2-AB/HPLC, dark colored lines = RapiFluor-MS/UPLC). FA2G1' and A2G1' denote the structural isomers of FA2G1 and A2G1, respectively.

Robustness of *Rapi*Fluor-MS N-glycan separations with glycan BEH amide columns

The robustness and resolving power of the HILIC column chromatography is critically important to successfully implementing this methodology. To this end, a test standard called *Rapi*Fluor-MS Glycan Performance Test Standard is available for method familiarization, system suitability, troubleshooting, and benchmarking studies. This standard contains a complex mixture of *Rapi*Fluor-MS N-glycans from human IgG that has been isolated from pooled human serum. Its composition of approximately 20 different major constituents makes it useful for evaluating the resolving power of a separation and the sensitivity of detection methods (Figure 11).



	Glycan	*Rapi*Fluor-MS Labeled Glycan Composition	M_i (Da)	M_{avg} (Da)
1	A2	C67H105O37N9	1627.6611	1628.5887
2	FA2	C73H115O41N9	1773.7190	1774.7299
3	FA2B	C81H128O46N10	1976.7984	1977.9224
4	A2G1	C73H115O42N9	1789.7140	1790.7293
5	A2G1'	C73H115O42N9	1789.7140	1790.7293
6	FA2G1	C79H125O46N9	1935.7719	1936.8705
7	FA2G1'	C79H125O46N9	1935.7719	1936.8705
8	FA2BG1	C87H138O51N10	2138.8512	2140.0630
9	FA2BG1'	C87H138O51N10	2138.8512	2140.0630
10	A2G2	C79H125O47N9	1951.7668	1952.8699
11	FA2G2	C85H135O51N9	2097.8247	2099.0111
12	FA2BG2	C93H148O56N10	2300.9041	2302.2036
13	FA2G1S1	C90H142O54N10	2226.8673	2228.1251
14	FA2G2S1	C96H152O59N10	2388.9201	2390.2657
15	FA2BG2S1	C104H165O64N11	2591.9995	2593.4582
16	FA2G2S2	C107H169O67N11	2680.0150	2681.5203
17	FA2BG2S2	C115H182O72N12	2883.0949	2884.7128

Figure 11. RapiFluor-MS Glycan Performance Test Standard. An example fluorescence chromatogram obtained from an 8 pmole load of the standard and a separation with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. Peak identifications are provided. FA2G1', A2G1', and FA2BG1' denote the structural isomers of FA2G1, A2G1, and FA2BG1 respectively.

In line with its intended purpose, we have used the *Rapi*Fluor-MS Glycan Performance Test Standard to benchmark the chromatographic performance of four different columns containing amide bonded stationary phases designed for glycan separations. Two of the columns were UPLC-based and contained sub-2-µm particles while the remaining two were intended for use on HPLC instrumentation and contained 2.5 µm and 2.6 µm particles. Figure 12 shows representative chromatograms obtained with each of these columns run under equivalent conditions and linear velocities. Four glycan species spread across these separations were monitored to measure retention windows, average peak widths, and peak capacities. Notice that whether performing a separation with a phase intended for UPLC or HPLC chromatography, Glycan BEH Amide Columns provide exemplary resolving power and comparable selectivities thereby enabling the seamless transfer of this glycan separation between HPLC and UPLC platforms.[10]

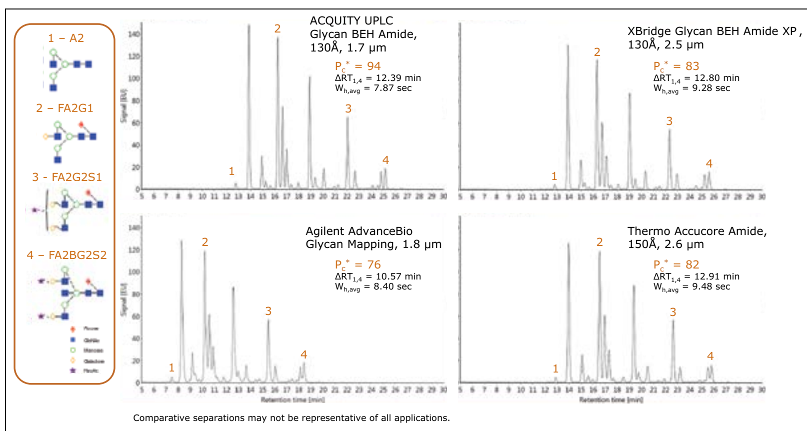


Figure 12. Chromatographic benchmarking of HILIC columns containing amide bonded stationary phases designed for glycan separations. Fluorescence chromatograms of the RapiFluor-MS Glycan Performance Test Standard were obtained from an 8 pmole load of the standard and separations with 2.1 x 150 mm columns. All separations were performed at the same linear velocity on an ACQUITY UPLC H-Class Bio System. Four glycan species spread across the separations were monitored to measure retention windows, average peak widths, and peak capacities.

Separations of *Rapi*Fluor-MS labeled glycans with glycan BEH amide columns have also proven to be very robust. In demonstration of this, a single Glycan BEH Amide, 130Å, 1.7 µm Column was subjected to lifetime testing and 300 sequential runs. At every 20th run, *Rapi*Fluor-MS Glycan Performance Test Standard was separated in order to track any changes in the retentivity and selectivity of the column.

Chromatograms corresponding to the 1st and 300th runs are provided in Figures 13A and 13B, respectively. Quite clearly, near identical separations were obtained at the onset as well as at the end of this approximately 2-week constant use scenario, with no significant shifts in retention times of the labeled N-glycans having been observed throughout the testing (Figure 13C).

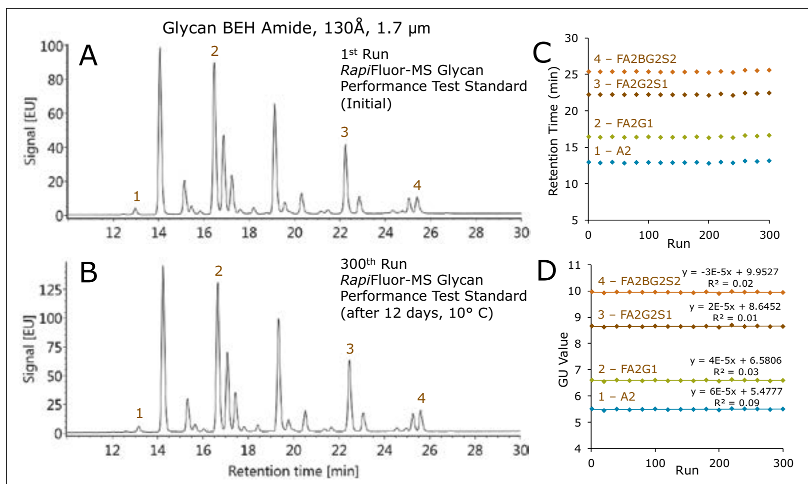


Figure 13. Robustness testing of an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm 2.1 x 150 mm Column for separations of RapiFluor-MS labeled N-glycans. Fluorescence chromatograms of the RapiFluor-MS Glycan Performance Test Standard were obtained at every 20th run from an 8 pmole load of the standard. Four glycan species spread across the separations were monitored to track the retentivity of the stationary phase and column. Fluorescence chromatograms are shown for the (A) 1st run and the (B) 300th run with the column. (C) Retention times as a function of run. (D) Glucose unit (GU) values as a function of run.

In this testing, LC calibrations were performed after every separation of the glycan mixture through application of a dextran ladder and assignment of glucose unit (GU) values. Separations with glycan BEH amide columns can be used in conjunction with glucose unit (GU) values as a means to calibrate HILIC-based glycan separations. Use of GU values minimizes subtle retention time variations between runs and between different instruments by expressing chromatographic retention in terms of standardized GU values.[11] To assign GU values, a dextran ladder (comprised of glucose multimers of increasing length) is used as an external calibrant. The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values.

The development of a dextran calibration ladder suitable for use with *Rapi*Fluor-MS labeled glycans was essential yet technically challenging. Given that dextran is a reducing sugar without a strong nucleophile, it cannot, unlike N-glycosylamines, be readily labeled with *Rapi*Fluor-MS Reagent. Because of the distinctive urea linkage imparted to N-glycans upon their derivatization with rapid tagging reagents, *Rapi*Fluor-MS labeled N-glycans have very unique fluorescence maxima at approximately 265 nm (excitation) and 425 nm (emission) (Figure 14A). In a novel labeling approach, we have prepared a *Rapi*Fluor-MS Dextran Calibration Ladder by first reductively aminating dextran with ethanolamine and then labeling it with *Rapi*Fluor-MS.

The resulting urea-linked dextran derivatives exhibit identical fluorescence properties to those of *Rapi*Fluor-MS labeled N-glycans. Furthermore, the obtained dextran is tuned for desired HILIC retention because of the hydroxyl group being incorporated through ethanolamine. A representative fluorescence chromatogram for this novel dextran ladder is provided in Figure 14B, and an example cubic spline fit of the retention data is shown in Figure 14C.

The impact of implementing GU value calibration is exemplified in Figure 13D, where the retention time data throughout the Glycan BEH Amide lifetime testing are reported in GU values. In comparing the retention time data shown in Figure 13C to the GU data in Figure 13D, one can see that the subtle fluctuations in retention times across the 2-week lifetime testing are compensated for by the GU calibration. In fact, RSDs for the GU value data are reduced by a factor of 2 compared to the RSDs in the retention time data.

Also, linear regression analysis of the GU value data shows that there is essentially no drifting in the HILIC retention data once calibrated using a dextran ladder. This analysis therefore clearly demonstrates the value of GU calibration with respect to improving the quality of reported data.

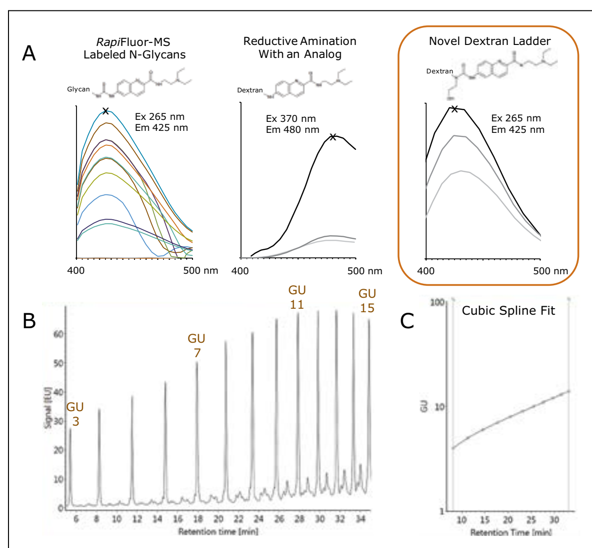


Figure 14. Assignment of Glucose Unit (GU) values with RapiFluor-MS labeling. (A) Chemical structures and fluorescence spectra of RapiFluor-MS labeled N-glycans versus dextrans derivatized with RapiFluor-MS-like labels. The novel dextran ladder that has been commercialized as the RapiFluor-MS Dextran Calibration Ladder is highlighted. (B) An example fluorescence chromatogram for the RapiFluor-MS Dextran Calibration obtained for a 0.5 µg mass load with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. (C) Calibration curve resulting from cubic spline fitting.

CONCLUSIONS

In this application note, we have demonstrated the robustness of *Rapi*Fluor-MS N-glycan preparations and Glycan BEH Amide HILIC Column chromatography. The *Rapi*Fluor-MS N-Glycan Kit enables analysts to perform a high yielding sample preparation with quantitative recovery that ensures accurate and repeatable profiling of N-glycans that is highly comparable to HPLC, 2-AB based methodologies. Moreover, it has been demonstrated that Glycan BEH Amide Columns afford exemplary resolving power and ruggedness for separations of *Rapi*Fluor-MS labeled N-glycans. Additionally, this separation can be readily transferred between UPLC and HPLC platforms. To further ensure success with these new methodologies, two standards have been commercialized, and their use to facilitate *Rapi*Fluor-MS analyses has been demonstrated. The *Rapi*Fluor-MS Glycan Performance Test Standard has been used for benchmarking studies, while the novel *Rapi*Fluor-MS Dextran Calibration Ladder has been employed to enhance the reproducibility of chromatographic retention time data. In summary, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and supporting standards and columns can significantly reduce the burdens associated with N-glycan profiling while providing accurate, reproducible, and sensitive analyses.

Acknowledgement

We would like to thank Peter De Vreugd and Mark Eggink from Synthon Biopharmaceuticals BV for providing data on the comparability of released N-glycan analyses with *Rapi*Fluor-MS and UPLC versus 2-AB and HPLC.

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[TECHNOLOGY BRIEF]

GlycoWorks HILIC SPE Robust Glycan Sample Preparation

Matthew A. Lauber, Jennifer L. Fournier, Stephan M. Koza, and Kenneth J. Fountain

GOAL

To demonstrate the benefits of using HILIC as an ideal separation mode for glycans as well as the robustness of HILIC in an SPE format.

BACKGROUND

Reversed-phase (RP) chromatography, though universally accepted for a majority of compounds, is not particularly well suited for analytes that are hydrophilic in nature. When subjected to RP chromatography, polar compounds are often poorly retained or separated with non-optimal selectivity. Alternatively, Hydrophilic Interaction Chromatography (HILIC) can be used to successfully improve retention of very polar species, such as the glycans encountered during the characterization of protein therapeutics. There are over 40 highly cited published papers using HILIC chemistry for the separation of glycans (search on SciFinder June 2014). One reason for this is that this mode of separation is ideal for these types of compounds due to their highly polar nature. Polar analytes can be strongly and selectively retained onto a HILIC stationary phase when a low polarity mobile phase is used. The concept can be simplified by describing it as like-attracts-like.[2] Specifically, the retention of glycans onto a HILIC stationary phase can be explained in terms of the hydrogen bonding as well as ionic and dipole-dipole interactions that occur while the glycans partition into an immobilized water layer.

This technique can be exploited as a form of solid phase extraction (SPE) with the goal to clean up and concentrate the analyte of interest, in this case glycans.

Glycans are highly polar biomolecules, making them amenable to Hydrophilic Interaction Chromatography (HILIC) based Solid Phase Extraction (SPE).

Through sequential load wash and elution steps, one can successfully purify glycans while removing many of the less polar "contaminants" found in deglycosylation and post-derivatization mixtures. Overall, HILIC provides many benefits as an attractive mode of separation for glycans.[1]

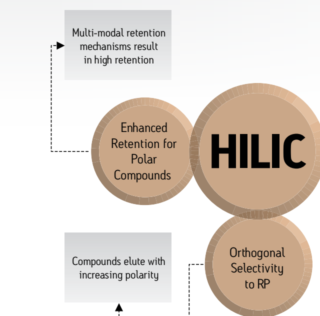

Figure 1. Benefits of HILIC SPE.

THE SOLUTION

HILIC method robustness

In the case of GlycoWorks™ SPE, a silica-based aminopropyl sorbent was chosen from several tested due to its highly polar nature. This stationary phase readily and selectively retains polar compounds such as glycans. In addition, this sorbent possesses a weakly basic surface allowing the added potential to exploit ion exchange/repulsion properties.

The eluent pH and ionic strength can impact retention on this HILIC phase and cannot be ignored. For instance, as the ionic strength of the eluent is increased, ionic interactions between the stationary phase and the solutes are disrupted, resulting in ion exchange playing a lesser role in retention.[1]

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In addition, as the mobile phase pH is altered, the charge state of the stationary phase surface is impacted (by nature of the aminopropyl ligand and the base particles' silanol activity). At higher pH values the ionization of basic analytes and the aminopropyl ligand is reduced, however the ionization of the surface silanols is increased. Because of this, it is critical to choose an an eluent that will have a fixed, stable pH every time an assay is performed. Method development taking into account all of these factors is crucial for successful assay reproducibility.[1]

The first step to HILIC SPE is conditioning with aqeuous mobile phase, which establishes a layer of polar solvent on the stationary phase surface. It is into this aqueous layer that the glycans will partition when they are loaded under low polarity solvent conditions. Subsequently, the adsorbed glycans are washed with solvent to ensure less polar compounds are removed from the sample. Thereafter, the glycans are eluted off the stationary phase with a strongly polar solution, in this case a high concentration of water with a buffer in order to minimize the ionic interactions. The use of ammonium salts of formic acid or acetic acid (ammonium formate or ammonium acetate) are preferentially used due to their volatility. Refer to the application note Optimization of GlycoWorks HILIC SPE for the Quantitative and Robust Recovery of N-Linked Glycans from mAb-Type Samples and one of its corresponding figures (Figure 2) for a demonstration on how an eluent for GlycoWorks HILIC SPE was optimized for the reproducible elution of 2-AB labeled glycans.[3]

In this work, 2-AB labeled glycans were loaded onto a 96-well HILIC µElution plate according to the protocol provided in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual. Various eluents were then employed for elution of the labeled glycans and recoveries for each major species in the test mixture were subsequently determined. These data were compared alongside the recoveries of the glycans from just the lyophilization and reconstitution steps that were performed after the HILIC SPE procedure in preparation of the samples for HILIC-FLR. By means of this development work, a 100 mM NH4OAc, 5% ACN eluent was selected as the optimal elution condition, since it is a pH stable solution and it provided high as well as relatively unbiased analyte recoveries.

SPE robustness

Many times, due to the nature of solid phase extraction devices, especially in the case of micro-elution plates, well to well flow rate variation can occur. At times, it can be observed that one well will exhibit a faster flow than another. Such well-to-well flow rate differences can be further amplified by more viscous solvent such as highly aqueous solvents. Other variables that can contribute to flow variability include improper seating of the plate on the vacuum manifold, introduction of air when pipetting into a well and changes in operating pressures when vacuum driven SPE is performed. Optimization of individual vacuum manifolds can be critical to achieving consistent well-to-well flow rates. Nevertheless, it is almost impossible to entirely eliminate well to well flow rate differences so the question was asked, what impact does this have on the resulting reproducibility of oligosaccharide recovery? If the method is robust, this mechanical variability should be inconsequential.

To this end, the GlycoWorks Care and Use protocol was employed along with the Dextran Calibration Ladder (PN 186006841) to test the impact of variable flow rates on sample recovery (GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual).[4] As an initial step, 500 µL of water was pulled through wells on a GlycoWorks HILIC SPE uElution plate. A vacuum setting of 5 inches of Hg was used to drive flow through one well at a time.

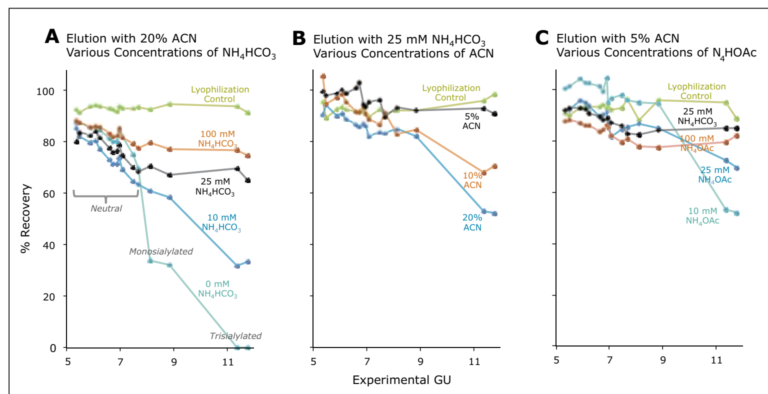

Figure 2. Recovery of 2-AB labeled N-glycans (Figure 2) from GlycoWorks HILIC SPE 96-well µElution Plates (30 pmol of glycans processed). Percent recoveries as a function of experimentally determined glucose units (GU) are shown for various elution conditions. Values are based on the average of three replicate analyses.

Meanwhile, the time to draw the 500 µL through each well was recorded, enabling a flow rate metric for each well to be determined. Sample was thereafter processed, without the use of any special practices. Figure 3A shows the measured flow rates. The color gradient denotes faster flow rates with darker colors and slower flow rates with lighter colors.

Figure 3B illustrates the fluorescence chromatograms obtained for oligosaccharide samples processed through wells with varying flow rate characteristics.

Overall, the GlycoWorks HILIC SPE plate exhibited less than a 3-fold variation in flow rate across a set of 48 wells when operated with a vacuum manifold (Figure 3A).

When these data were viewed in the context of oligosaccharide recovery, however, it became clear that any differences that do exist in flow rate characteristics from well-to-well do not negatively impact recovery, whether absolute or relative, as demonstrated in Figure 4.

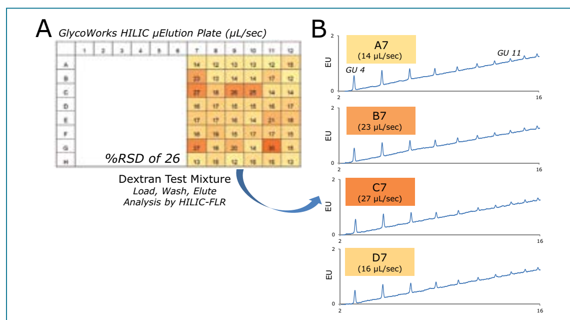

Figure 3. Well-to-well reproducibility testing of the GlycoWorks HILIC µElution Plate. (A) Flow rates (µL/sec) observed during an aqueous conditioning step. (B) HILIC-Fluorescence (FLR) chromatograms obtained using eluate of the dextran test mixture from wells A7, B7, C7, and D7.

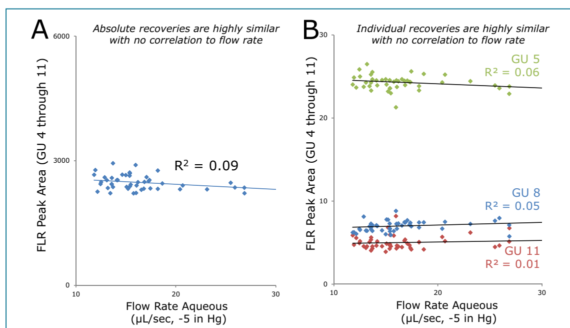

Figure 4. Well-to-well reproducibility of the GlycoWorks HILIC µElution Plate. (A) Combined fluorescence peak area for dextran test mixture eluted from 48 different wells displayed as a function of flow rate. (B) Peak areas of Glucose Unit (GU) 5, 8, and 11 from the test mixture plotted as a function of flow rate.

SUMMARY

GlycoWorks HILIC SPE offers a robust, reliable solution for cleanup and concentration of glycans from complex matrices. Its application during the sample preparation of 2-AB labeled glycans helps ensure that an analyst reliably obtains successful results when studying protein glycosylation.

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Quality Control and Automation Friendly GlycoWorks *Rapi*Fluor-MS N-Glycan Sample Preparation

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APPLICATION BENEFITS

- *Rapi*Fluor-MS™ glycan labeling procedure with larger volume, simplified liquid transfer to improve ease of use and automatability.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Intact mAb Mass Check Standard

ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm, Column

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

MassLynx® 4.1 Software

GlycoWorks Rapid Buffer, 5 mL

KEY WORDS

HILIC Chromatography, UPLC®, HPLC, method transfer, N-glycans, *Rapi*Fluor-MS, GlycoWorks

INTRODUCTION

Recently, Waters® introduced a novel labeling reagent, *Rapi*Fluor-MS (RFMS), that provides a fast, efficient, and reproducible sample preparation workflow and unsurpassed fluorescent and MS sensitivity for released N-glycan profiling.[1,2] This initial methodology was designed to accommodate the lowest possible glycoprotein sample concentration and, as result, calls for several low volume (1.2 to 7 µL) liquid transfers. Looking to minimize the impact of pipetting volume inaccuracies, we have redesigned this sample preparation to make pipetting volumes larger (≥10 µL) and thereby reduce the variation in the absolute quantities of analytes and reagents that get delivered during the denaturation, PNGase F deglycosylation, and RFMS labeling steps of the procedure.

In the following work, we demonstrate that RFMS labeled glycan samples prepared using this alternative sample preparation scheme are comparable to those produced by the previously published flexible volume procedure. By virtue of its simplification and use of larger volumes, this protocol should be an excellent fit for adoption of RFMS into Quality Control (QC) environments and automated platforms.

EXPERIMENTAL

Method conditions

LC system: ACQUITY UPLC H-Class Bio System

Detection: ACQUITY UPLC FLR Detector with analytical flow cell

Wavelength: 265 nm Excitation, 425 nm Emission

Column: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)

Column temp.: 60 °C

Sample temp.: 10 °C

Injection volume: 10.0 µL

Mobile phase A: 50 mM ammonium formate (pH 4.4) LC-MS grade water, from a 100 x concentrate (p/n 186007081)

Mobile phase B: LC-MS grade acetonitrile

Gradient:

Time	Flow rate (mL/min.)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

Sample vials: Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL (p/n 186002640)

Data management: MassLynx 4.1 Software

MS conditions for *Rapi*Fluor-MS released N-glycans

MS system: Xevo G2-XS QTof

Ionization mode: ESI+

Analyzer mode: Resolution (~ 40,000)

Capillary voltage: 2.2 kV

Cone voltage: 75 V

Source temp.: 120 °C

Desolvation temp.: 500 °C

Source offset: 50 V

Desolvation gas flow: 600 L/Hr

Calibration: NaI, 1 µg/µL from 100–2000 *m/z*

Acquisition: 700–2000 *m/z*, 0.5 sec scan rate

Lockspray: 300 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds

Data management: MassLynx 4.1 Software

RESULTS AND DISCUSSION

Comparison of GlycoWorks *Rapi*Fluor-MS protocols

The GlycoWorks *Rapi*Fluor-MS sample preparation procedure (Table 1) was developed to allow for maximum flexibility with respect to the concentration of the sample being prepared for released N-glycan analysis.[3] By altering the addition of water, this method is capable of preparing samples with concentrations as low as 0.66 mg/mL. While this procedure was designed with significant molar excesses of the critical reagents, such as denaturant, enzyme, and the RFMS label, to produce reproducible results,[4] a potential drawback of this procedure is that several of the aliquoted volumes are well below 10 µL. As such, the methodology is not as amenable for adoption into certain QC laboratories, depending on their internally imposed method requirements, or for use in specific robotic platforms. Pipetted volume accuracy and precision increases with volume and, based on the International Organization for Standardization (ISO) requirements for mechanical pipette accuracy and precision, it is at 10 µL or more that the lowest permissible systematic and random errors are obtained (Figure 1, Adapted from Reference 5). It should be noted that these maximum permissible errors are doubled for the use of multi-channel pipettes. For this reason, some laboratories prefer to avoid procedures with pipetted volumes lower than 10 µL.

Component	Flexible volume standard tube (1 mL tube)	Flexible volume PCR tube (200 µL tube)
2.0 mg/mL sample	7.5 µL	7.5 µL
5% *Rapi*Gest[1]	6.0 µL	3.0 µL
Water	15.3 µL	3.3 µL
PNGaseF	1.2 µL	1.2 µL
Total volume of released N-glycan sample	30 µL	15 µL
RFMS[2]	12.0 µL	6.0 µL
Total volume of the labeled N-glycan sample	42 µL aliquot	21 µL aliquot
ACN dilution	358 µL	179 µL
Total volume of HILIC SPE Load	400 µL	200 µL

Table 1. Aliquoted volumes for GlycoWorks RapiFluor-MS Kit flexible-volume protocols for 1 mL and 200 µL tubes.

RapiGest reconstitution: 10 mg with 200 µL buffer or 3 mg w/60 µL buffer.

RFMS reconstitution: 23 mg in 335 µL DMF and 9 mg in 131 µL DMF (68.7 µg/µL) for Standard Protocol or 23 mg in 168 µL DMF and 9 mg in 66 µL DMF (136.4 µg/µL) for PCR Protocol.

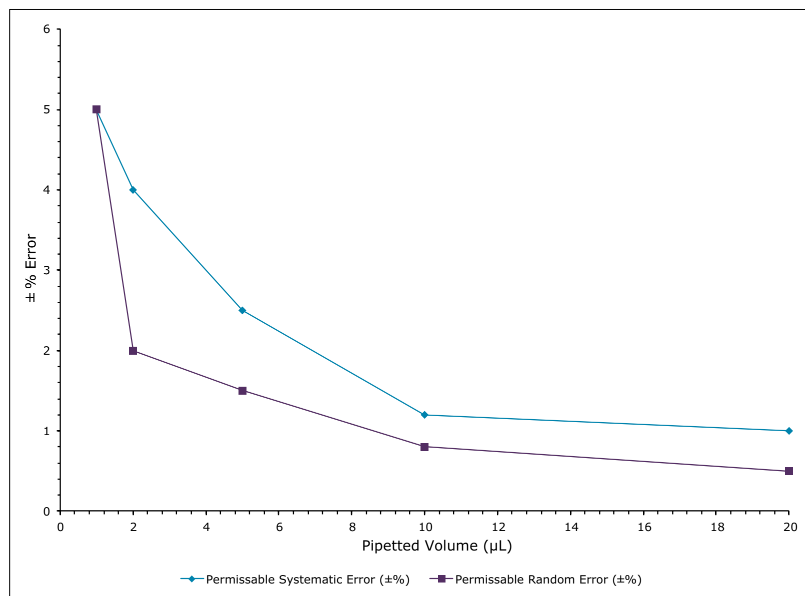

Figure 1. Trends in the maximum permissible pipette volume errors based on ISO 8655.

To provide a protocol with transfer volumes of 10 µL or more, the standard RFMS sample preparation procedure was reconsidered. The primary changes made to the deglycosylation procedure were that the addition of water was removed, and more dilute solutions of *Rapi*Gest™ SF surfactant and PNGase F were used. The final recommended conditions are presented in Table 2. This revised procedure is designed to give optimal results using a 10 µL sample of glycoprotein at a concentration of 1.5 mg/mL (15 µg of glycoprotein), however, samples that are more or less concentrated can still potentially produce quality results. It should be noted that at significant deviations from the optimal sample quantity, i.e. <5 µg and >30 µg, the labeling reaction can produce undesirable side reactions or low yields, so it is recommended to assess these situations on a case-by-case basis.

The only fundamental difference in this revised procedure is that the concentration of *Rapi*Gest SF is slightly higher (1.5%) during the denaturation step versus the previous procedure (1.0%). This increase is not predicted to cause any deleterious effects and may provide some benefit for certain glycoprotein samples that are particularly resistant to denaturation. In the following step, PNGase F deglycosylation, concentrations of the principal components (glycoprotein, *Rapi*Gest SF, and PNGase F) are equivalent to the standard, flexible volume procedure.

In modifying the labeling step, the aliquoted amount of the RFMS label solution was decreased from 12 to 10 µL to be consistent with the other lowest pipetted volumes of the procedure. To account for this volume change, the concentration of the RFMS reagent was increased proportionally such that the final ratio of RFMS to glycoprotein remains equivalent. In addition to the protocol using the 1 mL reaction tubes provided in the kit, this revised procedure, like the previous procedure, has also been adapted for use with a 200 µL thermocycler tube. If using a thermocycler with this new QC and automation friendly protocol, it is necessary to divide the final released and labeled glycan sample into two aliquots, or to transfer sample to a larger tube, prior to dilution and SPE clean-up.

Component	Automated /QC standard tube (1 mL tube)	Automated/QC PCR tube (200 µL tube)
1.5 mg/mL sample	10 µL	10 µL
3% *Rapi*Gest[1]	10 µL	10 µL
Water	0 µL	0 µL
PNGaseF (diluted)[2]	10 µL	10 µL
Total volume of released N-glycan sample	30 µL	30 µL
RFMS[3]	10 µL	10 µL
Total volume of the labeled N-glycan sample	40 µL aliquot	Divide into 2 x 20 µL aliquots
ACN dilution	360 µL	2 x 180 µL
Total volume of HILIC SPE Load	400 µL	200 + 200 = 400 µL

Table 2. Aliquoted volumes for GlycoWorks RapiFluor-MS Kit automation and QC volume protocols for 1 mL and 200 µL tubes.

[1]RapiGest reconstitution: 10 mg with 200 µL buffer + 135 µL water or 3 mg w/60 µL buffer + 40 µL water.

[2]PNGase F dilution (contents of vial 30 µL + 220 µL water).

[3]RFMS reconstitution: 23 mg in 280 µL DMF or 9 mg in 110 µL DMF (82.5 µg/µL).

Comparing *Rapi*Fluor-MS labeled N-glycan profiles

To compare the standard, flexible volume procedure with its newly designed, QC and automation-friendly analog, analyses of the Waters Intact mAb Mass Check Standard were performed. Samples from this murine monoclonal antibody were prepared and analyzed following the two different protocols along with 1 mL sample tubes and single-channel pipettes. A comparison of representative chromatograms for each of these sample preparations are presented in Figure 2. The labeled peaks were integrated and the quantitative results are presented in Figure 3. The glycan species observed in this profile were assigned using online ESI-MS detection with a Xevo G2-XS QTof Mass Spectrometer (Table 3). As can be clearly seen, the two procedures produce both qualitative and quantitative results that are comparable and reproducible for peaks with relative abundances as low as 0.06%.

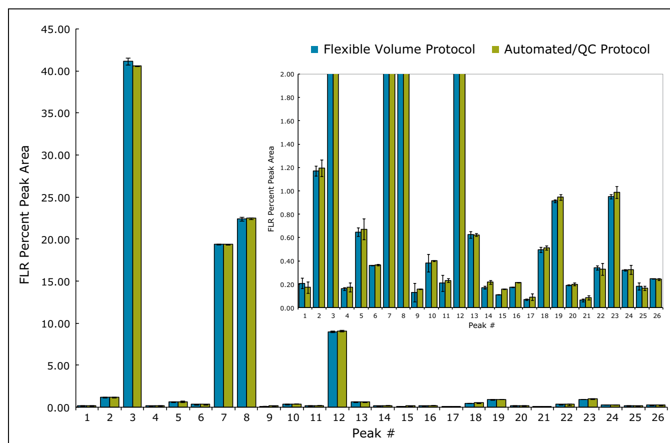

Figure 2. Quantitative comparison of RFMS labeled glycans prepared using GlycoWorks RapiFluor-MS Kit flexible volume protocol and proposed automation and QC volume protocol. Inset shows zoomed view of results.

Peak #	Peak ID
1	FA1
2	A2
3	FA2
4	M5
5	FA1G1 A2G1
6	A2G1 (iso)
7	FA2G1
8	FA2G1 (iso)
10	FA2G1B
11	FA2G1B (iso)
12	FA2G2
13	FA2G1Ga1
14	FA2BG2
18	FA2G2Ga1
19	FA2G2Ga1 (iso)
22	FA2G2Sg1
23	FA2G2Ga2
24	Fa2G2GaSg1
25	Fa2G2GaSg1 (iso)
9, 15, 16, 17, 20, 21, 26	unidentified

Table 3. Figure 1 peak identifications based on mass (Xevo G2-S QTof).

CONCLUSIONS

The GlycoWorks *Rapi*Fluor-MS N-glycan sample preparation procedure has been successfully adapted to be more amenable to automation and QC use by adjusting pipetted volumes to ≥10 µL. This supplemental procedure requires a glycoprotein sample concentration of 1.5 mg/mL to obtain optimal results. As an added benefit, the dispensed aliquots of the sample and principal reagents are equivalent in volume (10 µL), thereby providing greater assurance that the relative amounts of these components will be equivalent regardless of the systematic accuracy of the pipetting device that is used, which should result in greater intra-laboratory and inter-laboratory reproducibility.

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5. ISO 8655-2:2002, pg. 6.

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Profiling Released High Mannose and Complex N-Glycan Structures from Monoclonal Antibodies Using *Rapi*Fluor-MS Labeling and Optimized Hydrophilic Interaction Chromatography

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Optimized LC method to improve the HILIC profiling of common mAb N-glycan species.
- Improved resolution of high mannose structures, in addition to sialylated species and N-glycan structures containing alpha-linked galactose units.
- The use of *Rapi*Fluor-MS™ High Mannose Standard in system suitability testing and peak identification.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Intact mAb Mass Check Standard

*Rapi*Fluor-MS High Mannose Standard

ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

UNIFI® 1.7 Software

MassLynx® 4.1 Software

KEY WORDS

ACQUITY UPLC H-Class Bio System, ACQUITY UPLC Glycan BEH Amide Column, glycans, glycosylated protein, glycoprotein, Glycosylation, N-Linked glycans, HILIC, *Rapi*Fluor-MS labeling, Intact mAb Mass Check Standard, IgG, monoclonal antibody (mAb), high mannose N-glycans, *Rapi*Fluor-MS High Mannose Standard

INTRODUCTION

Glycan characterization is at the forefront of the biopharmaceutical industry, since most protein therapeutics possess N-glycosylation, if not also O-glycosylation. The most common therapeutic modality, an IgG monoclonal antibody (mAb), is, for instance, typically N-glycosylated at two conserved sites in its Fc domain. Not surprisingly, the nature of the N-glycans on a mAb can impact its circulation half-life and efficacy. As a result, it is particularly important that the N-glycans of a mAb be well characterized and routinely monitored.

A powerful strategy for such an analysis involves releasing N-glycans enzymatically, labeling them with a reagent to improve their detectability, and profiling them using hydrophilic interaction chromatography (HILIC).[1] Recent developments in released N-glycan profiling, made possible by the novel *Rapi*Fluor-MS labeling reagent, have eclipsed conventional N-glycan techniques by simplifying the steps in the procedure, reducing the overall sample preparation times, and enabling unprecedented sensitivities for both fluorescence and mass spectrometric detection.[2] However, regardless of how quickly a sample can be prepared or with what sensitivity it can be detected, it is imperative for N-glycan profiling to exhibit optimal chromatographic resolution. With there being many different sample types, it is often necessary to consider tailoring liquid chromatographic (LC) methods to ensure that robust and optimal chromatographic performance can indeed be achieved.

In this application note, we highlight the development of an LC method that optimizes the chromatographic resolution for the released N-glycans that are commonly found on mAbs, including the high mannose N-glycan structures that are known to negatively affect circulation half-life as well as indicate aberrant cell culture conditions. In this work, N-glycans from a mAb were rapidly released with PNGase F, labeled with *Rapi*Fluor-MS and profiled by HILIC using sensitive fluorescence and mass spectrometric detection. The separation method was optimized to improve the resolution of high mannose glycans, those terminated with N-glycolyl neuraminic acid (Sg) (a member of the broad class of sialic acids), and species containing alpha-linked galactose monosaccharides. Within this work, the utility of this newly developed LC method is demonstrated by means of system suitability testing with high mannose spiked samples.

Sample description

The Intact mAb Mass Check Standard (p/n 186006552) was reconstituted in water to a concentration of 2 mg/mL. N-glycans were released from a 15-μg aliquot of this murine mAb and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606) following the instructions provided in its care and use manual (715004793). *Rapi*Fluor-MS-labeled N-glycans were prepared for injection at a concentration of 0.5 pmol/μL (as a mixture in a solvent composed of 90 μL SPE eluate, 100 μL dimethylformamide, and 210 μL acetonitrile).

*Rapi*Fluor-MS High Mannose Standard (p/n 186008317) was reconstituted in water to produce a 5 pmol/μL solution. A series of spiked samples were then prepared by mixing *Rapi*Fluor-MS labeled glycans from Intact mAb Mass Check Standard with the *Rapi*Fluor-MS High Mannose Standard and water. In this way, four spiked samples containing 0.45 pmol/μL of sample-derived N-glycans were prepared along with varying concentrations of the high mannose glycans. Spiking produced samples with mannose-5 (M5) at a relative abundance ranging from approximately 0.2% to 2%.

*Rapi*Fluor-MS Dextran Calibration Ladder (p/n 186007982) was reconstituted with 100 μL of water to produce a 0.5 μg/μL solution. The GU assignments were calculated using a cubic spline fitting method and UNIFI 1.7 Software.

LC conditions for *Rapi*Fluor-MS Released N-Glycans

Chromatographic separations were performed using the following conditions, unless otherwise noted:

Universal N-Glycan Profiling Method

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 60 °C

Flow rate: 0.4 mL/min

Injection volume: 10 μL for DMF/ACN-diluted samples or 1 μL for aqueous samples

Column: ACQUITY UPLC Glycan BEH Amide, 1.7 μm, 2.1 x 150 mm (p/n 186004742)

Fluorescence detection: Ex 265 nm / Em 425 nm, 2 Hz

Mobile phase A: 50 mM aqueous ammonium formate, pH 4.4 (LC-MS grade water; from a 100x ammonium formate concentrate (p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

mAb N-Glycan Profiling Method

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 45 °C

Flow rate: 0.5 mL/min

Injection volume: 10 μL (DMF/ACN-diluted samples), 1 μL (aqueous samples)

Column: ACQUITY UPLC Glycan BEH Amide 1.7 μm, 2.1 x 150 mm (p/n 186004742)

Fluorescence detection: Ex 265 nm / Em 425 nm, 2 Hz

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate (p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.00	0.5	20	80	
3.00	0.5	27	73	6
35.0	0.5	37	63	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	20	80	6
47.6	0.5	20	80	6
55.0	0.5	20	80	6

MS conditions for *Rapi*Fluor-MS Released N-Glycans

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr
Calibration:	NaI, 0.1 µg/µL from 100–2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
Lockspray™:	100 fmol/µL human Glu-fibrinopeptide B prepared in a solution composed of 70:30:0.1% water/acetonitrile/formic acid, sampled every 90 seconds
Data management:	MassLynx 4.1 Software, UNIFI 1.7 Software

RESULTS AND DISCUSSION

A Universal N-Glycan Profiling Method

The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit facilitates the robust analysis of many, very diverse N-glycans. Accordingly, we first aimed to establish a separation method for an ACQUITY UPLC Glycan BEH Amide Column that can be universally applied to all types of N-glycans, from small biantennary structures up to highly sialylated, tetraantennary species. It is very useful to run this so-called 'universal N-glycan profiling method' when analyzing new samples. Given that this method is the basis of an upcoming glucose unit (GU) database for *Rapi*Fluor-MS labeled glycans, it will also be the technique recommended for future workflows involving GU based peak assignments.

Being a generic tool, the universal N-glycan profiling method is not optimized for any particular N-glycan sample, including the N-glycans obtained from the Intact mAb Mass Check Standard that is provided as a control sample in each GlycoWorks *Rapi*Fluor-MS N-Glycan Kit. Since this standard is a mAb expressed from a murine cell line, it is a relevant surrogate to many mAb therapeutics and will, in fact, produce a highly similar N-glycan profile. The universal N-glycan profiling method produces chromatograms where the mAb glycans elute in only the first half of the analytical gradient, as shown in Figures 1A and 1B. Using online, mass spectrometric detection, at least 14 different N-glycans can be readily identified (Table 1). In addition to having been eluted in a narrower retention window, several of these glycans are only partially resolved and consequently are difficult to monitor, and reliably quantitate, on an LC system that is not fully optimized for low extra-column dispersion. Such peaks, or those that are unresolved, require the use of MS detection and an analysis of extracted ion chromatograms (Figures 1C and 1D) to be deciphered. Notable critical pairs exhibiting at least partial co-elution include M5/A2G1 and FA2G2Sg1/FA2G2Ga2. Given the significance of monitoring M5 and immunogenic glycans, like those containing the noted N-glycolyl neuraminic acid (Sg) and alpha-linked galactose monosaccharides, these separations were optimized for increased resolution between species containing these types of sugars.

	Glycan	*Rapi*Fluor-MS labeled glycan composition	Mi (Da)	2+	3+
1	A2	$C_{67}H_{105}O_{37}N_9$	1627.66	814.84	543.56
2	FA2	$C_{73}H_{115}O_{41}N_9$	1773.72	887.87	592.25
3	M5	$C_{63}H_{99}O37N_7$	1545.61	773.81	516.10
4	FA1G1	$C_{71}H_{112}O_{41}N8$	1732.69	867.35	578.57
	A2G1	$C_{73}H_{115}O_{42}N_9$	1789.71	895.86	597.58
5	A2G1	$C_{73}H_{115}O_{42}N_9$	1789.71	895.86	597.58
6	FA2G1	$C_{79}H_{125}O_{46}N_9$	1935.77	968.89	646.26
7	FA2G1	$C_{79}H_{125}O_{46}N_9$	1935.77	968.89	646.26
8	FA2G2	$C_{85}H_{135}O_{51}N_9$	2097.82	1049.92	700.28
9	FA2G1Ga1	$C_{85}H_{135}O_{51}N_9$	2097.82	1049.92	700.28
10	FA2G2Ga1	$C_{91}H_{145}O_{56}N_9$	2259.88	1130.95	754.30
11	FA2G2Ga1	$C_{91}H_{145}O_{56}N_9$	2259.88	1130.95	754.30
12	FA2G2Sg1	$C_{96}H_{152}O_{60}N_{10}$	2404.92	1203.46	802.65
13	FA2G2Ga2	$C_{97}H_{155}O_{61}N_9$	2421.93	1211.97	808.32
14	FA2G2Ga1Sg1	$C_{102}H_{162}O_{65}N_{10}$	2566.97	1284.49	856.66

Table 1. RapiFluor-MS labeled N-glycans from the Intact mAb Mass Check Standard, a murine monoclonal antibody.

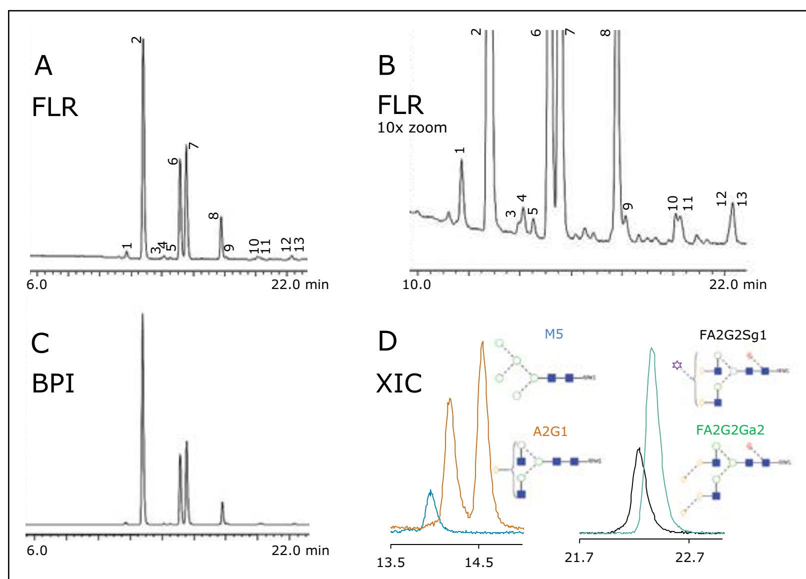


*Figure 1. (A) Fluorescence (FLR) chromatogram of RapiFluor-MS labeled N-glycans from Intact mAb Mass Check Standard obtained using the universal N-glycan profiling method and a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column. (B) Fluorescence chromatogram scaled with a 10x zoom to show low abundance N-glycans (C) Base peak intensity (BPI) chromatogram of Rapi*Fluor*-MS labeled N-glycans obtained with the universal N-glycan profiling method (D) Extracted ion chromatograms (XICs) of critical pairs to highlight issues with partial co-elution. N-glycan samples corresponding to 0.38 µg of the Intact mAb Mass Check Standard were analyzed in each experiment.*

Developing a Higher Resolution mAb N-Glycan Analysis Method

The significant co-elution of the members of these critical glycan pairs necessitated the development of an LC method specifically tailored for N-glycans released from mAbs. Modifying a HILIC separation by reducing the slope of the gradient can produce more resolution between labeled N-linked glycans with similar partition coefficients. As a result, the first change made to the method was to reduce the gradient slope while retaining the overall run time. A new gradient running from 26%–37% mobile phase A (versus 25% to 46%) indeed showed improvement in peak resolution. In another step, the retention time of the glycans was reduced by increasing the flow rate of the separation from 0.4 to 0.5 mL/min. This flow rate adjustment shifted the mAb N-glycan profile to be well within the gradient window, while at the same time, the maximum pressure of the analysis was maintained well below the pressure limit of the system. Increasing the flow rate of the separation also yielded an improvement in the resolution of the critical pairs (Figures 2A and 2B), but caused an average of a 90% increase in peak widths. This peak broadening was attributed to poor band formation when the injected sample approached the head of the column. To improve band formation, the eluent strength at the onset of the separation was reduced. The gradient was changed to have two segments, an initial ramp from 20% to 27% mobile phase A over 3.2 minutes followed by 27% to 37% mobile phase A over 31.8 minutes. Indeed, these changes facilitated better band formation at the head of the column and obtaining correspondingly sharper glycan peaks (Figure 2C). Despite improving the method in multiple ways, the two noted critical pairs of *Rapi*Fluor-MS labeled N-glycans remained only partially resolved. One final adjustment to the running conditions proved highly effective in improving the resolution of these critical pairs.
Lowering the column temperature from 60 °C to 45 °C increased their separation to the point that near baseline resolution could be achieved (Figure 2D). With these final conditions, half-height resolution of the M5/A2G1+FA1G1 and the FA2G2Sg1/FA2G2Ga2 peaks were found to be 1.61 and 1.13, respectively. The extent of separation has been improved for all major species throughout the mAb profile, except for the alpha-linked galactose isomers of the FA2G2Ga1 glycan eluting at approximately 25 minutes.

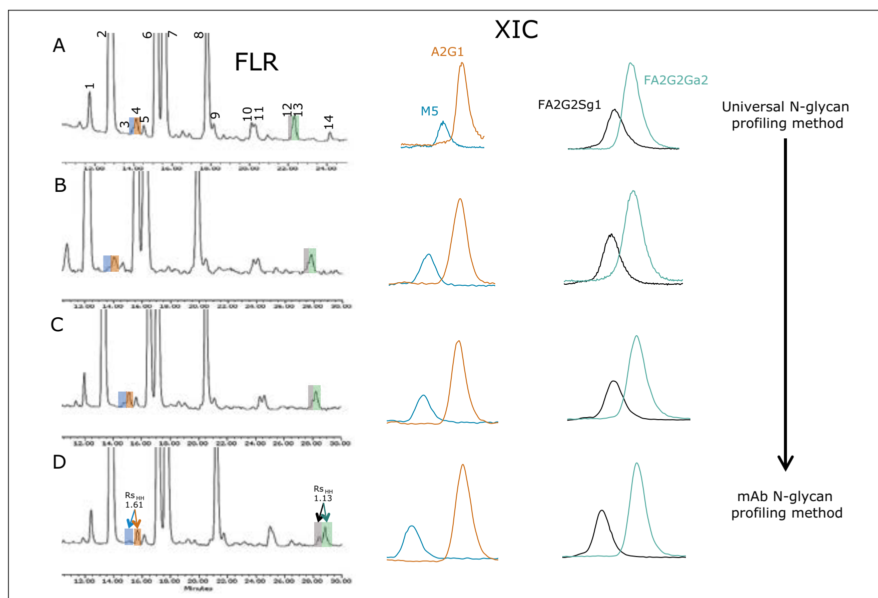
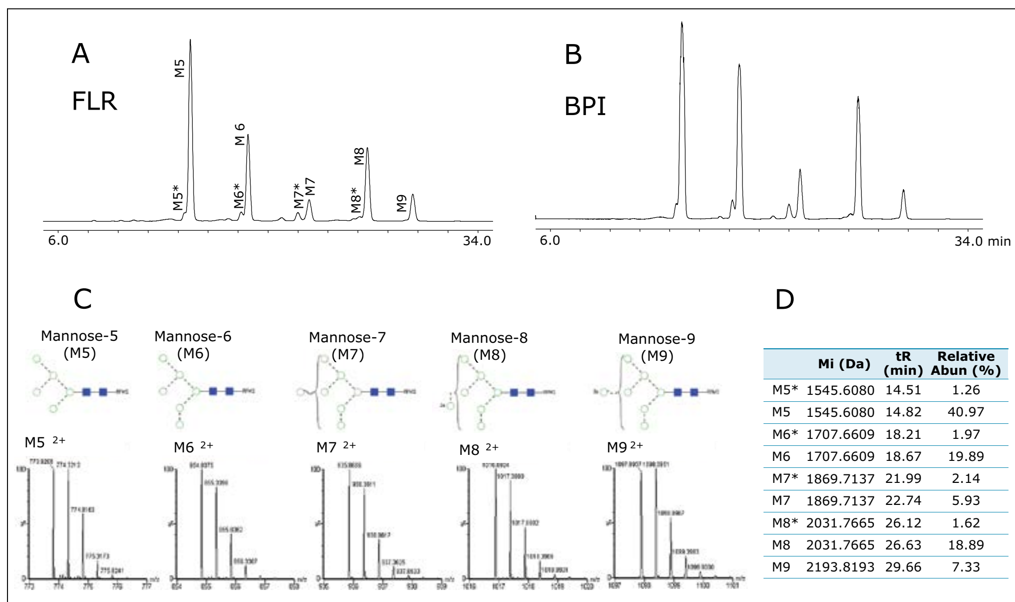


Figure 2. (A) Fluorescence (FLR) chromatogram and extracted ion chromatograms (XICs) obtained with the universal N-glycan profiling method and a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column. (B) FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60 °C column temperature, and a 35 minute gradient from 26% to 37% H_2O (C) FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60 °C column temperature, and a two-step gradient of 20% to 27% H_2O in 3.2 min followed by 27% to 37% H_2O in 31.8 min. (D) FLR chromatogram and XICs obtained as listed in (C) except with a column temperature of 45 ˚C (the mAb N-glycan profiling method). N-glycan samples corresponding to 0.38 µg of the Intact mAb Mass Check Standard were analyzed in each experiment. RsHH denotes peak resolution measured at half-height (HH).

Using the *Rapi*Fluor-MS High Mannose Standard to Demonstrate System Suitability

The resolution gains afforded by the new mAb N-glycan profiling method allows for better monitoring of high mannose structures. To this end, we have employed a new proficiency standard, the *Rapi*Fluor-MS High Mannose Standard, to demonstrate its ability to precisely monitor high mannose structures. This new standard contains *Rapi*Fluor-MS-labeled M5, M6, M7, M8, and M9. When separated on its own, it is evident that the *Rapi*Fluor-MS High Mannose Standard is a relatively simple mixture (Figure 3).



	Mi (Da)	tR (min)	Relative Abun (%)
M5*	1545.6080	14.51	1.26
M5	1545.6080	14.82	40.97
M6*	1707.6609	18.21	1.97
M6	1707.6609	18.67	19.89
M7*	1869.7137	21.99	2.14
M7	1869.7137	22.74	5.93
M8*	2031.7665	26.12	1.62
M8	2031.7665	26.63	18.89
M9	2193.8193	29.66	7.33

Figure 3. (A) Fluorescence (FLR) and base peak intensity (BPI) chromatograms (B) of the Waters RapiFluor-MS High Mannose Standard as obtained using a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column and the mAb N-glycan profiling method. Asterisks denote isomers of M5, M6, and M7. (C) ESI mass spectra of each major component. (D) Representative chromatographic data. Approximately 5 pmoles of high mannose N-glycans were analyzed in these experiments. An asterisk (*) denotes a linkage isomer.

As a result, it can potentially be used to support the identification of high mannose species when MS detection may not be available. Moreover, this high mannose glycan mixture is well suited for use in spiking studies, which can be performed to establish system suitability. In this work, *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard were spiked with varying concentrations of the *Rapi*Fluor-MS High Mannose Standard (as outlined in the experimental section). Four *Rapi*Fluor-MS labeled glycan samples were prepared with M5 relative abundances ranging from 0.2% to 2.0% and analyzed as illustrated in Figure 4A. In these samples, M5, M6, and M8 are readily detected, while M7 and M9 are not due to their lower relative abundances in the spiking standard. The high resolution of the method allows for better integration of the high mannose glycan species. This can be clearly demonstrated by plotting the fluorescence peak areas of M5, M6, and M8 as functions of the spiking level. The linearity of these data ($R^2 \geq 0.974$) underscores the suitability of this technique for monitoring high mannose structures (Figure 4B). These spiking experiments also demonstrate that the Intact mAb Mass Check Standard is effectively free of high mannose species and that the M5 previously monitored during the development of the separation is near the limit of quantitation of this method.

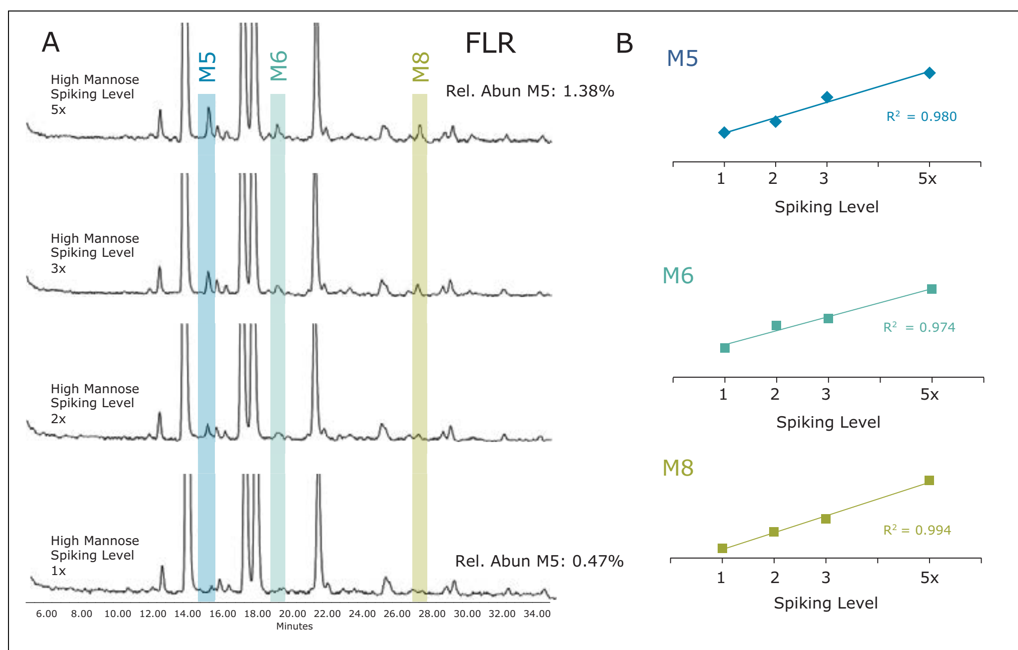


Figure 4. (A) Fluorescence (FLR) chromatogram obtained for RapiFluor-MS N-glycans prepared from Intact mAb Mass Check Standard spiked with varying concentrations of the RapiFluor-MS High Mannose Standard. Top to bottom: spiking levels are 5x, 3x, 2x, and 1x. (B) Peak area of high mannose structures at different spike concentrations. N-glycan samples corresponding to 0.34 µg of the Intact mAb Mass Check Standard were analyzed in each experiment. Separations were performed using the mAb N-glycan profiling method and an ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column.

GU Values from the Universal N-Glycan Profiling Method Versus the mAb N-Glycan Profiling Method

As mentioned earlier, the universal N-glycan profiling method is a generic tool for all N-glycan sample types. It will also be the method recommended for workflows involving assignment of new glycan peaks based on matching GU values to data in an upcoming *Rapi*Fluor-MS GU database, which is currently being constructed in collaboration with the National Institute for Bioprocessing Research and Training (NIBRT).

With this in mind, it is important to recognize that GU values, regardless of labeling strategy, are method specific. So although the mAb N-glycan profiling method can be used with GU values, it will not generate GU values that are meaningful for searching a NIBRT database based on the universal N-glycan profiling method. GU values still have merit for glycan analyses, even if they are not used for database matching. Use of GU values minimizes subtle retention time variations between runs and between different instruments by expressing chromatographic retention in terms of standardized GU values.[3] To assign GU values, a dextran ladder, consisting of glucose multimers of increasing length, is used as an external calibrant. The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values. Chromatographic data collected from separations of the *Rapi*Fluor-MS Dextran Calibration Ladder are provided in Table 2, one set of data obtained with the universal N-glycan profiling method and the other with the new, mAb N-glycan profiling method. Not surprisingly, differences between the methods led to shifts in the retention times of the individual glucose multimers. Therefore, GU values derived for the mAb N-glycans are also shifted, as shown in (Table 2). For the most strongly retained species, the FA2G2Ga1Sg1 glycan, there is, in fact, a GU shift of +0.37. Clearly, it is important to give consideration to how GU values are generated and how they are to be used. The universal N-glycan profiling method is the appropriate method for GU database searching. Nevertheless, GU values can be used along with the mAb N-glycan profiling method as replacements to standard retention times to improve the robustness of data reporting.

		Universal N-Glycan Profiling	**mAb N-Glycan Profiling**
Component	Name	Glucose Units	Glucose Units
1	A2	5.49	5.54
2	FA2	5.82	5.91
3	M5	6.19	6.24
4	FA1G1 + A2G1	6.23	6.37
5	A2G1	6.38	6.49
6	FA2G1	6.69	6.72
7	FA2G1	6.85	6.86
8	FA2G2	7.43	7.69
9	FA2G1Ga1	7.55	7.81
10	FA2G2Ga1	8.25	8.57
11	FA2G2Ga1	8.30	8.60
12	FA2G2Sg1	9.06	9.39
13	FA2G2Ga2	9.11	9.49
14	FA2G2Ga1Sg1	9.88	10.25

Table 2. Glucose unit values for the RapiFluor-MS labeled N-glycans from the Intact mAb Mass Check Standard and the RapiFluor-MS High Mannose Standard, as obtained with the universal N-glycan profiling method versus the mAb N-glycan profiling method. Glucose unit (GU) values were assigned using cubic spline fitting and UNIFI 1.7 Software.

CONCLUSIONS

The N-linked glycosylation of mAbs can impact their circulation half-life and efficacy. Therefore, it is particularly important for the N-glycans of a mAb to be well characterized and routinely monitored. By labeling mAb N-glycans with *Rapi*Fluor-MS, high sensitivity detection by both fluorescence and MS is made possible. The sample loading condition, gradient steepness, flow rate, and separation temperature of the universal N-glycan profiling method were adjusted to create a mAb N-glycan profiling method that was able to better resolve the Man5/A2G1+FA1G1 and FA2G2Sg1/FA2G2Ga2 critical pairs. The mAb N-glycan profiling method yielded a half-height resolution of 1.61 for M5/A2G1+ N-glycans and FA2G2Sg1/FA2G2Ga2 of 1.13. By improving the resolution of these critical pairs of N-glycans, we have provided additional separation space for monitoring high mannose structures. To this end, the *Rapi*Fluor-MS High Mannose Standard was used in a series of spiking experiments to demonstrate the quantitative performance of this new gradient for analyzing high mannose N-glycan structures. Using the mAb N-glycan profiling method in conjunction with the new *Rapi*Fluor-MS High Mannose Standard and the *Rapi*Fluor-MS Dextran Ladder allows for the easier adoption of this system solution.

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[TECHNOLOGY BRIEF]

Enhancing the Peak Capacity of High Molecular Weight N-Glycan HILIC Separations with a Wide-Pore Amide Bonded Stationary Phase

Matthew A. Lauber and Stephan M. Koza

GOAL

To demonstrate the enhanced resolving power of the ACQUITY UPLC® Glycoprotein BEH amide, 300Å, Column for separations of high molecular weight, *Rapi*Fluor-MS™ labeled *N*-glycans.

Wide-pore glycoprotein BEH amide, 300Å, 1.7 µm columns for enhancing the resolution of tri- and tetra-antennary, *Rapi*Fluor-MS labeled *N*-glycans.

BACKGROUND

Protein glycosylation is frequently profiled by removing glycans from their counterpart glycoprotein and imparting them with a detectable chemical moiety, such as the fluorescence and MS-active *Rapi*Fluor-MS label.[1] High resolution separations of these released and labeled *N*-glycans can be obtained by UPLC® hydrophilic interaction chromatography (HILIC) with purposefully designed glycan BEH amide, 130Å columns.[2] Interestingly, glycosylation of proteins can be extremely diverse. While monoclonal antibodies tend to be modified with relatively low molecular weight (1 to 3 kDa) biantennary structures, numerous biotherapeutic proteins are expressed with comparatively high molecular weight (3 to 6 kDa) tri- and tetra-antennary structures. Such large and highly branched glycan structures exhibit large radii of hydration.

Consequently, the application of chromatography columns containing particles with standard average pore diameters (80 to 150Å) can limit the resolution with which these species can be separated. It is therefore advantageous to employ a stationary phase with a wide average pore diameter, wherein large structures will have access to the majority of the porous network and the surface area of the stationary phase. In addition, the large labeled glycan structures are less likely to experience restricted diffusion while migrating through the pores of a wide-pore material.[3-4] In this technology brief, we demonstrate the utility of an amide bonded stationary phase with an average pore diameter of 300Å (glycoprotein BEH amide, 300Å, 1.7 µm) and its ability to enhance the resolution of *Rapi*Fluor-MS labeled tri and tetra-antennary N-glycans derived from recombinant human Factor IX.

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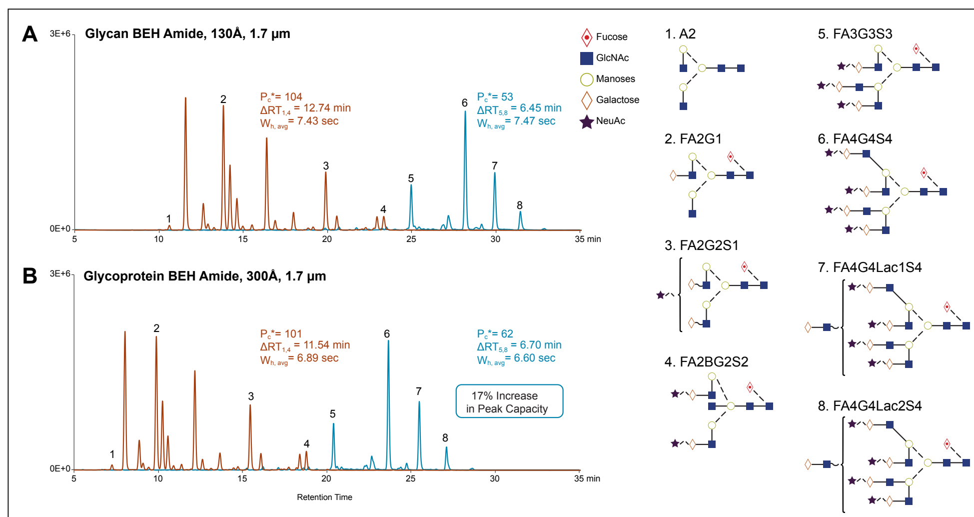

Figure 1. HILIC fluorescence chromatograms for RapiFluor-MS labeled N-glycans from pooled human IgG (orange) and recombinant Factor IX (blue). (A) Chromatograms obtained for glycans from 0.4 µg protein using an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. (B) Chromatograms obtained for glycans from 0.4 µg protein using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. Separations were performed according to the conditions in the GlycoWorks™ RapiFluor-MS Care and Use Manual (p/n 715004793). Peak capacities were calculated from half-height widths and retention windows derived from the labeled peaks.

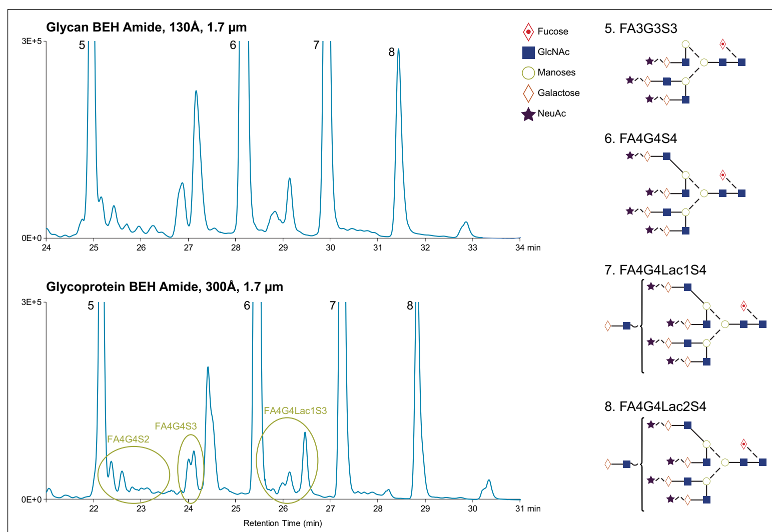

Figure 2. HILIC fluorescence chromatograms for RapiFluor-MS labeled N-glycans from recombinant Factor IX as obtained using 2.1 x 150 mm columns with various amide bonded stationary phases. Peak capacities were calculated from half-height widths and retention windows derived from the labeled peaks.

THE SOLUTION

N-glycans were prepared from both pooled human IgG as well as recombinant human Factor IX, a glycoprotein known to be modified with large, highly sialylated *N*-glycans.[5] Specifically, these samples were prepared using rapid deglycosylation, *Rapi*Fluor-MS labeling, and µElution HILIC SPE, as described in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit Care and Use Manual.[1,6] Glycan mapping of the resulting *Rapi*Fluor-MS labeled *N*-glycans was first performed with a glycan BEH amide, 130Å, 1.7 µm column, given that it is intended for general purpose glycan analyses and for use with GlycoBase database searching.[7] Figure 1A displays the fluorescence chromatograms obtained when HILIC-based chromatography is performed on a sample containing less hydrophilic IgG *N*-glycans (orange) versus a sample containing later eluting, more hydrophilic *N*-glycans from Factor IX (blue). In addition, Figure 1A displays the effective peak capacities for the retention windows of the two sample types. These results can be compared to Figure 1B, which presents the chromatograms and peak capacities obtained with a glycoprotein BEH amide column containing the 300Å, wide-pore stationary phase. While the effective peak capacities for the IgG *N*-glycans are comparable, a marked improvement in peak capacity of approximately 17% is apparent in the separations of the Factor IX *N*-glycans when the wide-pore glycoprotein BEH amide 300Å, 1.7 µm column is used. The resolving power of the wide-pore, BEH amide column for the large *N*-glycans is noteworthy, in that it facilitates resolving several low abundance species. Figure 2 highlights some of the impacted regions of the chromatogram where there are improvements in the resolution of FA4G4S2, FA4G4S3, and FA4G4Lac1S3 *N*-glycans. In summary, when smaller, biantennary *N*-glycans are to be separated, a glycan BEH amide, 130Å, 1.7 µm stationary phase is an ideal choice due to its high surface area and high retentivity. For the characterization of large, tri- and tetra-antennary *N*-glycans, it is, however, advantageous to use the wide-pore amide stationary phase. Moreover, the wide-pore amide column is intended specifically for large biomolecule separations: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm stationary phase is ensured to have consistent batch-to-batch performance through stringent quality control testing involving a separation of ribonuclease B (RNase B) glycoforms at the intact protein level (see reference 8 for an example of this chromatography).[8]

SUMMARY

High molecular weight, tri- and tetra-antennary *N*-glycans are highly branched structures that adopt relatively large radii of hydration in solution. To achieve optimal HILIC separations of these large structures, we propose a column with a wide-pore amide bonded stationary phase, a glycoprotein BEH amide, 300Å, 1.7 µm column. For large glycan species, this column provides increases in peak capacity over a conventional pore diameter column of approximately 17%. Improved resolving power is particularly useful in this separation space as it is typified by highly complex glycan profiles. Most notably, these improvements in resolution should be of significant utility in the characterization and routine monitoring of biopharmaceuticals that are expressed with large, highly complicated *N*-glycan structures, such as coagulation Factor IX, erythropoietin, and darbepoetin.

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Exploiting *Rapi*Fluor-MS Labeling to Monitor Diverse N-Glycan Structures via Fluorescence and Mass Detection

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Reduced sample preparation times for released N-glycan analyses
- Increased confidence in glycan monitoring by obtaining fluorescence and mass detection for every peak

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GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY® QDa® Mass Detector

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Column

Empower® 3 FR2 CDS

KEY WORDS

Glycan, mass detection, H-Class, ACQUITY, QDa, *Rapi*Fluor-MS

INTRODUCTION

Glycosylation is one of the most complex post-translational modifications of protein-based biotherapeutics. The efficacy of glycosylated therapeutics is directly related to the glycoprofile. The presence of undesired structures can lead to changes in PK/PD profiles, either positively or negatively, and have been associated with immunogenic responses. For these reasons glycosylation is often designated as a critical quality attribute (CQA). During the development process, the glycoprofile of candidate molecules is extensively studied and characterized. Characteristic profiles are then monitored through process development, commercialization, and post-approval studies to maintain product efficacy and safety.

In this application note, we present a streamlined approach to label released N-glycans with *Rapi*Fluor-MS and analyze the labeled N-glycans with the ACQUITY UPLC H-Class Bio System with fluorescent (FLR) and ACQUITY QDa Mass Detectors. This new monitoring workflow allows researchers to prepare samples from glycoprotein to UPLC-FLR/MS analysis in 30 minutes. In addition to reduced sample preparation times, *Rapi*Fluor-MS yields 14 times greater fluorescence response and 160 times greater MS response when compared to 2-AB. These improvements enable the use of FLR and mass detection with the ACQUITY QDa for routine analysis. In this application note we present the utility of *Rapi*Fluor-MS coupled with UPLC®-FLR-MS for monitoring labeled glycans ranging across a range of properties, masses, and relative abundance.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR and ACQUITY QDa Mass Detector
Column:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	2 µL
Data management:	Empower 3 FR2 CDS

FLR settings

Data rate:	5 points/sec.
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500–1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C
Ionization mode:	ESI+
Mobile phase A:	Acetonitrile (Pierce, LC-MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC-MS Grade, Waters ammonium formate concentrate)
Mobile phase C:	Acetonitrile (LC-MS grade)
Mobile phase D:	Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	% A	% B	%C	% D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

A sample of murine IgG1 mAb N-Glycans was prepared from Waters Intact mAb Mass Check Standard (p/n 186006552), which is included in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606). N-Glycans were also prepared from RNase B and bovine fetuin (Sigma Aldrich). Released and labeled N-glycan pools were generated using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit following the protocol provided in the Care and Use Manual (715004793). Following release and labeling, samples were dried using a CentriVap™ and reconstituted in 25 µl of a mixture of ACN/Water/DMF at a ratio of 22.5%:55.5%:22%, respectively. In each case the targeted mass load was 30 pmoles of released glycan. The ammonium formate mobile phase was prepared using Waters ammonium formate concentrate (p/n 186007081).

RESULTS AND DISCUSSION

N-glycosylation is a non-template driven process that generates a vast array of glycan structures that vary in size, charge, and extent of branching depending on the protein and expression system. To evaluate the capacity of the ACQUITY QDa to detect glycans both within and beyond its mass range, three glycoproteins (human IgG, RNAse B, and bovine fetuin) were selected to provide typically observed glycans ranging from neutral bi-antennary structures to tetra-sialylated structures. N-glycans from each protein were released using Rapid PNGase F and labeled with *Rapi*Fluor-MS following the provided sample preparation protocol. Labeled glycans were separated via UPLC-HILIC and detected using both an ACQUITY FLR and ACQUITY QDa.

As is evident in Figure 1, each glycan structure is chromatographically resolved using a single gradient method. In addition, each glycan structure observed in fluorescence (top panel) is also observed by the ACQUITY QDa Mass Detector (bottom panel), indicating the ability of the ACQUITY QDa to detect glycans across a range of possible structures and attributes when labeled with *Rapi*Fluor-MS. For traditional labeling technologies this is not possible due to poor ionization efficiency.

While it is useful that glycan structures can be observed by mass detection, it is important to understand the quality of the resulting spectra and the charge states of the glycan ions obtained within. To understand this aspect, we integrated peaks spanning a range of glycan properties and measured the relative abundances of species in each sample using FLR integrated data. The spectra shown in Figure 2 demonstrate the ability of the ACQUITY QDa to generate high quality spectra for glycan structures across a wide range of properties and masses. The data also demonstrate that both high and low abundance glycan structures can be readily detected. Our data indicates that high quality spectra are generated for structures present in the fluorescence profiles at abundances as low as 0.5% highlighting the sensitivity of ACQUITY QDa mass detection combined with the improved ionization efficiency afforded by *Rapi*Fluor-MS. Our data also demonstrate how the improved charging of glycan structures by the use of *Rapi*Fluor-MS allows small structures such as A2, as well as very large structures, such as the tetrasialylated A3G3S4, to be detected with the QDa.

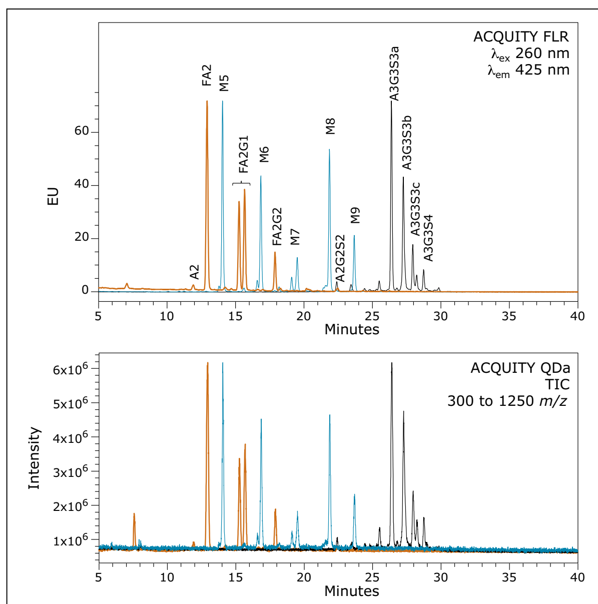

Figure 1. The ACQUITY QDa can detect an array of RapiFluor-MS labeled N-glycans. Glycans from human IgG (red trace), RNAse B (black trace), and bovine fetuin (blue trace) were released with Rapid PNGase F, labeled with RapiFluor-MS reagent. Individual glycan pools were then separated via HILIC and detected with both fluorescence (A) and mass detection (B).

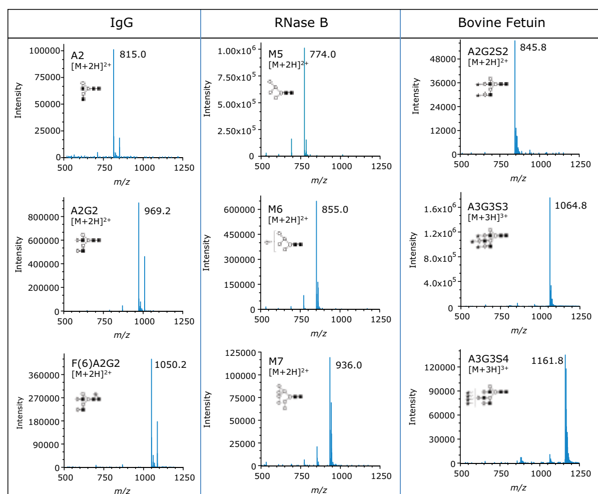

Figure 2. Spectra of selected RapiFluor-MS labeled glycans detected with an ACQUITY QDa Mass Detector. Glycans from murine IgG1 mAb, RNase B, and bovine fetuin were released with Rapid PNGase F and labeled with RapiFluor-MS. Shown are representative spectra for selected glycan structures separated in Figure 1.

CONCLUSIONS

Glycosylation of is a complex and critical aspect of most therapeutic proteins which must be well characterized. Often, the profile of N-glycans is identified as a critical quality attribute and as a result is monitored throughout the lifecycle of products. As discussed in this application note, preparation of samples with a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit can dramatically reduce sample preparation time and complexity. In addition, the use of *Rapi*Fluor-MS yields improved FLR sensitivity and dramatically improved MS sensitivity. Through improving glycan MS sensitivity *Rapi*Fluor-MS labeling permits the use of mass detection with the ACQUITY QDa and thereby affords greater confidence in peak monitoring across the range of structures encountered during biopharmaceutical development. Taken together, *Rapi*Fluor-MS labeling and HILIC-FLR-MS with ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Mass Detector offer an unparalleled solution for monitoring the N-glycan profiles of biotherapeutics.

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New Capabilities for Monitoring Released N-Glycans through the Combined Use of *Rapi*Fluor-MS Labeling, ACQUITY UPLC H-Class Bio System, and Serial Fluorescence/ACQUITY QDa Mass Detection

Eoin F.J. Cosgrave, Matthew A. Lauber, Robert Birdsall, and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Reduce sample preparation times for released N-glycan analyses
- Increase confidence in glycan monitoring by routinely obtaining mass information and fluorescence for every peak

WATERS SOLUTIONS

*Rapi*Fluor-MS™ Glycan Performance Test Standard

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

ACQUITY® QDa® Mass Detector

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC FTN

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Column

Empower® 3 CDS Software

KEY WORDS

Glycans, glycoforms, labeled glycans, peak monitoring, mass detection, fluorescence detection, IgG

INTRODUCTION

During the development of biopharmaceuticals, it is important to characterize and monitor glycoprofiles as they are often implicated as a product critical quality attributes due to their impact on safety, efficacy, and potency among other factors. It is well accepted that structural characterization of the glycoforms present is necessary, and that mass spectrometry (MS) often plays a large role in the identification of glycans.

Often, once the profile has been established, methods are transferred downstream which incorporate fluorescence detection. In many cases, there is a desire to obtain mass information for each detected peak even after characterization. These data have been difficult to obtain for a number of reasons, including a scarcity of mass spectrometers due to their cost and the requirement that MS specialized analysts are needed to generate meaningful and useful data.

In this application note, we present the combined use of *Rapi*Fluor-MS labeling reagent, ACQUITY UPLC H-Class Bio System, and serial fluorescence/ACQUITY QDa Mass Detector for the monitoring of released N-glycan profiles from IgGs. Overall, this new workflow allows scientists to rapidly prepare samples, from glycoprotein to analysis in 30 minutes.

In addition, *Rapi*Fluor-MS labeling yields unprecendented MS response,[1] which enables the use of the ACQUITY QDa for mass detection. We will discuss the improved sensitivity and charge state profile afforded by *Rapi*Fluor-MS, its general utility for fluorescence and mass detection, and the quality of ACQUITY QDa mass spectra obtained for a range of IgG glycan structures.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR and ACQUITY QDa Mass Detector
Column:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	2 µL

FLR settings

Data rate:	5 points/sec
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500–1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C
Ionization mode:	ESI+
Mobile phase A:	Acetonitrile (Pierce, LC/MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC/MS grade, Waters Ammonium Formate Concentrate)
Mobile phase C:	Acetonitrile (LC/MS grade)
Mobile phase D:	Acetonitrile (LC/MS grade)

Time	Flow rate (mL/min)	%A	%B	%C	%D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

SYNAPT® G2-S was used for assessment of *Rapi*Fluor-MS versus 2-AB N-glycan charge states. See Reference 1 for experimental details.

The *Rapi*Fluor-MS Glycan Performance Test Standard (p/n 186007983) was reconstituted in 25 µL of a mixture of DMF/acetonitrile/water at a ratio of 22.5%:55.5%:22%, respectively and used directly. For each analysis the injection volume was 2 µL, which corresponds to 32 pmol of released and labeled N-glycan on column. LC/MS-grade acetonitrile and water were purchased from Pierce. Ammonium formate was prepared using Waters Ammonium Formate Solution-Glycan Analysis (p/n 18600708) by pouring the entire contents of the solution into 1 L of water and mixed. The UPLC® System used was dedicated for applications which do not require non-volatile salts to reduce the likelihood of adduct formation in the mass detector.

RESULTS AND DISCUSSION

Addition of mass detection to an existing analytical workflow permits rapid and unambiguous identification of glycans. Historically, this has been a difficult task due to the need for high resolution instruments with appropriate sensitivity to obtain meaningful mass data. To overcome this issue, the novel labeling reagent, *Rapi*Fluor-MS, can been used. *Rapi*Fluor-MS dramatically increases both the MS sensitivity and charging of released N-glycans.

To demonstrate this, we compared the mass spectra of *Rapi*Fluor-MS labeled glycans to those of glycans labeled with a more traditional fluorescent label, 2-AB. This analysis was performed using time-of-flight mass spectrometry, which characteristically has a very wide mass range. The charge state characteristics of the different labeling technologies could thereby be objectively observed.

As shown in Figure 1, signal intensity improves dramatically when using *Rapi*Fluor-MS. Equally interesting is the shift in the charge states of the detected glycan ions that results from use of *Rapi*Fluor-MS labeling. As shown, *Rapi*Fluor-MS labeled FA2 near exclusively adopts an $[M+2H]^{2+}$ charge state, while more complex structures begin to adopt even higher $[M+3H]^{3+}$ charge states. In each case, at least one highly populated charge state falls well within the mass range of the ACQUITY QDa. Accordingly, *Rapi*Fluor-MS makes it feasible to use the cost effective, user-friendly ACQUITY QDa Mass Detector for N-glycan monitoring experiments.

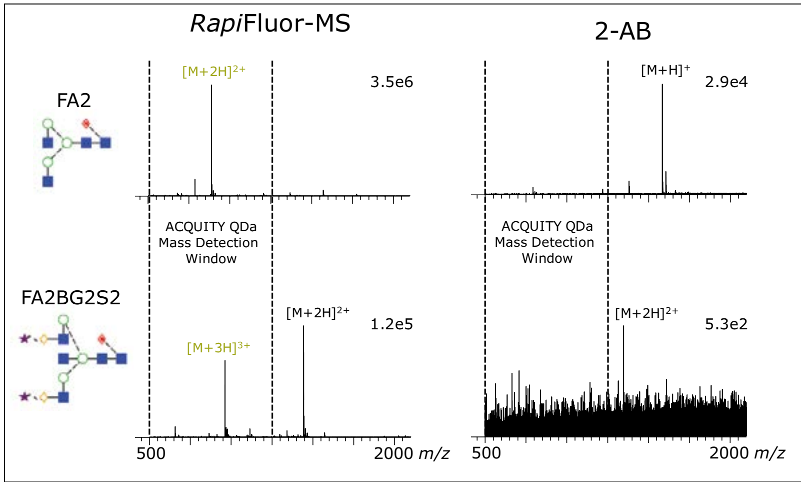

Figure 1. Charge States of RapiFluor-MS Labeled N-Glycans. Time-of-flight ESI+ mass spectra for two N-glycans labeled with RapiFluor-MS and 2-AB, respectively. The detected, protonated charge states that are within the ACQUITY QDa acquisition window are highlighted in green. The upper mass range of the ACQUITY QDa is indicated by the dashed line in each spectrum.

As discussed above, routine detection of N-glycans with the ACQUITY QDa is made possible by *Rapi*Fluor-MS labeling. Importantly, ACQUITY QDa mass detection can be paired with fluorescence detection to facilitate obtaining optical-based quantification along with corroborating data on peak homogeneity and mass information. To enable this data to be collected routinely, the design characteristics of the ACQUITY QDa are such that users without extensive mass spectrometry training are able to generate meaningful mass data easily.

To demonstrate this ability, we separated a sample of IgG released N-glycans labeled with *Rapi*Fluor-MS and monitored the eluting glycans with both FLR and ACQUITY QDa detectors. As shown in Figure 2, high quality data were obtained for both detector channels, with each species identified in the FLR also represented with ACQUITY QDa MS data such that peak assignments can be readily confirmed. Within Empower Software, it is possible to annotate peaks with component and mass information, which makes reviewing data simple, as exemplified in Figure 2.

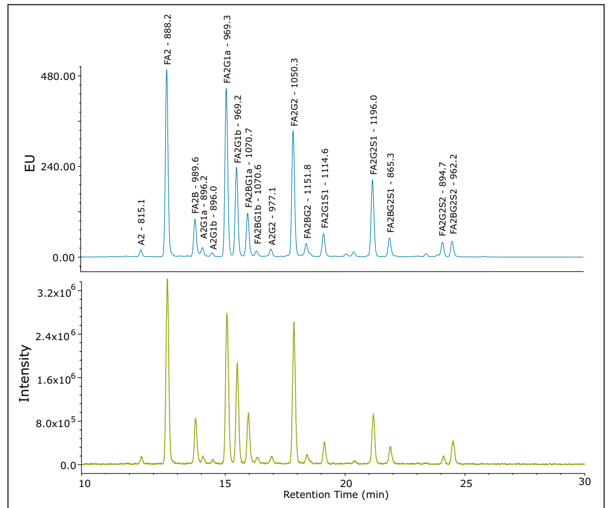

Figure 2. Fluorescence (top trace) and smoothed total ion chromatograms (bottom trace, 5 point mean smooth) of IgG glycans. Each peak is labeled with component name and base peak mass natively in Empower Data Management Software.

While the ability to detect N-glycan structures with the ACQUITY QDa is impressive, spectral quality is paramount for N-glycan monitoring, particularly when there is a need to interrogate the data in detail. We therefore reviewed the quality of MS data associated with peaks observed in the previously shown chromatograms. Figure 3 illustrates the spectra for each assigned peak in Figure 2. Notice that the ACQUITY QDa produced clean, easily interpretable mass spectra for the *Rapi*Fluor-MS labeled glycans, regardless of their relative abundance, molecular weight, or sialic acid content. Clearly, the ACQUITY QDa together with *Rapi*Fluor-MS can provide highly informative data that can be used to increase the confidence of assignments made during routine detection of N-glycans.

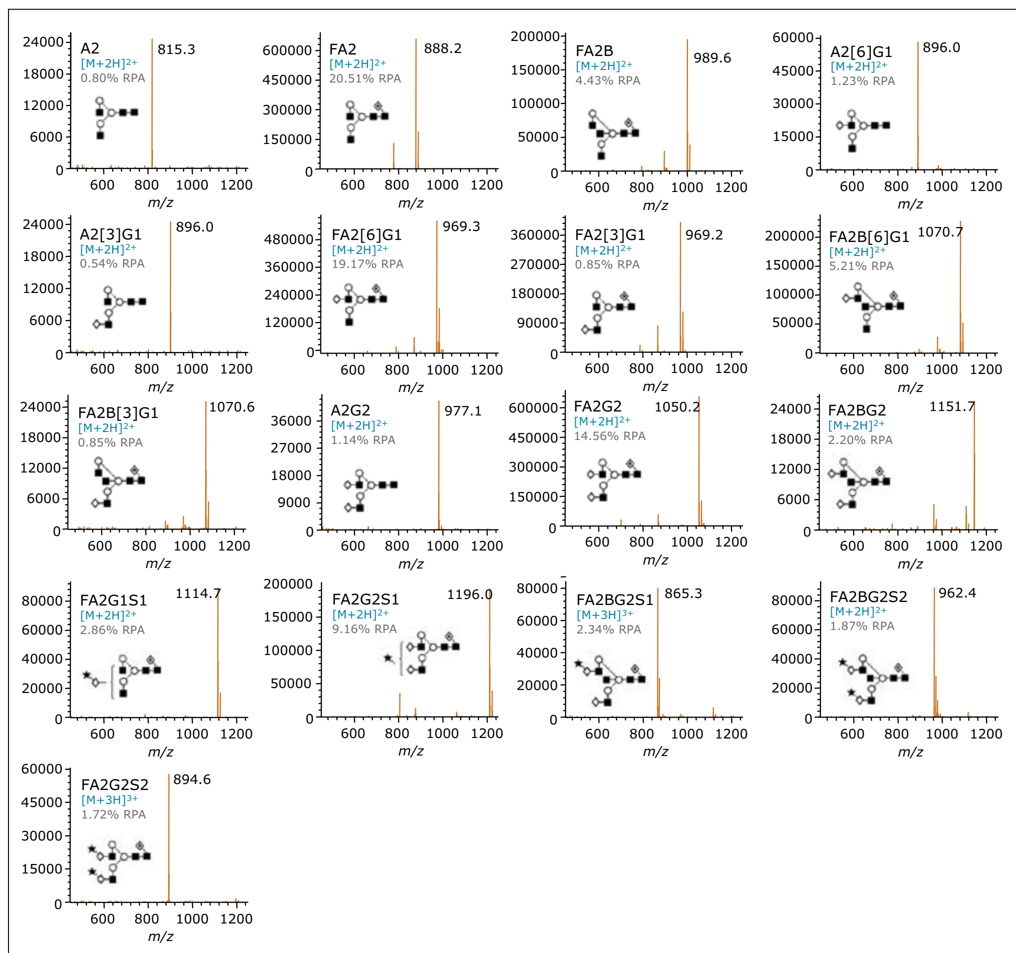

Figure 3. Combined spectra for each glycan structure identified in the chromatograms shown in Figure 2. Identified structures span from simple structures, such as A2, to more complex structures present in IgG samples, such as FA2G2S2. RPA = Relative Peak Area based on FLR integration.

CONCLUSIONS

Glycosylation is a complex and critical aspect of most therapeutic proteins that must be well characterized and monitored throughout product development and commercialization. As discussed in this application note, *Rapi*Fluor-MS can be used to dramatically reduce sample preparation times and complexity, to enhance FLR sensitivity, and to dramatically improve MS sensitivity. By improving glycan MS sensitivity, *Rapi*Fluor-MS labeling permits the use of mass detection with the ACQUITY QDa and thereby affords greater confidence in peak monitoring across the range of structures encountered during biopharmaceutical development.

Taken together, *Rapi*Fluor-MS labeling and HILIC-FLR-MS with the ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Mass Detector offer an unparalleled solution for monitoring the N-glycan profiles of biotherapeutics.

References

1. Lauber, M. A.; Brousmiche, D. W.; Hua, Z.; Koza, S. M.; Guthrie, E.; Magnelli, P.; Taron, C. H.; Fountain, K. J., Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent. Waters Application Note 720005275EN 2015.

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Rapidly Monitoring Released N-Glycan Profiles During Process Development Using *Rapi*Fluor-MS and the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Rapid feedback on glycoprofiles during production to ensure product quality
- Reduced sample preparation times for released N-glycans
- Increased throughput for N-glycan analysis
- Specificity for N-glycan species by incorporating mass detection

WATERS SOLUTIONS

*Rapi*Fluor-MS™ Glycan Performance Test Standard (p/n 186007983)

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class Bio System (FTN)

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Columns

Empower®3 Chromatography Data Software

Waters® Fraction Manager – Analytical

KEY WORDS

Glycans, mass detection, H-Class, ACQUITY, QDa, *Rapi*Fluor-MS, IgG

INTRODUCTION

As glycosylated biotherapeutics move through the development pipeline, the glycoprofile and N-glycan species present are characterized. In addition, as new protein therapeutics progress through development, manufacturing conditions are carefully studied and evaluated during scale-up to ensure consistent safety and efficacy in preparation for clinical studies, and eventual commercialization. As part of this process, the critical quality attributes are often monitored closely to ensure production batches remain within defined acceptance criteria, and to identify those parameters that are critical, often as part of a quality-by-design (QbD) approach. In particular, the N-glycan profile is often monitored closely due to the importance of glycans on the safety and efficacy of protein biotherapeutics.

Monitoring of released N-glycan profiles has historically been burdened with labor intensive sample preparation, which often takes several hours to days. This makes monitoring of the impact of manufacturing conditions on N-glycan profiles challenging. In addition, the analysis of released and labeled N-glycans frequently requires long analysis times. When monitoring of specific structures is desired, users often rely on optical detection for identification and quantification.

In this application note we present the use of *Rapi*Fluor-MS, a novel reagent for rapidly labeling released N-glycans. *Rapi*Fluor-MS dramatically reduces overall released N-glycan sample preparation times to 30 minutes, while improving fluorescence signal by up to 14x and MS signal by up to 1000x, compared to traditional labeling techniques. In conjunction with reduced sample preparation times, we geometrically scaled a highly resolving chromatographic method to one having a total cycle time of 10 minutes. Finally, we incorporated the ACQUITY QDa Detector to monitor specific glycan species using selected ion recording (SIR), which provides a selective means of monitoring species, even if they co-elute. We will discuss how the combination of *Rapi*Fluor-MS and the ACQUITY QDa Detector provides a powerful solution for obtaining meaningful data rapidly and efficiently.

EXPERIMENTAL

Released N-glycans were prepared from commercially available trastuzumab following the protocol provided within the *Rapi*Fluor-MS sample preparation kit. High mannose species used in spiking studies were isolated from RNase-B following release and labeling with *Rapi*Fluor-MS. Mannose species were chromatographically separated and collected using the Waters Fraction Manager – Analytical. Collected samples were dried down using a CentriVap and reconstituted in water. For each analysis the mass load was approximately 32 pmol of released and labeled N-glycan on column. LC-MS grade acetonitrile and water were purchased from Pierce. Ammonium formate was prepared using Ammonium Formate Solution-Glycan Analysis (p/n 186007081) by pouring the entire contents of the solution into 1 L of water and mixed. The UPLC® system used was dedicated for applications which do not require non-volatile salts to reduce the likelihood of adduct formation in the mass detector.

LC conditions

LC system: ACQUITY UPLC H-Class Bio

Detectors: ACQUITY UPLC FLR
ACQUITY QDa

Columns: High resolving method:
ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 μm, 2.1 mm x 150 mm (p/n 186004742)

High throughput method:
ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 μm, 2.1 mm x 50 mm (p/n 186004740)

Column temp.: 60 °C

Sample temp.: 10 °C

FLR settings

Data rate: 5 points/sec

Excitation wavelength: 265 nm

Emission wavelength: 425 nm

QDa settings

Sample rate: 5 points/sec

Mass range: 500 – 1250 Da

Cone voltage: 15 V

Capillary voltage: 1.5 kV

Probe temp.: 400 °C

Mode: Positive ion

Mobile phase A: Acetonitrile (Pierce, LC-MS Grade)

Mobile phase B: 50 mM ammonium formate, pH 4.4, (LC-MS Grade, ammonium formate concentrate)

Mobile phase C: Acetonitrile (LC-MS Grade)

Mobile phase D: Acetonitrile (LC-MS Grade)

Gradient table high resolution method:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

Gradient table high throughput method:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.800	75	25	0	0
5.8	0.800	54	46	0	0
6.1	0.400	0	100	0	0
6.6	0.400	0	100	0	0
7.1	0.400	75	25	0	0
8.0	0.800	75	25	0	0
10.0	0.800	75	25	0	0

Data management

Empower 3 Chromatography Data Software (CDS)

RESULTS AND DISCUSSION

During characterization of released N-glycans, a highly resolving method is often used to provide accurate identification and quantification of the species present in samples. While these methods can be effectively scaled, there is a corresponding loss in resolution as overall run time decreases when using the same chromatograph and particle size column. Often, some loss in resolution will be tolerated if the benefit of speed is achieved, however critical structures must remain clearly identifiable. As shown in Figure 1, moving from a higher resolving 55 min method to a 10 min high throughput method preserves much of the resolution between N-glycan species, however there is loss of resolution between the indicated peaks when moving to the shorter method. This loss of resolution complicates accurate monitoring by optical detection as there is no ability to discriminate between two species.

Due to the improved MS response, we introduced the use of the ACQUITY QDa Detector as part of the detector stream to selectively monitor each of the species present in the sample. By using the SIR function of the ACQUITY QDa Detector we were able to collect independent chromatographic traces for each of the components to overcome the challenge of using optical only detection. As shown in Figure 2, we can clearly discriminate between different glycoforms by using selected ion recording. For each species the corresponding peak, or peaks for species with resolved positional isomers, can be clearly identified and integrated for quantification.

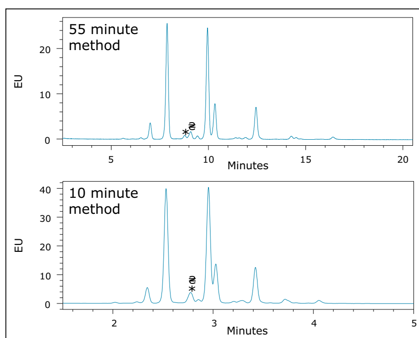

Figure 1. A high resolution separation (top chromatogram) was scaled to a high throughput method by scaling the gradient geometrically while reducing column length and flow rate. While resolution is reduced, selectivity remains constant.

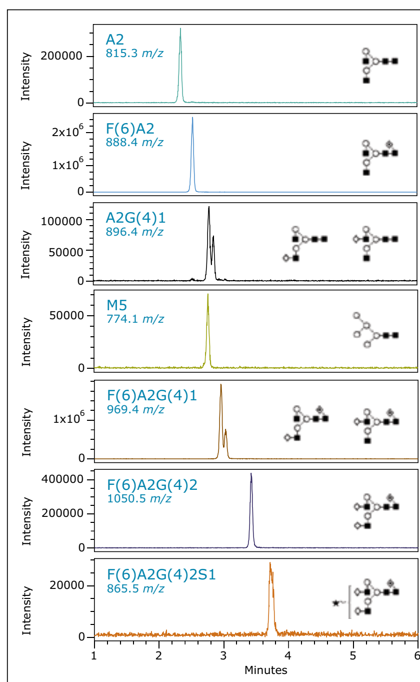

Figure 2. Selected ion chromatograms for N-Glycan species separated using high throughput method. Co-eluting M5 and A2G(4)1are easily discriminated by mass detection.

With a high throughput method developed and the ability to selectively monitor particular species with mass detection, we investigated the linearity of response for the target N-glycan species Mannose 5 (M5). As described in the experimental section, the *Rapi*Fluor-MS labeled M5 species was isolated from the labeled N-glycan pool of RNAse-B. After collection, the collected material was dried and reconstituted in water. The reconstituted sample was added to a *Rapi*Fluor-MS labeled released N-Glycan sample from trastuzumab at various levels. We investigated the linearity of the response by selectively monitoring the peak area of M5 in relation to the volume added to the sample. As shown in Figure 3, the chromatographic reproducibility was quite good. In addition, the peak area for each volume added was highly linear (Figure 4), strongly indicating that the mass detector provides a response suitable for quantification.

After determining the linearity of response for spiked M5 species, we simulated a bioreactor process in which the relative amount of M5 was increasing. For this study we selected the A2G1 species as the reference for relative quantification and spiked in increasing amounts of M5. As shown in Figure 5, the abundance of A2G1 (right column) remains largely constant over the course of the study while the M5 species (middle column) increases as expected with increased spiking levels. In addition, the FLR trace (left column) demonstrates an increase in peak area for these two species (labeled peak), however in the absence of mass information the precise cause of this increase cannot be determined.

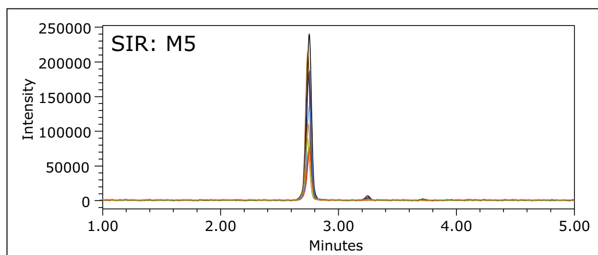

Figure 3. Overlay of chromatograms over a range of M5 spike levels. Spiked amounts ranged from 1-6 µL of reconstituted M5. Absolute concentrations were not determined.

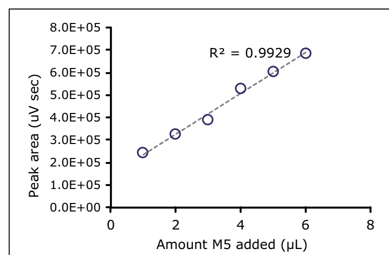

Figure 4. Plot of amount of M5 added vs. peak area for spike M5 samples (data shown in Figure 3).

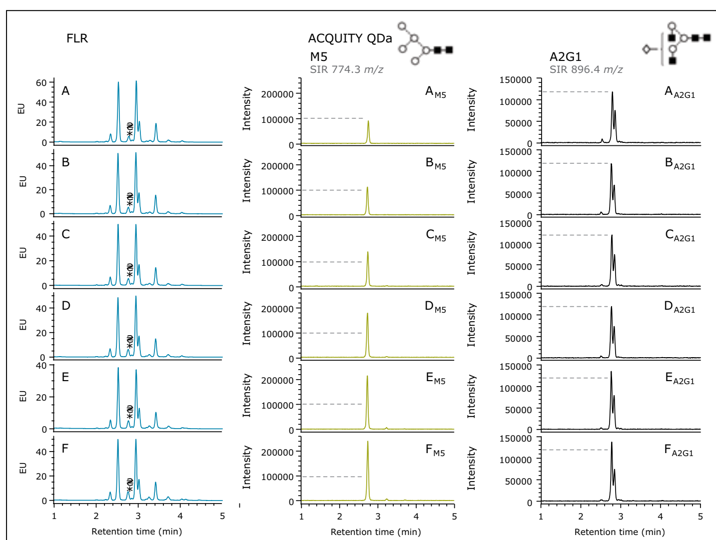

Figure 5. SIR for co-eluting glycan structures. Left: fluorescence profiles of trastuzumab N-glycans with increasing M5 (A to F). The indicators denote the retention times for co-eluting glycans M5 and A2G1. Middle: SIR of M5 for each of the glycan samples A to F. Right: SIR for the co-eluting structure, A2G1. Use of ACQUITY QDa SIR enables the quick determination of glycan structure responsible for changing peak area in fluorescence profiles.

CONCLUSIONS

For routine high throughput assays, *Rapi*Fluor-MS with the ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Detector provides a novel approach for accurately monitoring released N-glycan species. Reduced sample preparation times and greatly improved MS response when coupled with the ACQUITY UPLC and ACQUITY QDa enable the ability to more closely monitor released N-glycan profiles, something which has previously not been possible. While FLR detection was used in this example, for high throughput methods requiring only relative quantification this may not be needed as each species can be monitored with the ACQUITY QDa Detector. As discussed here, complete sample preparation and analysis can be completed in 40 minutes. In addition to reproducible sample preparation, separation and quantification are reproducible and quantitative, allowing scientists to make meaningful decisions rapidly.

Reference

1. Lauber MA, Brousmiche DW, Hua Z, Koza SM, Guthrie E, Magnelli P, Taron CH, and Fountain KJ. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent. 2015, Waters Application Note P/N 720005275EN.

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Applying a Novel Glycan Tagging Reagent, *Rapi*Fluor-MS, and an Integrated UPLC-FLR/QTof MS System for Low Abundant N-Glycan Analysis

Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A novel glycan labeling reagent, *Rapi*Fluor-MS,™ significantly enhances both FLR and MS signals. Improvement from MS detection allows better detection for minor glycan forms.
- The Xevo® G2-XS QTof Mass Spectrometer combines an off-axis ion guide, StepWave,™ with a novel collision cell design to provide significant increases in sensitivity for *Rapi*Fluor-MS labeled glycans.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Glycan BEH Amide Column

ACQUITY UPLC FLR Detector

Xevo G2-XS Mass Spectrometer

KEY WORDS

Automated N-Glycan analysis

INTRODUCTION

UPLC-FLR/MS(MS) analysis of released N-glycans labeled with a fluorescent tag has become routine with high-performance LC and MS instrumentations. Glycans labeled with commonly used fluorescent tags, such as 2-AB and 2-AA, can be detected by fluorescent (FLR) detection with ultra-high sensitivity. Unlike an FLR detector, mass spectrometry is known to be less sensitive to detect native or tagged glycans, especially low abundant ones, due to their poor ESI performance. The limited dynamic range of this approach has restricted the use of this combined workflow for glycan characterization.

To overcome the low MS ionization efficiency associated with conventional labels and confidently assign lower-level glycans, a novel tag, *Rapi*Fluor-MS has been developed by Waters. *Rapi*Fluor-MS contains a rapid tagging reactive group, an efficient fluorophore, and a functional group that imparts high ionization efficiency.[1] Complete tagging of glycans can be achieved in less than 5 minutes using this novel reagent.

Initial results with this glycan label show significant enhancement in both FLR and MS(MS) signals compared to 2-AB.[1] The increased sensitivity enables the detection and identification of very low level glycans, at 0.1%, with sufficient MS signal. In this study, we demonstrate the benefits of combining *Rapi*Fluor-MS with an integrated UPLC-FLR/QTof MS system for detailed characterization of the minor glycoforms from the human IgG and mouse IgG1 samples.

EXPERIMENTAL

Sample preparation

The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit Care and Use manual (p/n 715004793en) contains a detailed sample preparation procedure for the deglycosylation of N-glycans from biotherapeutics, followed by the *Rapi*Fluor-MS labeling step and glycan extraction using an SPE device. The entire sample preparation procedure took 30 minutes.

LC conditions

All chromatographic mobile phases are prepared using LC/MS compatible solvents and reagents.

System: ACQUITY UPLC H-Class

Detector: ACQUITY UPLC FLR

Column: ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)

Column temp.: 60 °C

Mobile phase A: 50 mM ammonium formate (pH 4.4)

Mobile phase B: 100% acetonitrile

UPLC HILIC LC gradient table:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
40.0	0.4	49	51	6
41.5	0.2	100	0	6
44.5	0.2	100	0	6
48.1	0.2	25	75	6
52.6	0.4	25	75	6
60.0	0.4	25	75	6

FLR settings:

General Settings

Mode: 2D Channels | Sampling rate: 2 points/sec

Data channels: 1 | Filter time constant: Normal 1.0 Sec

On inject start: Auto zero

Gain: Enable 1

Data units: Emission

Lamp state: Lamp on

Mode Parameters

	Name	Excitation (nm)	Emission (nm)	Data Mode	Comment
1	Channel A	265	425	Channel A	No Comment

MS conditions

System: Xevo G2-XS QTof MS: ESI+ in sensitivity mode (resolution ~ 30,000)

Capillary voltage: 3.0 kV

Cone voltage: 80 V

Source temp.: 120 °C

Desolvation temp.: 300 °C

Desolvation gas flow: 800 L/h

LockSpray

Capillary voltage: 3.0 V

Cone voltage: 40 V

Scan time: 0.5 s

Interval: 20 s

GFP solubilized in 0.1% formic acid with 50:50 (MeCN: H_2O) at 200 fmol/µL was infused, *m/z* = 785.8421 (z = 2) was used for lock mass calibration.

Collision induced dissociation

MS/MS analyses were performed in continuum mode from 100–2000 *m/z* with collision induced dissociation (CID) to generate glycan fragmentation data. Ions with 2+ and 3+ charge states were selected for fragmentation. Customized collision energy tables that were charge state and mass specific were used for optimized fragmentations; the approximated CE range was between 15 to 40 eV. Data Dependent Acquisition (DDA) was used with duty cycle times of 1.6 sec and 0.5 sec for MS and MS/MS modes. The two most abundant precursors were selected for fragmentation.

Data management

UNIFI Scientific Information System v1.7.1

RESULTS

Previous work showed that the *Rapi*Fluor-MS labeling reagent improves N-glycan MS ionization in positive ion mode. More than two order of magnitude MS sensitivity increase was observed when compared to 2-AB label.[1] Combined with highly sensitive Xevo G2-XS QTof Mass Spectrometer, we are now able to detect minor glycoforms with high confidence.

Figure 2 shows an example of analyzing the *Rapi*Fluor-MS labeled N-Glycans released from 0.5 µg of human IgG on UPLC/FLR/QTof MS system. Comparable FLR and MS response across a broad range of glycans was easily achieved.

The MS and MS/MS fragmentation spectra were also shown as an example in Figure 2 for a minor glycoform, A2G2S1, which is present at 0.1% level. The MS spectrum shows doubly charged ions with minor sodium adduct ions in the raw MS spectrum.

We observed a similar fragmentation pathway for the *Rapi*Fluor-MS labeled glycans compared to the 2-AB labeled glycans. The MS/MS fragmentation of A2G2S1 showed that glycosidic bond cleavage from both reducing and non-reducing end was the dominant fragmentation pathway. The observed sequential neutral losses from the non-reducing end stops at the first GlcNAc residue at the reducing end with the *Rapi*Fluor-MS label attached. Also, the counter fragment ions from the non-reducing end, oxonium ions, were readily observed.

In addition to human IgG, we also tested the *Rapi*Fluor-MS labeled glycans released from a mouse IgG1 sample. It is well known that N-glycolyneuraminic acid and alpha (1-3) galactose containing N-glycans on mAbs generated from murine cell lines are glycans with immunogenic epitopes. These glycans present analytical challenges, due to 1) their low abundance in the glycan mixture, and 2) difficulty to characterize them structurally due to poor MS and MS/MS signals from using the conventional labels.

Figure 1. Biopharmaceutical System Solution with UNIFI for glycan analysis.

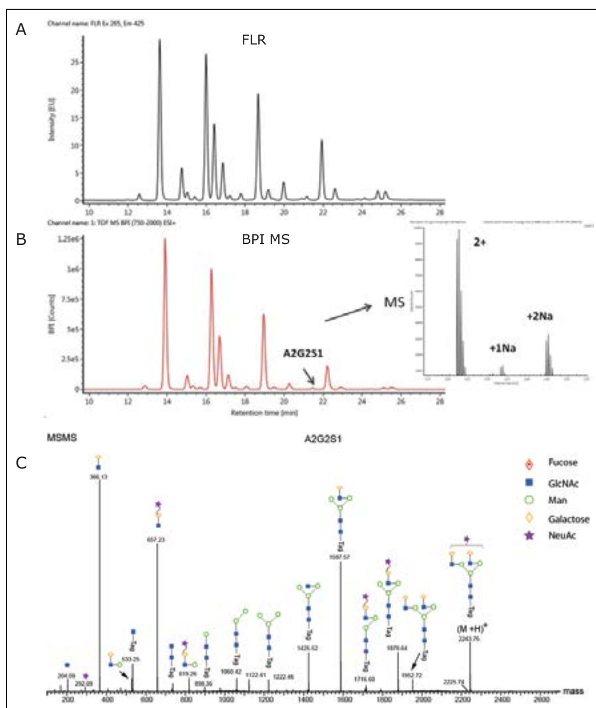

Figure 2. UPLC/FLR/MSMS analysis of RapiFluor labeled human IgG N-glycans. A) FLR data channel. B) BPI MS data channel. The MS spectrum of a low intensity ion was inserted (A2G2S1). The dominant ions were doubly charged with minor sodium adduct ions. C) Deconvoluted MS/MS spectrum of A2G2S1 was displayed.

Figure 3 shows an example of a UPLC/FLR/QTof MS analysis of the mouse IgG1 glycans that contain these immunogenic epitopes. Structural informative fragments (with asterisks) are observed for a low abundant immunogenic glycan, FA2Gal1Sg1, which is present at about 0.1% level. The fragment ion at *m/z* of 528.2 suggests this glycan contains alpha-gal when this ion was the most dominant fragment ion in the entire spectrum; also another diagnostic ion at *m/z* of 2260.8 was generated from losing one NeuGc from the precursor ion. This glycan was also observed in FLR chromatogram of 2-AB labeled glycans without sufficient MS signals to obtain good quality CID fragmentation (data not shown). With *Rapi*Fluor-MS labeling chemistry, sufficient amount of precursor ions were obtained for subsequent MS/MS fragmentation.

Overall, we demonstrated that *Rapi*Fluor-MS labeling chemistry enhances MS and MS/MS sensitivity to obtain high quality precursor and fragmentation ion spectra. Therefore, rich structural information for low abundant glycan species are achieved using this approach.

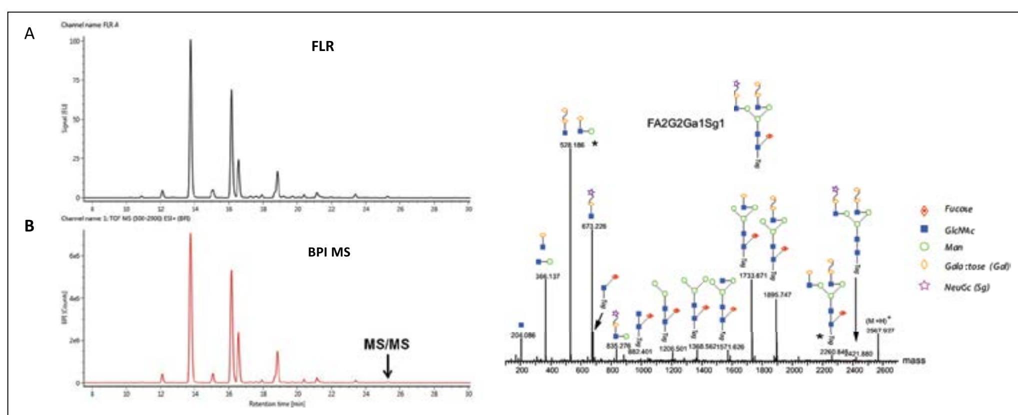

Figure 3. UPLC/FLR/MSMS analysis of RapiFluor-MS labeled mouse IgG1 N-glycans. A) FLR data channel. B) BPI MS data channel. One of the last eluting glycans were selected for MS/MS fragmentation. The deconvoluted fragmentation data from FA2Ga1Sg1 was displayed in C); "Ga" stands for galatose and "Sg" stands for NeuGc. Structurally informative fragments (with asterisks) are observed for this low abundant ion (< 0.1% relative abundance). Fragment ion at m/z of 528.2 suggests this glycan contains alpha-gal when this ion was the most dominant fragment ion in the entire spectrum; also another diagnostic ion at m/z of 2260.8 was generated from the loss of one NeuGc from the precursor ion.

CONCLUSIONS

LC/FLR analysis of N-glycans released from protein therapeutics is performed routinely in analytical laboratories around the world. For scientists who want to add MS characterization capability to their glycan analysis, they often struggle with low MS signals and poor quality MS/MS fragmentation for mass confirmation and structure elucidation using conventional FLR labels such as 2-AB and 2-AA. To address these challenges, Waters offers enabling technologies that include the novel *Rapi*Fluor-MS labeling chemistry for rapid glycan sample preparation, and a UPLC/FLR/QTof MS system controlled by UNIFI Scientific Information System. The improved FLR and MS sensitivity from the *Rapi*Fluor-MS label and the QTof MS with StepWave Technology allow confident identification and characterization of minor but critical glycoforms from mAbs.

References

1. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent. Waters and New England Biolabs application note (p/n 720005275en.)
2. GlycoWorks *Rapi*Fluor-MS Kit Care and Use Manual (p/n 715004793en.)

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[TECHNOLOGY BRIEF]

Released N-linked Glycan Analysis Using the Glycan Application Solution with UNIFI

Ying Qing Yu
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate two fit-for-purpose glycan analysis workflows for comprehensive N-linked glycan profiling and structural elucidation within the Waters® Glycan Application Solution with UNIFI®

BACKGROUND

The vast majority of biotherapeutics are glycosylated. Glycans attached to the proteins play a critical role in the serum half-life, efficacy, and safety of the biotherapeutic drug. In recent years, Waters has launched a series of innovative analytical tools to address the challenges faced in N-glycan analysis. This began with the launch of the ACQUITY UPLC® BEH Amide Column (1.7 µm particle size) for enhanced chromatographic separations of glycans under HILIC mode.[1] Early in 2015, Waters then introduced a new GlycoWorks™ sample preparation kit that provides fast, easy N-glycan sample preparation from enzymatic glycan release to labeling and clean up. This kit includes a novel fluorescent labeling reagent, *Rapi*Fluor-MS,™ enabling highly sensitive mass spectral detection of the labeled glycans.[2,3] The advancements in sample preparation, chromatographic separation, and enhanced ESI MS is now further complemented with an equally enterprising Informatics solution – the UNIFI Scientific Information System – to streamline glycan data acquisition, processing, and reporting.[4]

Comprehensive N-linked glycan analysis using the Glycan Application Solution with UNIFI.

- ACQUITY UPLC H-Class Bio System
- ACQUITY UPLC Column Manager
- ACQUITY UPLC FLR Detector
- Xevo® G2-XS QTof MS
- UNIFI Scientific Information System
- GlycoWorks *Rapi*Fluor-MS N-Glycan Kit
- ACQUITY UPLC Glycan BEH Amide Column

UNIFI® SCIENTIFIC INFORMATION SYSTEM

Figure 1. Glycan Application Solution with UNIFI for RapiFluor-MS labeled glycan analysis.

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THE SOLUTION

Two workflows available with the Glycan Application Solution with UNIFI are featured: 1) Glycan FLR with MS confirmation for profiling and mass confirmation; 2) Glycan DDA workflow via exporting of processed MS/MS data to SimGlycan (Premier Biosoft) for identification and structural elucidation.

Workflow 1: Glycan FLR with MS confirmation

The heart of this workflow is a scientific library containing calibrated chromatographic retention times (in glucose units, GU[5]) and accurate mass values for fluorescently labeled glycan structures. N-glycan identification using the scientific glycan library is illustrated in Figure 2. The assignment is based on accurately matched retention times in GU (calibrated using a fluorescently labeled dextran ladder) and accurate mass measurements from a Xevo G2-XS QTof MS.[6] Currently, a comprehensive 2AB-glycan GU library containing 319 unique N-glycan structures from therapeutic proteins is available with the Glycan Application Solution with UNIFI. A new scientific library based on the *Rapi*Fluor-MS labeling technology is currently under joint development by Waters and NIBRT. UNIFI software also allows users to create customized glycan scientific libraries which can be constructed directly by entering experimental GU values and importing structures from GlycoWorkbench. In addition, this workflow automatically calculates relative percentage value for each glycan component based on integrated fluorescent intensity for robust quantitation.

Workflow 2: Glycan DDA

Figure 3 shows the Glycan DDA workflow: Glycan MS/MS information using a Data Dependent Acquisition (DDA) mode was first acquired, followed by peak processing to convert all ions to singly charged "ion sticks." The processed data can then be exported in either .mzML or .LCS file format into SimGlycan software for identification and fragment ion annotation.

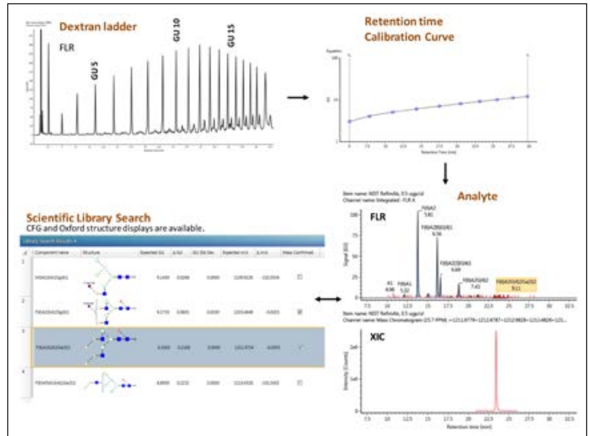

Figure 2. Workflow 1: Glycan FLR with MS confirmation.

Figure 3. Workflow 2: Glycan DDA workflow in UNIFI Software. Collision-induced dissociation (CID) of glycans in data dependent acquisition (DDA) mode is processed and exported to SimGlycan.

Analytical method for Glycan Application Solution in UNIFI

Sample preparation: N-glycans were prepared using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003713)

System: Biopharmaceutical Platform with UNIFI

LC settings for *Rapi*Fluor-MS labeled glycans

Column: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7µm, 2.1 mm x 150 mm (p/n 186004742)

Column temp.: 60 °C

Mobile phase A: 50 mM ammonium formate (pH 4.4, LC-MS grade)

Mobile phase B: 100% acetonitrile (LC-MS grade)

Gradient:

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
0.0	0.4	25%	75%	6
35.0	0.4	46%	54%	6
36.5	0.2	80%	20%	6
39.5	0.4	25%	75%	6
43.1	0.4	25%	75%	6
55.0	0.4	35%	75%	6

Fluorescent: λex = 265 nm, λem = 425 nm

Xevo G2-XS QTof MS settings

Capillary voltage: 3.0 kV

Sample cone: 30 V

Source temp.: 120 °C

Desolvation temp.: 300 °C

Desolvation gas: 800 L/hr

Recommend settings for DDA

Charge state recognition: 2+, 3+, and 4+

Collision energy ramping

Low mass start: 10 V, low mass end: 15 V

High mass start: 44 V, high mass end: 50 V

MS scan: 0.5 sec, MS/MS scan: 0.5 sec

SUMMARY

Released glycan analyses are traditionally done using either optical or MS only analytical systems, and the data interpretation can be very challenging due to a lack of integrated analytical systems. The Glycan Application Solution with UNIFI features two independent analytical glycan workflows. These workflows allow scientists to characterize and profile glycans using both optical (fluorescent) and MS (MS) data within an integrated UPLC/FLR/QTof MS system. Combined with the novel *Rapi*Fluor-MS glycan labeling technology and the sophisticated UNIFI Software, scientists are now able to identify and quantify low abundant, potentially immunogenic glycan structures with higher confidence.

References

1. "Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 um sorbent." *J. Chrom. B.* 878 (2010) 403–408.
2. "Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection." *Anal. Chem.* 2015, 87 (10), 5401–5409.
3. "Applying a Novel Glycan Tagging Reagent, *Rapi* Fluor-MS, and an Integrated UPLC-FLR/QTof MS System for Low Abundant N-Glycan Analysis." Waters Application Note (p/n 720005383EN).
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5. Mattew P. Campbell, Louise Royle, Catherine M. Radcliffe, Raymond A. Dwek and Pauline M. Rudd. "GlycoBase and autoGU: tools for HPLC-based glycan analysis." Bioinformatics Applications Note, vol. 24, no, 9, 2008, 1214–1216.
6. "Biopharmaceutical Platform Solution with UNIFI: A Holistic Workflow for Acquiring, Processing, and Reporting Fluorescent-Labeled Glycans." Waters Application Note (p/n 720004619en).

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A Holistic Workflow for Acquisition, Processing, and Reporting Fluorescent-Labeled Glycans Using the Biopharmaceutical Platform Solution With UNIFI

Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

We present a fully integrated HILIC UPLC® FLR detection workflow that is carried out within a comprehensive compliant-ready platform that integrates informatics and instrument control. This platform enables laboratories to perform routine biotherapeutic glycan analysis with greater speed, accuracy, and consistency than by using disparate laboratory processes.

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI

ACQUITY UPLC® H-Class System

ACQUITY® UPLC BEH Glycan Column

ACQUITY UPLC FLR Detector

UNIFI® Scientific Information System

Glycobase Database

2-AB Dextran Calibration Ladder

GlycoWorks™ Reductive Amination Single Use Sample Preparation Kit

KEY WORDS

2-AB labeled glycans, dextran ladder, Glycan Units

INTRODUCTION

One of the challenges of managing the routine use of analytics in biopharmaceutical laboratories is the number of approaches used to characterize a biomolecule – and the complexity of controlling instruments and processing data collected from different structural levels.

The Waters® Biopharmaceutical System Solution with UNIFI integrates high-resolution biotherapeutic analyses with bioinformatics that are designed to support routine workflows used in the development process. The system enables researchers to acquire, process, report, and share mass spectrometry and chromatography data including intact mass analysis of proteins, peptide mapping, and glycan profiling – with a single software platform that can also manage multiple systems in a networked workgroup.

In this application note, we illustrate a dedicated workflow for the acquisition, processing, and automated reporting of data from fluorescent-labeled (such as 2-aminobenzamide, or 2-AB) released glycan samples. Sample data was acquired using a HILIC UPLC® separation and fluorescent (FLR) detection methodology. Normalized retention time for the various glycans was achieved using a calibrated *Rapi*Fluor-MS Dextran Calibration Ladder (p/n 186007982).

Data acquisition, processing, reporting and management were achieved under control of the UNIFI Scientific Information System. The HILIC UPLC FLR System used for glycan data acquisition was part of a compliant-ready UNIFI workgroup, consisting of two UPLC/QTof MS systems and multiple UPLC optical detection (FLR and UV) systems. This workgroup configuration enabled centralized instrument control, processing, and data integration on a common server for the methods, data, and reports acquired from the networked systems. We demonstrate that such an integrated laboratory and data workflow provides exceptional efficiencies for routine released glycan profiling of biotherapeutics.

EXPERIMENTAL

LC conditions

System: ACQUITY UPLC H-Class System

Column: ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)

Column temp.: 40 °C

Mobile phase A: 50 mM ammonium formate (pH 4.4)

Mobile phase B: Acetonitrile

Informatics

UNIFI Scientific Information System

FLR settings

General Settings

Mode: 2D Channels

Sampling rate:

Data channels: 1

Filter time con

On inject start: Auto zero

Gain: Enable

1

Data units: Emission

UPLC HILIC LC gradient table

	Time (min)	Flow Rate (mL/min)	Composition A (%)	Composition B (%)	Composition C (%)	Composition D (%)	Curve
1	0.00	0.561	30.0	70.0	0.0	0.0	Initial
2	1.47	0.561	30.0	70.0	0.0	0.0	6
3	24.81	0.561	47.0	53.0	0.0	0.0	6
4	25.50	0.561	70.0	30.0	0.0	0.0	6
5	26.50	0.400	70.0	30.0	0.0	0.0	6
6	29.50	0.400	70.0	30.0	0.0	0.0	6
7	30.00	0.561	30.0	70.0	0.0	0.0	6
8	35.00	0.561	30.0	70.0	0.0	0.0	6

Biopharmaceutical Platform Solution with UNIFI

- ACQUITY UPLC H-Class System
- ACQUITY UPLC BEH Glycan Column
- ACQUITY UPLC FLR Detector
- UNIFI Scientific Information System
- 2-AB Dextran Calibration Ladder (p/n 186006841)
- GlycoWorks Reductive Amination Single Use Sample Preparation Kit (p/n 176003119)

Sample preparation

The 2-AB Dextran Calibration Ladder (p/n 186006841) and the GlycoWorks Reductive Amination Single Use Sample Preparation Kit (p/n 176003119) are glycan standards available from the Waters Corporation. The dextran ladder is used to calibrate and normalize labeled glycan retention times for exceptional day-to-day, system-to-system, and lab-lab reproducibility. The retention time for polyglucose 4–12 peaks were used to produce a fifth order polynomial calibration curve (Glucose Unit or GU vs. Retention Time). All analyte peaks are reported and searched using this calibrated GU value.

RESULTS AND DISCUSSION

Workflow for routine released glycan determination

The majority of the therapeutic proteins are glycosylated, and the attached glycans have significant impact on the efficacy and safety of the biotherapeutic. The International Conference on Harmonization Guideline Q6B requires the analysis of carbohydrate content, structural profiles, and characterization of the glycosylation site(s) within the polypeptide chain(s).

The most widely adopted analytical workflow for routine N-linked glycan characterization involves labeling the enzymatically released glycans with a fluorescent tag (typically 2-aminobenzamide, or 2-AB), resolving the labeled glycans by hydrophilic interaction liquid chromatography (HILIC UPLC), and detecting the labeled glycan peaks with a fluorescence detector. The assignment of glycan peaks during routine analysis is fundamentally based on matching their retention time to established values. For non-routine analysis, glycosidase arrays or MS analysis are employed to give tentative assignments or resolve ambiguous peak assignments.

In order to best control method variation (between runs, days, instruments, scientists, and labs) glycan profiles from the HILIC separation are always calibrated and normalized against a 2-AB Dextran Calibration Ladder (glucose homopolymer). Glycan peaks in an unknown sample can be assigned a Glucose Unit (GU) value from the GU vs. Retention Time calibration curve using the dextran ladder, which is typically fitted with a fifth-order polynomial or cubic spline calibration line.[1]

The new streamlined workflow in UNIFI Software, v. 1.6, (Figure 1) enables users to automatically collect one or more dextran ladder standard data sets, process the chromatograms, generate dextran calibration curve, and apply curves directly to unknown samples. Assigned GU values for peaks in an unknown are searched through Glycobase 3.1+, an integrated UPLC GU released glycan database, developed by Waters in collaboration with the National Institute for Bioprocessing Research and Technology (NIBRT) (https://glycobase.nibrt.ie/glycobase/browse_glycans.action).

Targeted component search is enabled within a more information-rich integration of Glycobase in the next version of UNIFI Software, v. 1.6.1. The integrated Glycobase database will reference hundreds of glycan GU entries along with supporting structural, mass, and exoglycosidase digestion pathway information.

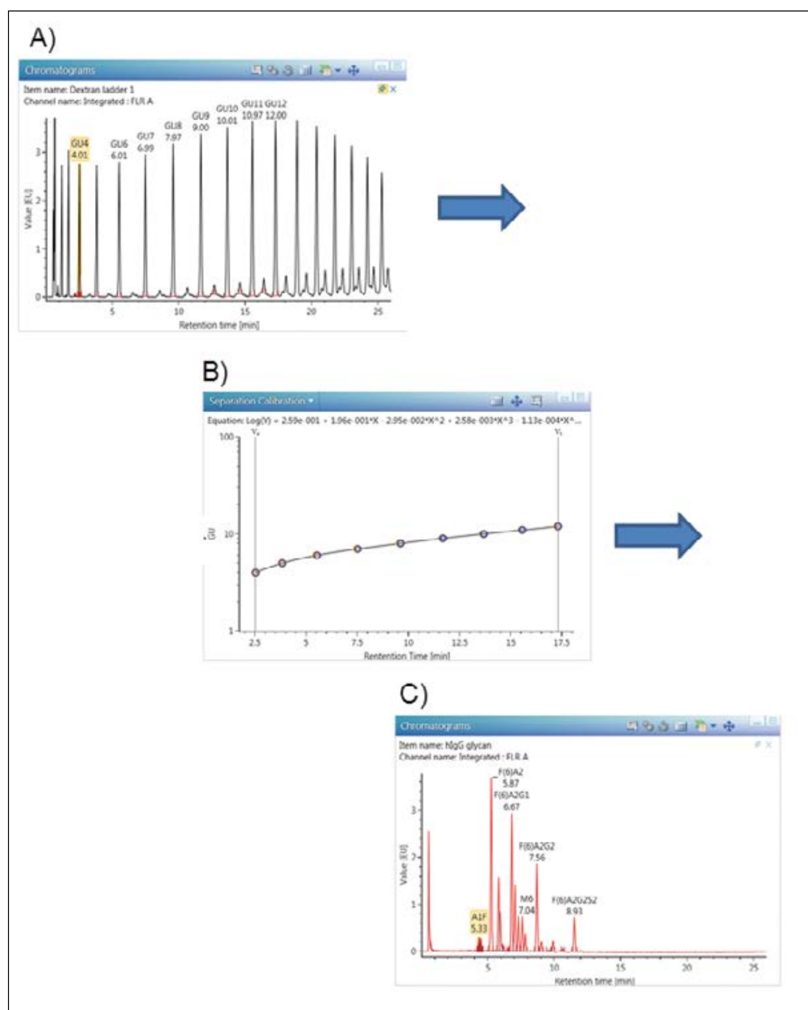

Figure 1. Glycan analysis workflow. A) 2-AB Dextran Calibration Ladder was used as a separation standard. B) The Glucose Unit (4-12) calibration from the separation standard was fitted by a fifth order polynomial curve. C) Glycan peaks from an unknown sample were assigned GU values from this curve. The measured GU value was searched using the Glycobase entries to assign structures from GU values.

UNIFI method generation

Figure 2 depicts the key elements of producing an automated and holistic glycan acquisition, processing, and reporting method within UNIFI. The instrument settings define the instrument modules required for the analysis, and define method parameters for HILIC UPLC separation and fluorescence detection. The processing setting defines peak integration and dextran ladder calibration settings. A comprehensive report can be generated automatically following acquisition and processing using one or more report templates that can be readily modified. Modifications to the processing and reporting settings within the method are possible post-acquisition, and are audit trailed for simplified documentation and compliance purposes.

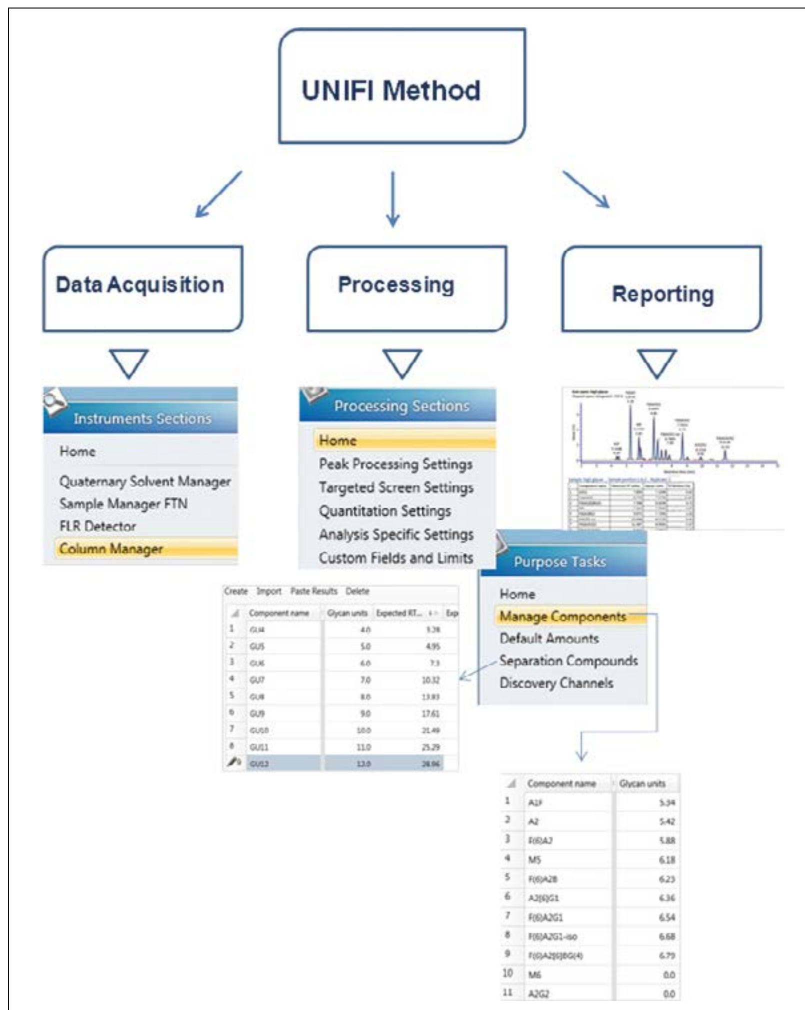

Figure 2. The UNIFI released glycan analysis method.

Figures 3 through 5 detail how a scientist can use UNIFI Software to navigate through processed and raw chromatographic results. A typical dextran calibration curve shown in Figure 3 is applied to the GlycoWorks Reductive Amination Single Use Sample Preparation Kit (p/n 176003119), a mixture of N-glycans from mAb (Figure 4). Reproducibility of the results is depicted in Figure 5. The ability of UNIFI to automatically summarize data from multiple injections of standards and unknown samples is highlighted in these displays.

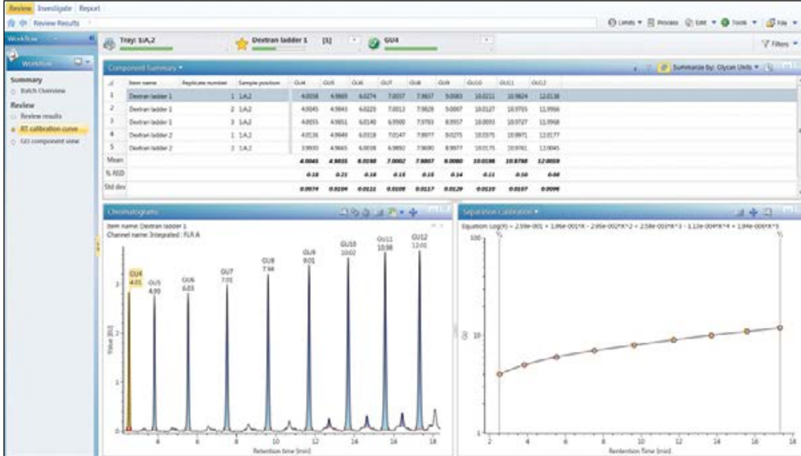

Figure 3. In UNIFI Software, the review panel summarizes GU values (GU 4–12) obtained across 5 injections of the 2-AB Dextran Calibration Ladder standard. The chromatogram of the first injection and calibration curve results for all injections are displayed below the summary table.

Figure 4. A component summary table in the review page that compares the GU value for all the identified glycans across a triplicate injection of a glycan mixture sample. Summary calculations for individual glycans across triplicate analysis of the glycan test standard are presented in the component summary table.

Figure 5. The Investigate panel of the experiment demonstrates glycan profile reproducibility for five HILIC UPLC FLR analyses of the glycan mixture.

Figure 6 provides a snapshot of a typical glycan analysis report; information such as the sample list, retention time calibration curve fitting for 2-AB Dextran Calibration Ladder, and calculated GU values for unknown glycan samples are organized within single report template. Valuable information such as the relative abundance and the GU value for each glycan component were also summarized with appropriate statistical analysis.

Figure 6. A typical UNIFI released glycan report.

CONCLUSIONS

UNIFI is the first comprehensive software that seamlessly integrates UPLC chromatography, optical detection, high resolution mass spectrometry, and integrated informatics within one platform. Released glycan analysis is one of the latest application workflows to be offered in UNIFI Software, v. 1.6.

This new UNIFI Application Solution enables a scientist in regulated or unregulated laboratory environments to acquire, process, and report qualitative and quantitative glycan information along with high confidence and minimal user intervention.

Reference

1. Campbell, M.P.; Roylez, L.; Radcliffe, C.M.; Swek, R.A.; Rudd, P.M. GlycoBase and autoGU: tools for HPLC-based glycan analysis. *Bioinformatics*. 2008, 24:9, 1214–1216.

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N-linked Glycan Characterization and Profiling: Combining the Power of Accurate Mass, Reference Glucose Units, and UNIFI Software for Confident Glycan Assignments

Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The integrated UPLC/FLR/QTof MS analytical technologies available with the Waters Biopharmaceutical Platform Solution with UNIFI® improve a biopharmaceutical organization's ability to deliver well-characterized glycosylated biotherapeutics to market, from discovery through QC. The solution allows routine assignment of N-linked glycan structures using data from time-aligned FLR and MS channels along with database-driven assignment of glycans based on retention time. This enables the profiling of released glycans for individual analysis or to facilitate multi-batch or biosimilar/innovator comparability studies.

WATERS SOLUTIONS

UNIFI Scientific Information System

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Glycan BEH Amide Column

ACQUITY UPLC FLR Detector

Xevo® G2-S QTof

GlycoWorks™ Reductive Amination Single Use Sample Preparation Kit

2-AB Dextran Calibration Ladder

2-AB Glycan Performance Test Standard

KEY WORDS

Biosimilar, etanercept, Waters Glycan GU Library, glucose units

INTRODUCTION

The Waters® Biopharmaceutical Platform Solution with UNIFI is comprised of industry-leading UPLC bioseparations columns and analytical instrumentation, along with optical detection and mass spectrometry, for comprehensive biopharmaceutical characterization and analysis. Data acquisition, processing, bioinformatics, and reporting tools are integrated and automated within UNIFI Scientific Information System's compliant-ready architecture.

In this application note, we detail a new workflow for a glycan assay, using FLR with mass confirmation, available in the latest version of the Biopharmaceutical Platform Solution with UNIFI. The practical use of this workflow for fluorescent labeled (2-AB) N-linked released glycan characterization is illustrated using a biosimilar/innovator biotherapeutic comparability study.

The analytical platform used for this study is comprised of an ACQUITY UPLC H-Class Bio System and an ACQUITY UPLC Fluorescent Detector in-line with a Xevo G2-S QTof Mass Spectrometer. This Glycan Application Solution with UNIFI enables the assignment and profiling of 2-AB labeled released N-linked glycans based on searches of calibrated retention time in glucose units (also known as GU) and accurate mass data within the Waters Glycan GU Library, which is integrated within UNIFI Scientific Information System version 1.7 and higher.

Accurate mass analysis proves a valuable technique for confirming GU based assignments and distinguishing cases where multiple glycan structures could be assigned to a single peak. Other complementary data for confirming these assignments (e.g. glycan DDA MS/MS data and exoglycosidase array studies) can also be collected on the Biopharmaceutical Platform Solution with UNIFI, and will be addressed in future application notes.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class Bio System
Column:	ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)
Column temp.:	40 °C
Mobile phase A:	50 mM Ammonium Formate (pH 4.4)
Mobile phase B:	Acetonitrile
Note:	LC-MS grade water and acetonitrile was used for this experiment

MS conditions

MS system:	Xevo G2-S QTof MS
Mode:	ESI+ in sensitivity mode
Capillary voltage:	3.0 kV
Cone:	80 V
Source temp.:	120 °C
Desolvation temp.:	300 °C
Desolvation gas flow:	800 L/h
Scan time:	0.5 s
Interval:	20 s

Data acquisition, processing, and reporting

UNIFI Scientific Information System

In this work, we illustrate the features of the platform for glycan analysis:

- The analytical workflow moves seamlessly from acquisition through data processing, with FLR and MS data channels being acquired and time-aligned automatically, for a routine and repeatable approach to data processing and reporting.
- The Waters Glycan GU Library allows confident assignment of the glycan structures based on retention time (in GU) with accurate mass confirmation.
- The streamlined workflow continues through reporting of quantitative (relative %) and qualitative analysis of N-glycan profiles, enabling scientists to easily communicate this critical information without exporting information to external data packages and thus reducing sources of data manipulation error. As a result, the laboratory's ability to maintain compliance and data integrity is enhanced.

ACQUITY UPLC FLR Detector settings

UPLC HILIC gradient table

	Time (min)	Flow Rate (mL/min)	Composition A (%)	Composition B (%)	Composition C (%)	Composition D (%)	Curve
1	0.00	0.400	30.0	70.0	0.0	0.0	Initial
2	2.06	0.400	30.0	70.0	0.0	0.0	6
3	34.80	0.400	47.0	53.0	0.0	0.0	6
4	36.00	0.250	80.0	20.0	0.0	0.0	6
5	39.00	0.250	80.0	20.0	0.0	0.0	6
6	40.00	0.400	30.0	70.0	0.0	0.0	6
7	45.00	0.400	30.0	70.0	0.0	0.0	6

Sample preparation and retention time calibration in GU values

The 2-AB Dextran Calibration Ladder (p/n 186006841) and the 2-AB Glycan Performance Test Standard (p/n 186006349) are glycan standards available from Waters Corporation. The 2-AB Dextran Calibration Ladder is used to calibrate and normalize labeled glycan retention times for exceptional day-to-day, system-to-system, and lab-to-lab reproducibility. This enables routine use of the Waters Glycan GU Library to produce primary glycan assignments.

The retention times for polyglucose 4–12 peaks were used to produce a fifth order polynomial calibration curve of GU vs. retention time. Peaks in experimental samples are automatically assigned and reported using this calibrated GU value. The 2-AB Glycan Performance Test Standard (p/n 186006349) contains a set of biantennary glycans, including high mannose and sialated structures, typical of many therapeutic mAbs commercializaed and in development today.

The GlycoWorks Reductive Amination Single Use Sample Preparation Kit (p/n 176003119) was used to generate 2-AB labeled released N-linked glycans from the innovator and a candidate biosimilar version of etanercept. The instructions were followed as detailed in the documentation package for the kit.

Fluorescent and MS chromatogram alignment

The fluorescent and MS chromatograms were aligned automatically during data acquisition using an experimentally derived value entered on the instrument console page. The time offset value depends on the length of the peak tubing (connection between the FLR and MS inlet) and the flow rate, and may vary system to system.

Critical settings

In the UNIFI processing method, settings are made for retention time calibration using 2-AB Dextran Calibration Ladder (p/n 186006841) (GU 4-12 is the typical range of calibration for mAb derived glycan samples). Both the fifth order and the cubic spine curve fit are applicable for retention time calibration.

RESULTS AND DISCUSSION

In this work, we provide specific details about the glycan UPLC-FLR/MS workflow (Figure 1) used with the Biopharmaceutical Platform Solution with UNIFI, including details of the analytical methods employed, the data review workflows employed, and reporting schemes required for efficient analysis of individual glycan samples and for more complex comparability studies.

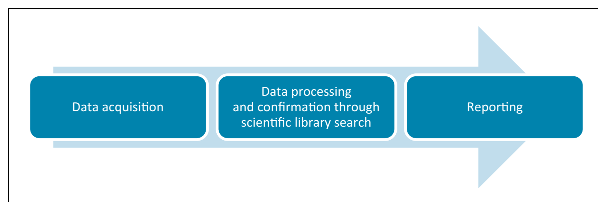

Figure 1. The glycan assay workflow, using FLR with MS confirmation.

Step 1: Data acquisition

Fluorescent-labeled glycans were separated using an ACQUITY UPLC H-Class Bio System with both FLR and MS detection, the latter using the Xevo G2-S QTof MS. The ACQUITY UPLC FLR Detector was directly interfaced with the QTof MS without any fluidic path modifications. The MS chromatogram is automatically time aligned with the FLR chromatogram as described above. An example of the UPLC-FLR/QTof MS chromatogram is shown in Figure 2.

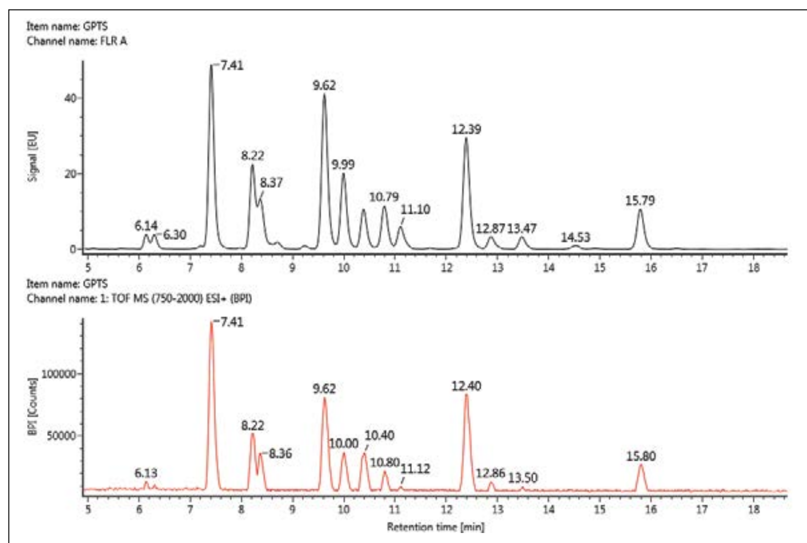

Figure 2. UPLC-FLR/MS chromatogram of 2-AB Glycan Performance Test Standard. The FLR chromatogram is shown at the top, and the BPI MS chromatogram is shown at the bottom. The BPI MS trace was time aligned with the FLR during data acquisition.

A 2-AB Dextran Calibration Ladder (p/n 186006841) was used as a retention time calibration standard. Typically, the samples are sandwiched in between dextran ladder injections. A fifth order curve, or cubic spline curve, for retention times vs. glucose unit values was automatically calculated using the average of all dextran ladders analyzed, and subsequently applied to the experimental glycan chromatograms during data processing. The benefit of using retention time calibration is to adjust the retention time shift to accommodate any variations in mobile phase preparation, instrument configuration, and other aspects of user and laboratory variability.

Figure 3 reviews the dextran ladder standard calibration result.

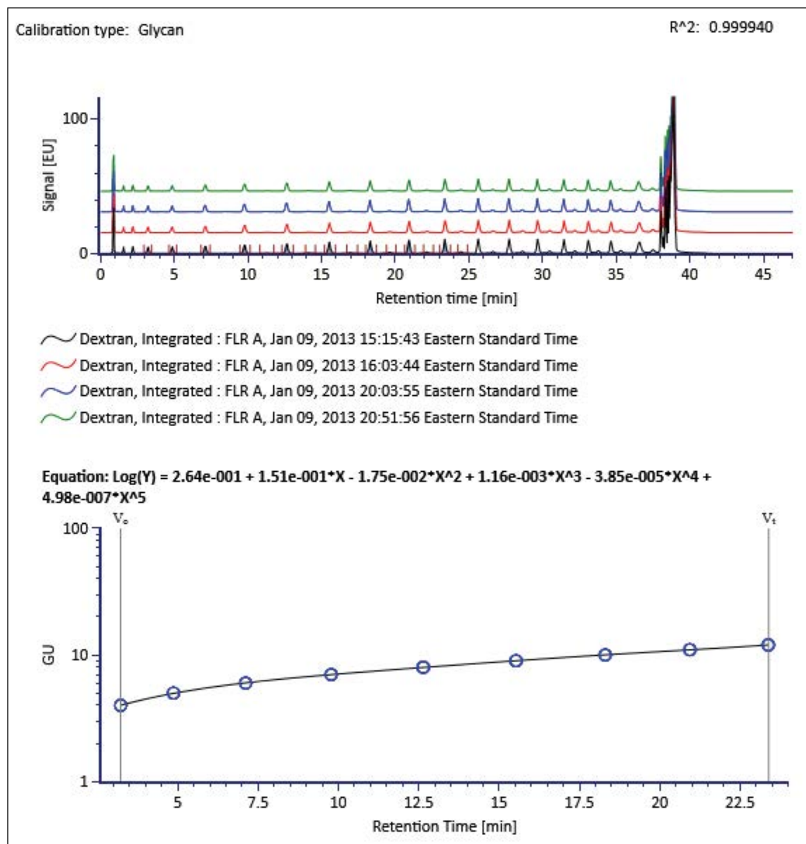

Figure 3. An example of dextran ladder calibration is shown. The top chromatogram shows the overlay of four injections of dextran ladder collected before and after two experimental sample runs. The bottom plot is the fifth order curve generated from these injections. The R^2 (0.999963) and overlayed data points highlight the excellent retention time correlations across these injections.

Step 2: Data processing and scientific library search

The Waters Glycan GU Library contains retention times (in GU) and mass information for 2-AB labeled N-linked glycans from a list of diverse glycoproteins as well as bulk human serum. The total number of unique glycans is currently 300. GU value, molecular formula, glycan structure, and monoisotopic mass are associated with each glycan entry. Scientists can search for a particular glycan or focus a search on specific classes of glycans in the library search.

An example is shown in Figure 4. The reagent selected is 2-aminobenzamide (2-AB), since the experimental GU values in the Waters Glycan GU Library are from 2-AB labeled proteins. The search criteria functions as a filter to narrow the range of GU search tolerance and by using restrictions for many types of glycan attributes. The Waters Glycan GU Library is the default library delivered with the Biopharmaceutical Platform Solution with UNIFI; however, a user can create their own GU library to search instead or in addition to the Waters library.

Library Search (Glycans)

Library Search will query Scientific Library to find all defined Glycan compounds that match the GU value for each component discovered in the FLR trace. You portion of the Glucose unit value will be used by analytical processing - the value portion will be replaced with the GU value computed for each discovered co

Enable library search

Reagent: 2AB (Used to adjust mass used for MS confirmation)

Duplicate Delete Clear

	Search criteria	Operator	Search value
1	Glucose unit	equals	7 ± 0.15
2	Glycan attributes	equals	Has not Antenna 3

Search in: Waters Glycan GU Library Show: 20 Results Search

	Name	Formula	GU value	GU value standard deviation	Monoisotopic mass...
1	A2G(4)1Ga1	C62H104N4O46	7.100	0.000	1640.5922
2	F(6)A2[3]BG(4)1	C70H117N5O50	7.084	0.019	1827.6766
3	M6 D3	C52H88N2O41	7.081	0.020	1396.4863
4	F(6)A2[6]BG(4)1	C70H117N5O50	6.950	0.036	1827.6766
5	M6	C52H88N2O41	7.000	0.063	1396.4863
6	M6 D2	C52H88N2O41	7.020	0.060	1396.4863

Figure 4. Library search settings for Waters Glycan GU Library.

Waters Glycan GU Library

UNIFI Scientific Information System's automated data processing encompasses calculation of the GU value for integrated FLR peaks, determination of accurate mass values associated with the peaks, and the resulting scientific library search. The assignment is based on the following logic:

1. All glycans with experimental GU values (experimental vs. library) within the database GU search tolerance are associated with an FLR peak.
2. Among the potential assignments, those with accurate mass confirmation are given priority of assignment, with closest GU value assigned as the default candidate.
3. Since FLR is more sensitive than mass spectrometry, the very low-level glycans may have good FLR signal, but no or low MS signals. The assignment of these glycans may only be based on the GU value difference from the database.
4. In the case of coeluting glycans or glycans with identical GUs, the glycan that is most abundant (by mass spectrometry signal) gets the default assignment. Less abundant glycans (if present) are still represented in the alternative assignments (with mass confirmed checked).
5. When a GU value is not found in the library within the given search tolerance, such peaks are marked as "Discovered" components. Further investigation is needed to identify these peaks, and once the structures of these glycans are verified, a new library entry can be created.
6. Glycans that are structural isomers tend to have close GUs and identical mass. In such cases, the matching isomers will be marked as mass confirmed; the one that has the closest GU value will be highlighted as the top assignment. These may require glycosidase treatment or MS/MS analysis for direct assignment.

Figure 5 is a screen capture from UNIFI Scientific Information System's review tab, detailing the processed library search results. The FLR peaks are integrated and assigned with the best match. After reviewing the search result, an assignment can be changed to another glycan that has a similar GU value. This change is tracked by audit trail within the software.

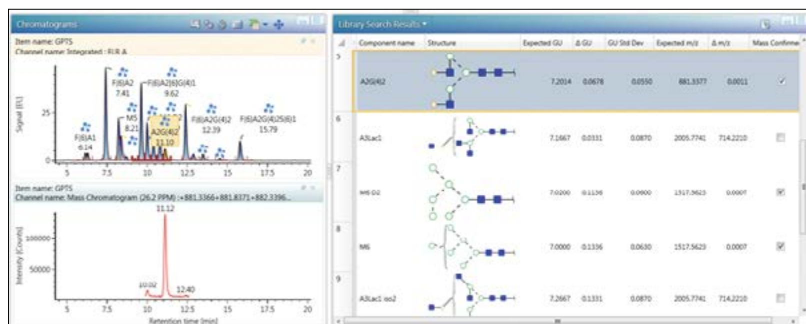

Figure 5. Waters Glycan GU Library search result from the review window. On the left is the processed FLR chromatogram and XIC MS of a highlighted glycan; on the right is the library search result of the selected glycan peak. Information such as the structure (with linkage assignment), expected GU, expected mass, ΔGU and Δmass are listed. In addition, the "Mass Confirmed" box is checked off if the mass of any of the candidate glycans is observed.

Practical application: Using UNIFI Scientific Information System to compare N-glycan profiles of an innovator biotherapeutic and a biosimilar candidate

Etanercept (trade name Enbrel) is a biotherapeutic mAb fusion protein for the treatment of rheumatoid arthritis and other autoimmune diseases; it is also one of the highest revenue biotherapeutics on the market today. Many biotechnology companies are actively working to creating biosimilar versions of etanercept.

In this study, we compared the 2-AB labeled N-glycan profile from one biosimilar candidate to that of the innovator using the Glycan Application Solution with UNIFI and its FLR/MS workflow. We observed that the biosimilar's N-glycan profile is highly similar to that of the innovator's, but some points of difference can be detected.

For example, high mannose structures were observed at higher abundance in the biosimilar candidate, including some extended mannose (e.g. Man 6 and Man 8) structures detected only in the biosimilar candidate (Figure 6). We also observed that the biosimilar candidate contains the following glycans, F(6)A2[6]BG(4)1, F(6)A2[3]G(4)1S(3)1, F(6)A3G(4)3S(3,3)2, and A2G(4)2S(6)1 in relative abundance that is greater than 0.1%, however, these sialylated glycans are either absent or below the 0.1% threshold in etanercept.

The cause of the N-glycan profile differences is most likely due to the variations in cell culture conditions. Bioassays and clinical experience are likely required to establish the extent to which these differences would affect the safety or efficacy of the biosimilar candidate.

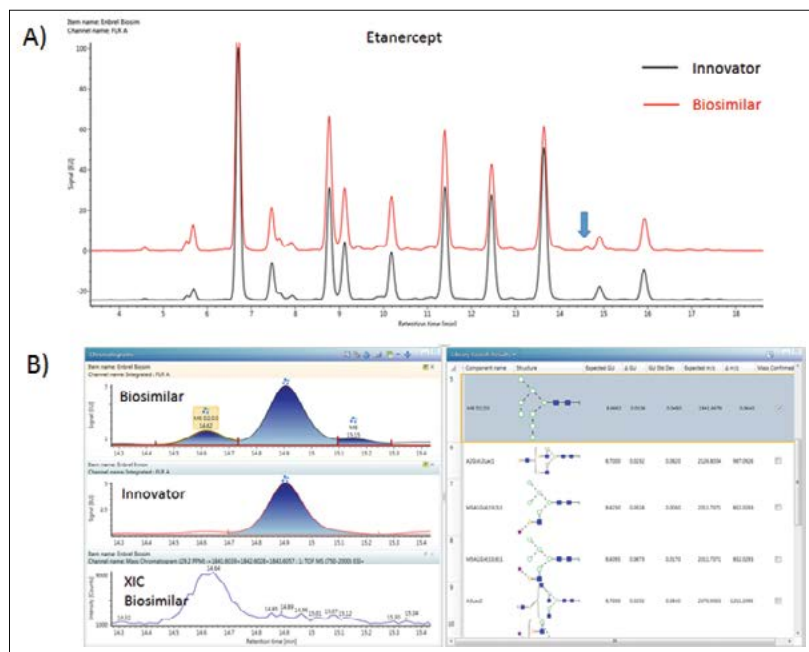

Figure 6. A) Overlay chromatogram of the N-glycans from the innovator and a biosimilar candidate etanercept. The blue arrow highlights the Man8 glycan that is only observed in the biosimilar candidate. B) The Waters Glycan GU Library search result shows that the marked peak from (A) is assigned to Man8. The library search result for the highlighted peak, Man8, XIC of Man8 provides further evidence for the correct structural assignment display in the chromatogram window.

Reporting

UNIFI Scientific Information System includes application-specific reporting templates. The default glycan assay report templates provide a sound basis for reporting details on individual samples as well as comparative data. The templates can also be readily modified to suit the reporting needs of a specific glycan analysis project.

Figure 7 gives an example of the type of information captured by a generic glycan analysis report using the innovator/biosimilar etanercept N-glycan analysis as an example.

Figure 7. An example of a UNIFI report for the N-glycan profiling and comparison between innovator/biosimilar etanercept glycans. Key information such as sample list, retention time calibration curve, the scientific library search result for each identified glycan, summary table on relative % amount for innovator/biosimilar N-glyans, UPLC-FLR/MS chromatograms, and component tables. The report template is customized to fit the analysis type.

CONCLUSIONS

Glycan characterization has remained a challenging aspect of biotherapeutic characterization compared to techniques such as intact mass or peptide map analysis, which most labs consider routine today. The addition of the glycan UPLC-FLR/MS workflow and use of the experimentally derived Waters Glycan GU Library within the Glycan Application Solution with UNIFI have addressed the desire for compliant-ready, automated, high-confidence glycan structure assignments by enabling rapid acquisition, review, and communication of individual glycan profile results, and the larger sets of glycan analyses used for comparability studies.

Additional capabilities with the Biopharmaceutical Platform Solution with UNIFI, such as glycan/glycopeptide DDA MS/MS analysis and the ability to execute exoglycosidase arrays, certainly complement this new workflow, providing additional orthogonal results that enable the characterization of even the most complex biotherapeutic glycoproteins.

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Transferring *Rapi*Fluor-MS Labeled N-Glycan HILIC Separations Between UPLC and HPLC

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Seamless scalability and transfer of *Rapi*Fluor-MS™ labeled glycan separations between UPLC and HPLC instrumentation
- ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm and XBridge® Glycan BEH Amide ***XP***, 130Å, 2.5 µm Columns provide high resolution UPLC® and HPLC glycan separations

WATERS SOLUTIONS

ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Columns

XBridge® Glycan BEH Amide ***XP***, 130Å, 2.5 µm Columns

Alliance® HPLC System

ACQUITY UPLC H-Class Bio System

Empower® 3.0 Software

MassLynx™ 4.1 Software

*Rapi*Fluor-MS Glycan Performance Test Standard

KEY WORDS

HILIC Chromatography, UPLC, HPLC, method transfer, N-glycans, *Rapi*Fluor-MS

INTRODUCTION

In 2009, Waters introduced a revolutionary UPLC HILIC Column designed specifically for achieving high resolution glycan separations. This column technology was based on stationary phase constructed from 1.7 µm diameter, 130Å pore size, ethylene bridged hybrid (BEH) particles with an optimized amide ligand bonding that has exhibited exceptional resolution for a broad range of N-glycans ranging from small neutral structures to highly sialylated extended structures.[1] In addition to this UPLC-based column, Waters has also introduced HPLC-based XBridge Glycan BEH Amide Columns based on 2.5 µm particles and has demonstrated that these columns provide selectivity for 2-AB labeled N-glycans comparable to that observed in UPLC separations.[2] Most recently, Waters has introduced a novel labeling reagent, *Rapi*Fluor-MS, that provides both a fast and efficient sample preparation workflow and unsurpassed fluorescent and MS sensitivity.[3]

In the following work, we demonstrate that Glycan BEH Amide Columns packed with 1.7 µm and 2.5 µm particle sizes afford scalability between *Rapi*Fluor-MS labeled glycan separations performed under UPLC and HPLC-compatible conditions. Using standard LC method transfer principles to account for differences in particle diameter (dp), column length, and column internal diameter, we show that comparable chromatographic profiles and relative quantitation can be achieved with the larger particle size column at HPLC-compatible pressures, albeit with an increase in sample load, mobile phase use, and most importantly, analysis time.

EXPERIMENTAL

Method conditions

LC system: Alliance HPLC or ACQUITY UPLC H-Class Bio System

Detection: Alliance HPLC 2475 Fluorescence (FLR) Detector ACQUITY UPLC FLR Detector with analytical flow cell

Wavelength: 265 nm excitation, 425 nm emission

Columns: ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm, (p/n 186004742)

XBridge Glycan BEH Amide ***XP*** Column, 130Å, 2.5 µm, 3.0 x 150 mm (p/n 186008040) and XBridge Glycan BEH Amide XP Column, 130Å, 2.5 µm, 3 mm x 75 mm (p/n 186008039) in series. Columns connected by 0.005 x 1.75 UPLC SEC Connection Tubing (p/n 186006613)

Column temp.: 60 °C

Sample temp.: 10 °C

Injection volume: 1.2 µL UPLC, 3.7 µL HPLC

Mobile phase A: 50 mM Waters Ammonium Formate Solution – Glycan Analysis (p/n 186007081), pH 4.4 (LC-MS-grade water, from a 100X concentrate)

Mobile phase B: LC-MS-grade acetonitrile

Gradients:

Time UPLC/HPLC (min)	Flow rate UPLC/HPLC (mL/min)	%A (50 mM amm. formate, pH 4.4)	%B (Acetonitrile)
0.0/0.0	0.40/0.56	25	75
35.0/77.2	0.40/0.56	46	54
36.5/80.5	0.20/0.28	100	0
39.5/87.1	0.20/0.28	100	0
43.1/95.1	0.20/0.28	25	75
47.6/105.0	0.40/0.56	25	75
55.0/121.3	0.40/0.56	25	75

Table 1.

Sample vials: Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL Volume (p/n 186002640)

Data management: MassLynx 4.1 Software
Empower Pro 3.0 Software

Sample description

The *Rapi*Fluor-MS Glycan Performance Test Standard (p/n 186007983) was diluted in water to a concentration of 20 pmole/µL.

RESULTS AND DISCUSSION

Calculating the transfer of glycan HILIC methods between UPLC and HPLC columns

There are two primary considerations to be made when transferring a HILIC-based N-glycan separation method from one LC system and column to another. Most importantly, the surface chemistry and pore size of the particles in the two columns must be comparable. Once appropriate columns have been chosen, the separation must then be appropriately scaled with respect to particle size. Generally this can be accomplished by maintaining a comparable ratio between the length of the column and the size of the particle, L/dp. Once determined, alterations to the gradient can be calculated. In this example, the transfer between a 1.7 µm particle size, 2.1 mm x 150 mm ACQUITY UPLC Glycan BEH Amide Column to an XBridge Glycan BEH Amide Column with a 2.5 µm particle size required a column approximately 50% greater in length (225 mm) since the ratio of the particle sizes is 1.47 (i.e. 2.5/1.7). In practice, a 225 mm length can be easily constructed by combining 150 mm and 75 mm length columns with a suitable column connector. In addition to column length, it is also important to consider the optimal column I.D. HPLC systems invariably exhibit higher dispersion than UPLC systems (bandspread ~30 µL versus ~10 µL), so it is advisable to perform separations with relatively larger I.D. columns to ensure that the effect of extra-column band broadening is minimized. With a 3.0 mm HPLC column I.D. format, near optimal resolution separations can be achieved on an HPLC system, without the high mobile phase consumption rates typical of 4.6 mm I.D. column formats.

Having selected a 3.0 mm x 225 mm effective column dimension, we next calculated the appropriate gradient for the HPLC separation using general method transfer principals (refer back to Table 1 for the gradient).[4] Table 2 outlines column lengths, analysis times, and mobile phase as well as sample consumption corresponding to the use of various scaled methods and potential Glycan BEH Amide Column configurations. Clearly, this exercise highlights two of the significant advantages that UPLC separations provide: shorter analysis times (≥55% decrease) and decreased mobile phase usage (≥68% decrease). The UPLC separations also benefit from lower required sample loads (≥68% decrease), which can prove useful in cases where an analyst is sample limited. For these comparisons, mobile phase use was determined based on the gradient shown in Table 1. Based on these calculated results, the advantages of the UPLC format is evident as is the use of the XBridge Glycan BEH Amide ***XP*** Column, 2.5 µm, 3 mm I.D. Columns on an low band spread (29 µL) HPLC.

Particle size (µm)	Column length (mm)	Column I.D. (mm)	Flow rate (mL/min)	Run time (min)	Mobile phase (mL)	Sample (µL)
1.7	150	2.1	0.4	55	20	1.2
2.5	225	3.0	0.56	121.3	62	3.7
2.5	225	4.6	1.32	121.3	146	8.8
3.5	300	4.6	0.93	229.2	194	11.6

Table 2. Particle size, column lengths, flow rate, analysis times, and mobile phase as well as sample consumption corresponding to the use of various scaled methods and potential Glycan BEH Amide Column configurations.

Comparing UPLC and HPLC *Rapi*Fluor-MS labeled N-glycan profiles

The effectiveness of scaling from a 2.1 x 150 mm, 1.7 µm particle size, Glycan BEH Amide Column using an ACQUITY H-Class UPLC System to a total 225 mm length (150 mm + 75 mm) 2.5 µm particle size, 3.0 mm I.D., XBridge Column run on an Alliance HPLC System is demonstrated qualitatively in Figure 1. Both pairs of chromatograms show comparable profiles over normalized time ranges for the *Rapi*Fluor-MS Glycan Performance Test Standard (p/n 186007983), which represents the N-glycans released from a pooled human IgG sample. In this example, the analysis time difference is approximately 2.2-fold.

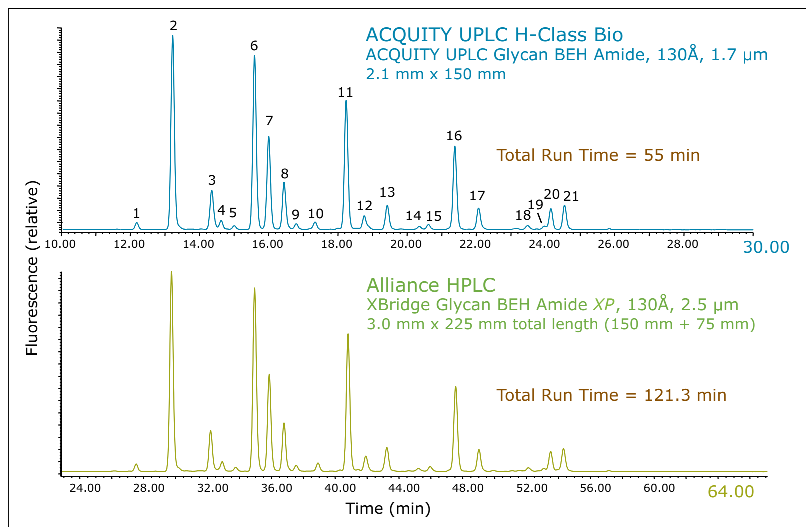

Figure 1. Comparison of UPLC and HPLC HILIC separations of RapiFluor-MS labeled N-glycans from the Glycan Performance Test Standard (p/n 186007983) diluted in water to a concentration of 20 pmole/µL. Injection volumes of 1.2 µL and 3.7 µL for the UPLC and HPLC analyses.

These separations were also compared quantitatively. Shown in Figure 2 is a comparison of the relative retention times for 21 of the most abundant N-linked oligosaccharides observed. Relative retention times were calculated off of Peak 1 (Figure 1) and corrected for the increased system and column dwell volumes of the HPLC separation (~1.2 minutes). Figure 3 illustrates the general comparability of the two separations with respect to the relative quantitation for the same 21 peaks evaluated for retention time. The majority of these values are well within 5% of each other with the most significant difference (~35%) being observed for Peak 19, which has a relative abundance of ~0.2% as determined by the HPLC analysis. If more precise quantitation of these low abundance species is required it would be advantageous to report these results relative to a reference material. Overall, these data demonstrate that the HILIC-based separation of *Rapi*Fluor-MS labeled glycans can be readily transferred between UPLC and HPLC formats. The comparability of the observed chromatographic profiles underscores the chemical comparability of the particle surfaces, as well as the comparability in pore characteristics.

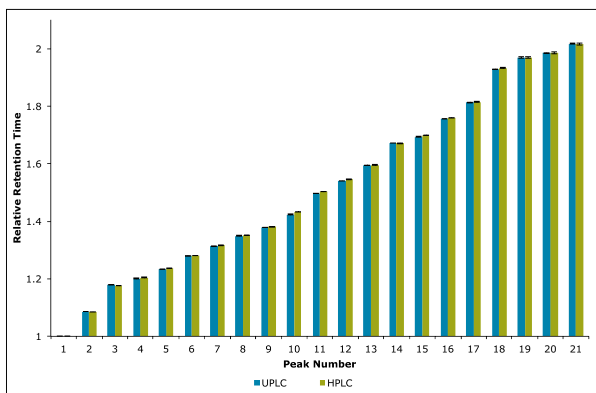

Figure 2. Comparison of UPLC and HPLC HILIC relative retention times (n=2) of RapiFluor-MS labeled N-glycans. Peak numbers are as labeled in Figure 1. Relative retention times were determined based on Peak 1.

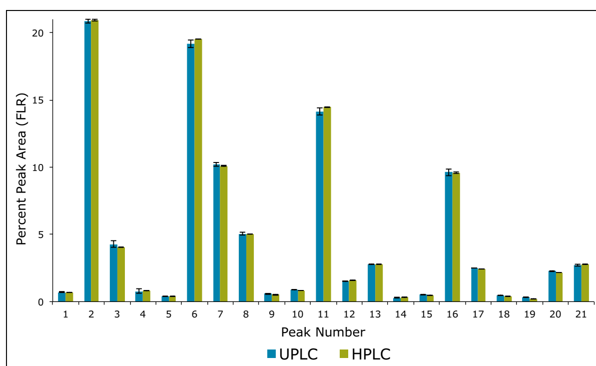

Figure 3. Comparison of UPLC and HPLC HILIC relative peak areas (n=2) of RapiFluor-MS labeled N-glycans. Peak numbers are as labeled in Figure 1.

CONCLUSIONS

These results demonstrate that a HILIC separation of *Rapi*Fluor-MS labeled N-linked oligosaccharides can be seamlessly transferred between UPLC and HPLC platforms when using the appropriate Glycan BEH Amide Columns. The advantage in using the UPLC-based separation is the capability to dramatically improve sample throughput while decreasing mobile phase use. Sample load requirements are also lowered. However, in the event that a laboratory encounters instrumentation limitations, it is beneficial to be able to easily transfer between UPLC and HPLC separations. Additionally, scaling from a UPLC to an HPLC platform can be useful if glycans must be fractionated and purified for structural analysis or to generate materials for method validation spiking studies.

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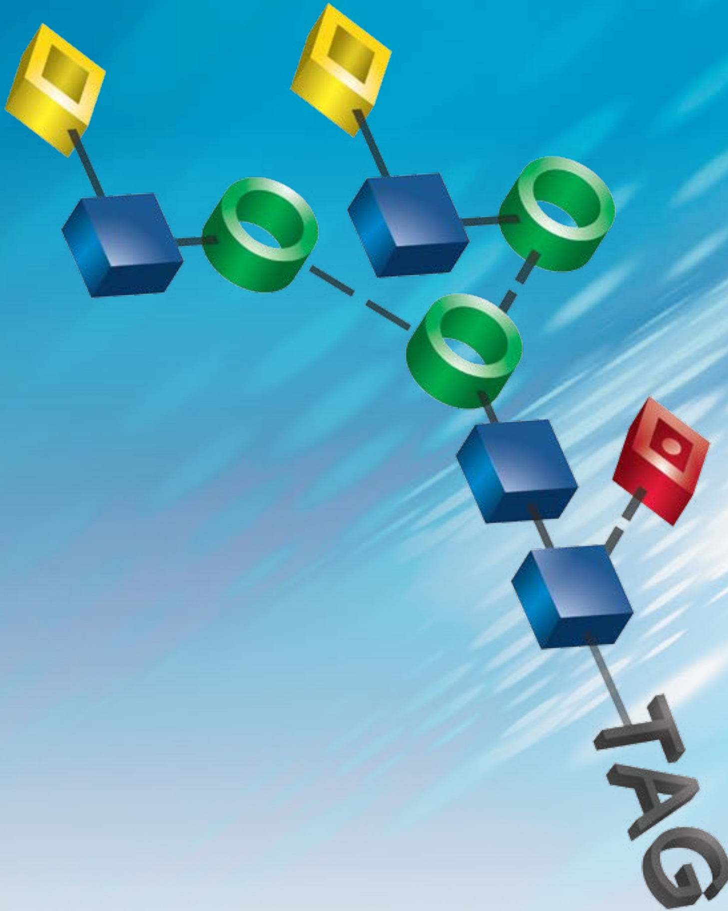
RELEASED N- AND O-LINKED GLYCANS



[TECHNOLOGY BRIEF]

Characterization of EPO N-Glycans using *Rapi*Fluor-MS and HILIC Profiling

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GOAL

To elucidate the complex N-glycans from erythropoietin using the GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit and hydrophilic interaction chromatography (HILIC).

BACKGROUND

Erythropoietin (EPO) is a highly glycosylated protein hormone that stimulates the production of red blood cells. EPO exhibits significant heterogeneity due to its multiple sites of glycosylation (three N-glycosylation sites at Asn 24, 38 and 83 and one O-glycosylation site at Ser 126) and the fact that each of these sites can bear various highly branched sialylated N-glycan structures.[1-2] As a consequence of these post-translational modifications, an EPO will have an apparent SDS-PAGE molecular weight between 30 and 40 kDa, 40% of which corresponds to glycan content. Not surprisingly, the glycosylation of an EPO has been found to impact its therapeutic characteristics, namely its stability, efficacy and potency.[1, 3-5] *In vivo* studies using glyco-engineered EPO have shown links between the safety and efficacy of a therapeutic EPO and several glycosylation associated critical quality attributes (CQA), perhaps with sialic acid content being the most important. Sialylated oligosaccharides have been shown to be associated with increased half-life in plasma as compared to desialylated forms which tend to be cleared within minutes.[1, 3-5]

The analysis of EPO N-glycans is facilitated by the high fluorescence and MS sensitivity afforded by *Rapi*Fluor-MS labeling.

Recombinant human EPO (rhEPO) expressed using Chinese hamster ovary (CHO) cells has been used efficiently in the treatment of anemia, since Epogen was approved by the FDA in 1989.[6] As patents for EPO therapeutics approach expiration, the market for biosimilar rhEPO is expected to increase exponentially. Accordingly, there is a need for efficient and accurate methods that can be used for the characterization of EPO glycosylation.

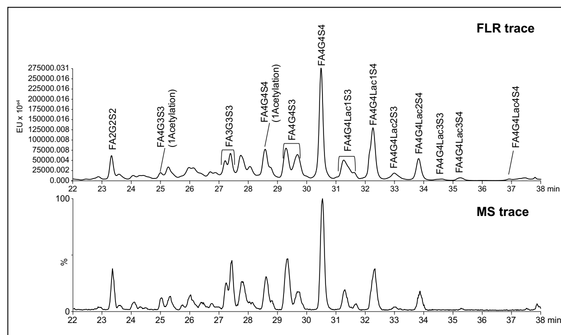

Figure 1. HILIC-FLR-MS of RapiFluor-MS labeled N-glycans. N-glycans from 0.5 µg of an rhEPO were separated using a 2.1 x 150 mm, ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm Column, mobile phases comprised of 50 mM ammonium formate (pH 4.4) (A) and acetontrile (B), and a column temperature of 60 °C. The separation was performed using a 35 minute gradient from 25% A to 46% A. Additional details on the method can be found in the GlycoWorks RapiFluor-MS Care and Use Manual (p/n 715004793).

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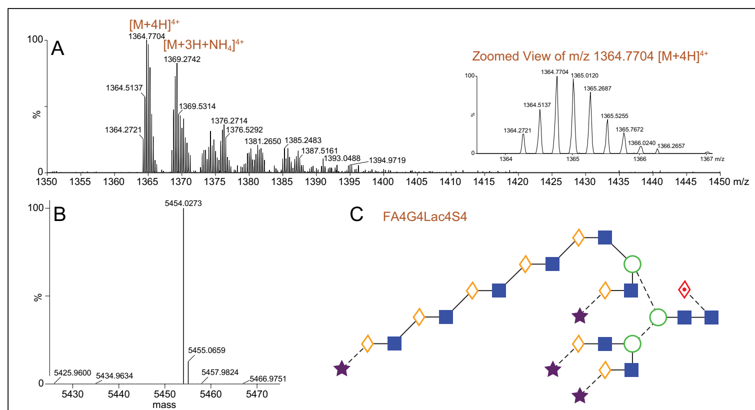

Figure 2. Mass spectrometric analysis of the RapiFluor-MS labeled FA4G4Lac4S4 glycan eluting at retention time 36.94 minutes. (A) Background subtracted ESI-MS mass spectrum showing an [M+4H]$^{4+}$ ion of FA4G4Lac4S4. (B) Charge deconvoluted and deisotoped mass spectrum. (C) Diagram of the identified FA4G4Lac4S4 glycan. (Proposed structure based on a previous study of Lac repeats.[7])

THE SOLUTION

N-glycans from rhEPO were released through fast enzymatic deglycosylation and rapidly labeled using a Waters GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003635).[7] The new *Rapi*Fluor-MS reagent has been designed to facilitate rapid labeling, improve fluorescence quantum yields and greatly enhance MS sensitivity.[7] In this sample preparation, the complex N-glycans of EPO were first made accessible for enzymatic deglycosylation by the use of *Rapi*Gest™ SF, an anionic surfactant. Subsequently, its N-glycans were released in approximately 5 minutes using Rapid PNGase F and an elevated incubation temperature of 50 °C. The resulting deglycosylation mixture, containing free N-glycans (glycosylamines), was then subjected to a 5 minute labeling reaction with *Rapi*Fluor-MS. Labeled N-glycans were thereafter efficiently extracted from the reaction mixture using a GlycoWorks HILIC µElution plate (p/n 186002780) and GlycoWorks SPE Elution Buffer (p/n 186007992). This process of going from glycoprotein to extracted, labeled N-glycans was accomplished in 30 minutes. In addition, this sample preparation allowed for the immediate analysis of the *Rapi*Fluor-MS labeled N-glycans via a HILIC separation with a 2.1 mm x 150 mm, ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Column (p/n 186004742) and an ACQUITY UPLC I-Class System. *Rapi*Fluor-MS N-glycan species eluting during these separations were serially detected by their fluorescence (FLR) and by positive ion-mode ESI-MS with a Xevo® G2-S QTof Mass Spectrometer.

Figure 1 presents chromatograms from the HILIC-FLR-MS analysis of EPO N-glycans as labeled with *Rapi*Fluor-MS. Notably, both the fluorescence and base peak intensity (BPI) MS chromatograms showed high signal-to-noise such that the presence of different N-glycan species could be readily confirmed. The major N-glycans species in this profile were identified using the accurate mass information in combination with data from previous observations of EPO N-glycans.[7] Previously, multidimensional chromatography strategies combining anion exchange chromatography and HILIC had been required to comprehensively characterize the N-glycans of EPO.[7] In this work, we have been successful in identifying EPO N-glycans by employing a one dimensional HILIC separation along with online ESI-Q-Tof MS detection. This is an approach that is facilitated by the improved fluorescence and MS sensitivity afforded by *Rapi*Fluor-MS labeling.[8] These new developments in N-glycan analysis aided in identifying tetra-antennary glycans with multiple sialic acids (three and four) as the most abundant species present on the analyzed rhEPO. The GlycoWorks *Rapi*Fluor-MS approach also helped in determining that tetra-antennary glycans with poly-N-acetyl lactosamine extensions were

also present in relatively high abundance. Relative quantification from the fluorescence profile, in fact, showed that that the tetra-sialylated, tetra-antennary glycan species (FA4G4S4) represents approximately 20% of the total N-glycan pool, while tetra-antennary glycans carrying one (FA4G4Lac1S4) and two (FA4G4Lac2S4) lactosamine extensions constitute approximately 12 and 4.5% of the total N-glycans, respectively. More interestingly, GlycoWorks *Rapi*Fluor-MS approach has yielded identifications of tetra-antennary structures with three (FA4G4Lac3S4) and four (FA4G4Lac4S4) lactosamine extensions at levels of 0.75% and 0.25%, respectively. Although some previous studies on EPO have reported one and two N-acetyl lactosamine extensions, few studies have reported detailed information on species containing four or more lactosamine repeats.[7] In this work, we have been able to successfully identify up to four repeats of poly-N-acetyl lactosamine using only a single dimension of separation and a gradient time of just 35 minutes (Figure 2). Moreover, it was possible to make confident identifications throughout this HILIC profile because of the enhanced fluorescence yields and the improvements in the ionization efficiencies of complex N-glycans that result from the use of the novel *Rapi*Fluor-MS labeling reagent.

SUMMARY

An approach combining the advantages of GlycoWorks *Rapi*Fluor-MS N-glycan sample preparation with the separation capabilities of UPLC HILIC has enabled us to perform a comprehensive analysis of the complex N-glycans present on a recombinant human erythropoietin (rhEPO). With the GlycoWorks *Rapi*Fluor-MS workflow, N-glycan samples were prepared in just 30 minutes. Most importantly, the samples were amenable to direct analysis by HILIC-ESI-QTof-MS analysis. *Rapi*Fluor-MS labeling not only reduced the burden of the sample preparation, but also enhanced the sensitivity of N-glycan detection, making it possible to obtain information-rich data and to elucidate the complicated N-glycan profile of an rhRPO. Because of these benefits, this new approach to N-glycan analysis could be used to hasten the development of EPO biosimilars.

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Comprehensive Characterization of the N and O-Linked Glycosylation of a Recombinant Human EPO

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Two facile strategies to elucidate information about both the N and O-linked glycosylation of EPO
- Unprecedented HILIC separations of high antennarity released N-glycans and intact protein glycoforms
- MS compatible HILIC to enable detailed investigations of sample constituents
- ACQUITY UPLC® Glycoprotein BEH Amide Column (300Å, 1.7 µm stationary phase) is QC tested via a glycoprotein separation to ensure consistent batch-to-batch reproducibility

WATERS SOLUTIONS

ACQUITY UPLC Glycoprotein BEH Amide, 300Å Column (patent pending)

Glycoprotein Performance Test Standard

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

SYNAPT® G2-S HDMS

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated protein, glycosylation, O-Linked, N-Linked, HILIC, *Rapi*Fluor-MS Labeling

INTRODUCTION

The immunoglobulin G (IgG) modality has paved the way for many efficacious protein-based therapies.[1] At the same time, numerous highly effective patient therapies have also been made possible by the production of recombinant, human hormones and enzymes. For example, erythropoesis stimulating therapeutics, like epoetin (EPO) alpha, have long been available for the treatment of anemia. Such a therapy for increasing patient red blood cell counts was first made possible by the commercialization of Epogen,® which has been available in the US market since its approval by the FDA in 1989.[2] And now, because the landscape of the biopharmaceutical industry continues to evolve and Epogen patents expired in 2013,[3] EPO drug products are targets for being developed into both international and domestic-market biosimilars.

Epoetin alpha has a relatively small primary structure, yet it has 3 sites of N-glycosylation and 1 site of O-glycosylation (Figure 1).[4] Because of its glycosylation, epoetin alpha has a molecular weight between 30 and 40 kDa even though its protein mass amounts to only 18 kDa. Interestingly, the glycosylation of epoetin is very much tied to its potency and serum half life. Two attributes of its glycan profile that are known to show positive correlations with *in vivo* activity include antennarity and sialylation.[5-7] As a result, it is critical for the glycosylation of an epoetin therapeutic to be well characterized. In addition, the significance of epoetin glycosylation suggests that detailed glycan profiling would be a path toward establishing a viable epoetin biosimilar.

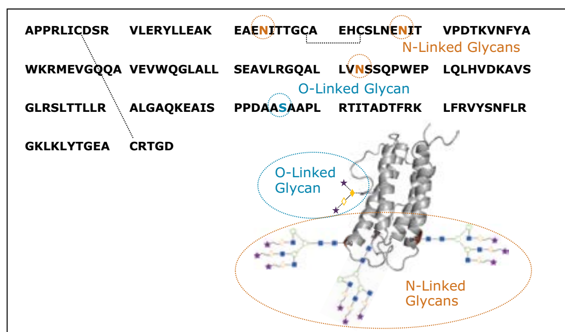

Figure 1. Sequence and structural information for recombinant, human epoetin alpha (rhEPO).

EXPERIMENTAL

Sample description

A recombinant, human epoetin alpha expressed from CHO cells (PeproTech, Rocky Hill, NJ) was reconstituted in 50 mM HEPES NaOH pH 7.9 buffer to a concentration of 2 mg/mL.

N-glycans were released from rhEPO and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and the instructions provided in its care and use manual (p/n 715004793). *Rapi*Fluor-MS labeled N-glycans were injected as a mixture of 90 µL SPE eluate, 100 µL dimethylformamide, and 210 µL acetonitrile.

To facilitate analysis of O-glycosylation, rhEPO was N-deglycosylated using the rapid deglycosylation technique outlined in the care and use manual of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 715004793).

Method conditions (unless otherwise noted)

Column Conditioning

New (previously unused) ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns should be conditioned via two or more sequential injections and separations until a consistent profile is achieved. The care and use manual of the column can be referred to for more information (p/n 720005408EN).

LC conditions for *Rapi*Fluor-MS Released N-Glycans

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 60 °C

Flow rate: 0.4 mL/min

Injection volume: 10 µL

Column: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard)

Fluorescence detection: Ex 265 nm / Em 425 nm, 2 Hz

Sample collection/ Vials: Sample Collection Module (p/n 186007988)

Polypropylene 12 x 32 mm Screw Neck vial, 300 µL volume (p/n 186002640)

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

MS conditions for *Rapi*Fluor-MS Released N-Glycans

MS system: Xevo G2-XS QTof

Ionization mode: ESI+

Analyzer mode: Resolution (~40 K)

Capillary voltage: 2.2 kV

Cone voltage: 75 V

Source temp.: 120 °C

Desolvation temp.: 500 °C

Source offset: 50 V

Desolvation gas flow: 600 L/Hr

Calibration: NaI, 1 µg/µL from 100–2000 *m/z*

Acquisition: 700–2000 *m/z*, 0.5 sec scan rate

Lockspray: 300 fmol/µL Human glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/ acetonitrile every 90 seconds

Data management: MassLynx® Software v4.1

LC conditions for Intact Protein HILIC of N-Deglycosylated rhEPO

LC system: ACQUITY UPLC H-Class Bio System

Sample temp.: 10 °C

Analytical column temp.: 45 °C

Flow rate: 0.2 mL/min

Fluorescence detection: Ex 280 nm/Em 320 nm (Intrinsic fluorescence), 10 Hz

Mobile phase A: 0.1% (v/v) TFA, H_2O

Mobile phase B: 0.1% (v/v) TFA, ACN

HILIC injection volume: 1.3 µL (A 2.1 mm I.D. HILIC column can accommodate up to an ~1 µL aqueous injection before chromatographic performance is negatively affected)

Columns: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column (p/n 176003702, with Glycoprotein Performance Test Standard)

Vials: Polypropylene 12 x 32 mm Screw Neck, 300 µL volume (p/n 186002640)

Gradient:

Time	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	25.0	75.0	6
21.0	35.0	65.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

MS conditions for for Intact Protein HILIC of N-Deglycosylated rhEPO

MS system: SYNAPT G2-S HDMS

Ionization mode: ESI+

Analyzer mode: Resolution (~20 K)

Capillary voltage: 3.0 kV

Cone voltage: 45 V

Source offset: 50 V

Source temp.: 150 °C

Desolvation temp.: 500 °C

Desolvation gas flow: 800 L/Hr

Calibration: NaI, 1 µg/µL from 500–5000 *m/z*

Acquisition: 700–4800 *m/z*, 1 sec scan rate

Data management: MassLynx Software v4.1

In this application note, we demonstrate the use of two facile strategies that can be used to detail the N and O-linked glycosylation of a recombinant, human epoetin (rhEPO). In this work, rhEPO N-glycans were rapidly released, labeled with GlycoWorks *Rapi*Fluor-MS and profiled by hydrophilic interaction chromatography (HILIC) using sensitive fluorescence and mass spectrometric detection. Then, in a second, parallel analysis, N-deglycosylated rhEPO was interrogated by intact protein HILIC to elucidate information on O-glycosylation.

RESULTS AND DISCUSSION

Released N-Glycan analysis of rhEPO using *Rapi*Fluor-MS labeling and HILIC profiling

The glycosylation of recombinant, human epoetin (rhEPO) has been investigated many times before.[4-5, 8-13] In large part, these previous studies have required relatively involved techniques. With this work, it was our objective to establish two facile and complementary, LC based approaches for the analysis of EPO, one capable of providing information about N-glycosylation and the other information about O-glycosylation.

A profile of the N-glycans from rhEPO can be readily obtained with a new sample preparation strategy involving the novel glycan labeling reagent, *Rapi*Fluor-MS. This sample preparation, based on the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, allows an analyst to rapidly release N-glycans and label them with a tag that provides enhanced sensitivity for fluorescence and electrospray ionization mass spectrometric (ESI-MS) detection.[14] In previous applications, *Rapi*Fluor-MS has been predominately used in the analysis of different IgG samples.[14-16] Nevertheless, using the protocol from the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, an analyst can successfully prepare samples from even heavily glycosylated proteins, such as rhEPO.

*Rapi*Fluor-MS labeled N-glycans have proven to be amenable to hydrophilic interaction chromatography (HILIC). Accordingly, HILIC-fluorescence-MS of *Rapi*Fluor-MS has emerged as a very powerful tool for detailing the N-glycosylation of proteins.[14]

To this end, a sample of *Rapi*Fluor-MS N-glycans derived from rhEPO was profiled using HILIC. A recently introduced widepore amide column, the ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7µm Column, was selected for this work to obtain high resolution N-glycan separations. This column was purposefully designed to facilitate HILIC separations of large molecules, such as glycopeptide and glycoproteins. However, the widepore particle architecture has also been shown to increase the peak capacity of highly branched, tri- and tetra-antennary N-glycans by 10–20%,[17] making it an ideal choice for the HILIC profiling of EPO N-glycans, which typically exhibit high antennarity. Figure 2A shows the HILIC fluorescence and base peak intensity (BPI) MS chromatograms of the *Rapi*Fluor-MS N-glycans resulting from 0.4 µg of rhEPO. Even with this relatively limited amount of sample, high signal-to-noise chromatograms are obtained. The sensitivity of the fluorescence trace allows for accurate, relative quantitation across the profile. The signal-to-noise of the MS chromatogram is also particularly noteworthy, though it should be noted that MS sensitivity decreases as N-glycan structures become larger. Nevertheless, the quality of these particular data is made possible by use of the *Rapi*Fluor-MS reagent in combination with the Xevo G2-XS QTof, a new generation MS instrument with improved transmission efficiency and sensitivity. This QTof technology provides unprecedented sensitivity as well as high mass resolution, as can be observed in the collection of mass spectra in Figure 2B that have been used to support the assignment of various N-glycan species.

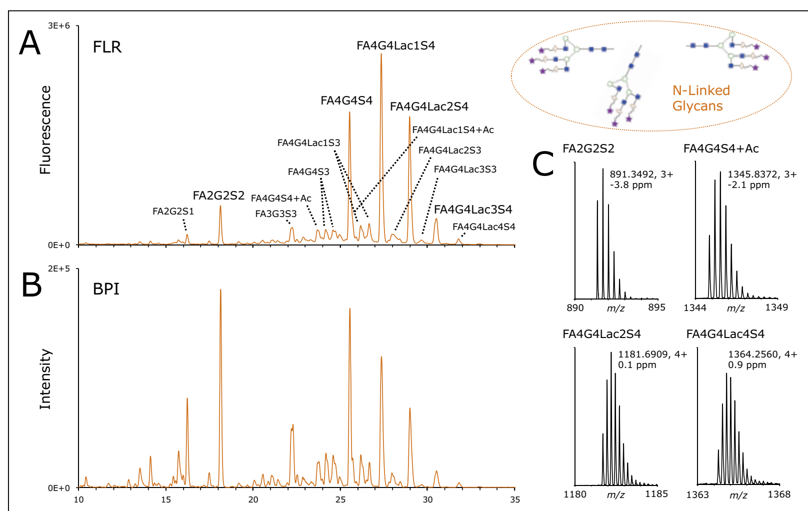

Figure 2. HILIC profiling of released N-glycans from rhEPO. (A) Fluorescence and (B) base peak intensity (BPI) chromatograms for RapiFluor-MS labeled N-glycans from rhEPO. Chromatograms obtained for glycans from 0.4 µg protein using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (C) MS spectra for four example N-glycan species. N-glycan assignments are listed according to Oxford notation. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues (Neu5Ac).[8]

The chromatographic and MS-level selectivity afforded by this analysis simplifies making N-glycan assignments such that the species of the rhEPO N-glycan profile were easily mapped (Figure 3).

The rhEPO analyzed in this study exhibits an N-glycan profile comprised primarily of tetra-antennary, tetrasialylated N-glycans (FA4G4S4) with varying N-acetyl lactosamine extensions. However, the profile also shows a highly abundant peak that corresponds to a disialylated, biantennary N-glycan (FA2G2S2). Given that the ratio of tetra-antennary to biantennary N-glycans has a positive correlation with the *in vivo* activity of an EPO,[6] this analysis has clearly produced valuable information. Other information that can be readily obtained from this N-glycan analysis includes the degree of sialylation and the extent to which structures are modified with lactosylamine extensions. Overall, these results demonstrate that a very-information rich N-glycan profile can indeed be obtained from a comparatively simple *Rapi*Fluor-MS N-glycan preparation and a corresponding HILIC-fluorescence-MS analysis.

RT (min)	Species	$MW_{Mono, Theo}$ (Da)	Observed *m/z*	*z*	$MW_{Mono, Obs}$ (Da)	Mass error (ppm)
16.21	FA2G2S1	2388.9201	1195.4659	2	2388.9172	1.2
18.12	FA2G2S2	2680.0155	894.3492	3	2680.0258	-3.8
22.24	FA3G3S3	3336.2432	1113.0924	3	3336.2554	-3.7
23.68	FA4G4S4 + Ac	4034.4813	1345.8372	3	4034.4898	-2.1
24.15/24.60	FA4G4S3	3701.3754	1234.7966	3	3701.368	2.0
25.52	FA4G4S4	3992.4708	1331.8309	3	3992.4709	0.0
25.7	FA4G4Lac1S4 + Ac	4399.6135	1467.5425	3	4399.6057	1.8
26.16/26.66	FA4G4Lac1S3	4066.5076	1356.5104	3	4066.5094	-0.4
27.34	FA4G4Lac1S4	4357.6030	1090.4097	4	4357.6097	-1.5
27.95	FA4G4Lac2S3	4431.6397	1108.9143	4	4431.6281	2.6
28.97	FA4G4Lac2S4	4722.7352	1181.6909	4	4722.7345	0.1
29.66	FA4G4Lac3S3	4796.7719	1200.2004	4	4796.7725	-0.1
30.50	FA4G4Lac3S4	5087.8674	1272.976	4	5087.8749	-1.5
31.77	FA4G4Lac4S4	5452.9996	1364.256	4	5452.9949	0.9

Figure 3. LC-MS data supporting the identification of various released N-glycan species. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues (Neu5Ac).[8]

Profiling the O-Glycosylation of Intact rhEPO using a Widepore Amide HILIC Separation

O-linked glycans can be challenging to characterize due to the paucity of high fidelity mechanisms to release them from their counterpart proteins. Released glycan analysis is an attractive approach for the characterization of N-glycans because of the simplicity and effectiveness of PNGase F deglycosylation. In place of using an analogous, universal glycosidase, analysts have resorted to releasing O-linked glycans by chemical means, such as alkaline beta elimination[18] or hydrazinolysis.[19] These release mechanisms can be challenging to implement and can very often produce artifacts, known as peeling products.

Rather than attempt a released O-glycan analysis of rhEPO, we looked to develop an alternative characterization strategy. A novel workflow was devised that first involved subjecting the rhEPO to rapid deglycosylation using GlycoWorks Rapid PNGase F and 1% *Rapi*Gest™ SF surfactant. In a 10-minute preparation, a sample of N-deglycosylated intact rhEPO was obtained that could then be profiled via a HILIC separation with an ACQUITY UPLC Glycoprotein BEH Amide Column. Figure 4 presents the chromatogram obtained in this analysis using intrinsic fluorescence detection and intact protein HILIC techniques that have been described in previous work.[20] The N-deglycosylated rhEPO analyzed in this study resolved into a series of approximately 10 peaks. Online ESI-MS provided highly detailed information, allowing for proteoforms of rhEPO to be assigned to the various chromatographic peaks. The two most abundant LC peaks were found to be represented by deconvoluted masses of 18893.8 and 19185.3 Da, which are consistent with N-deglycosylated rhEPO that has a C-terminal arginine truncation as well as trisaccharide and tetrasaccharide O-linked glycan modifications, respectively. More specifically, the mass shift observed for the lighter species is indicative of a glycan modification comprised of 1 hexose, 1 N-acetylhexosamine, and 1 N-acetylneuraminic acid. Meanwhile, the mass shift observed for the heavier species suggests a glycan modification comprised of the same structure with an additional N-acetyl neuraminic acid.

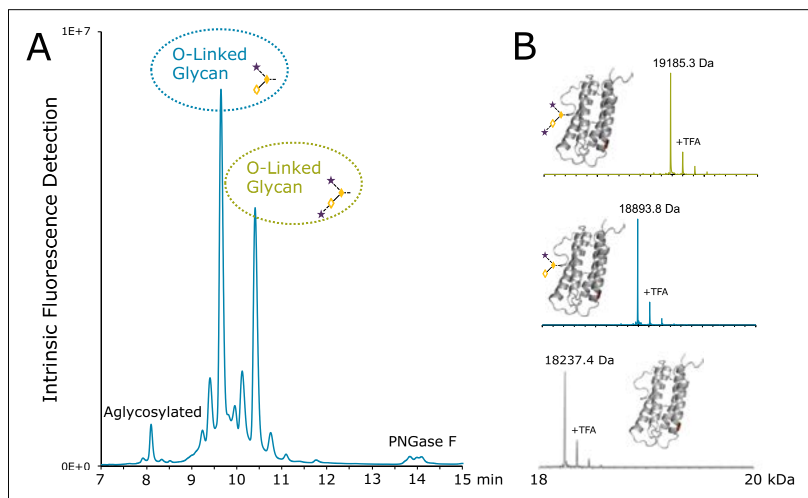

Figure 4. HILIC-fluorescence-MS analysis of N-deglycosylated, intact rhEPO. (A) Fluorescence chromatogram demonstrating O-linked glycan heterogeneity and occupancy. Chromatograms obtained from 0.7 µg protein using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Deconvoluted mass spectra corresponding to three of the major rhEPO proteoforms. Peak identifications, in addition to those denoted here, are tabulated in Figure 5.

Further investigation of the LC-MS data also showed that the proteoform of rhEPO that is aglycosylated with respect to the O-linked glycan eluted with a retention time of approximately 8.2 min. Moreover, these LC-MS data indicated there to be at least two additional O-linked glycoforms and even more C-terminal truncation proteoforms (Figure 5). Here, it is seen that this workflow can indeed be used to rapidly profile the O-linked glycosylation of an rhEPO, such that information is gained about both occupancy and heterogeneity.

RT (min)	Species	$MW_{Avg, Theo}$ (Da)	$MW_{Avg, Obs}$ (Da)	Mass Error (Da)
8.0	N-deglycosylated, –C-term GDR	18066.5	18065.2	-1.3
	N-deglycosylated, –C-term DR	18123.6	18122.4	-1.2
8.2	**N-deglycosylated, –C-term R**	**18238.7**	**18237.4**	**-1.3**
9.3	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1+Ac**	**18937.3**	**18936.2**	**-1.1**
9.5	N-deglycosylated, –C-term GDR +Hex1HexNAc1Neu5Ac1	18723.1	18722.3	-0.8
	N-deglycosylated, –C-term DR +Hex1HexNAc1Neu5Ac1	18780.1	18779.1	-1.0
9.7	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1**	**18895.2**	**18893.8**	**-1.4**
9.9	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2+Ac**	**19228.5**	**19227.3**	**-1.2**
10.0	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1 + O**	**18911.2**	**18910.0**	**-1.2**
10.2	N-deglycosylated, –C-term GDR +Hex1HexNAc1Neu5Ac2	19014.3	19013.7	-0.6
10.5	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2**	**19186.5**	**19185.3**	**-1.2**
10.8	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2 + O**	**19202.5**	**19201.2**	**-1.3**

Figure 5. LC-MS data supporting the identification of various N-deglycosylated rhEPO proteoforms. "–C-term" denotes the C-terminal truncation of the rhEPO; losses of different residues are noted. Hex, HexNAc, and Neu5Ac stand for hexose, n-acetylhexosamine, and N-acetylneuraminic acid. For example, Hex1HexNAc1NeuN5Ac1 corresponds to O-glycosylation involving 1 hexose, 1 N-acetylhexosamine, and 1 N-acetylneuraminic acid. "+O" denotes a mass shift indicative of the addition of an oxygen atom, such as an oxidation or an exchange of Neu5Ac for Neu5Gc.[8] Data supporting identifications of the most abundant rhEPO sequence variant (–C-term R) and its glycoforms are highlighted with bold text. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues (Neu5Ac).[8]

CONCLUSIONS

Several powerful tools have recently emerged for the analysis of glycans that are built upon LC-MS compatible hydrophilic interaction chromatography (HILIC). At the heart of these new glycan analysis workflows is a HILIC column that has been purposefully designed for large molecule separations. With this ACQUITY UPLC Glycoprotein BEH Amide Column, an analyst can achieve higher resolution separations of large, released N-glycans. And when this analysis is paired with *Rapi*Fluor-MS labeling, a technique is established that affords not only high resolution but also unprecedented sensitivity. This approach has been successfully applied to obtain highly detailed information about the N-glycosylation of a recombinant, human epoetin alpha (rhEPO). Given that N-glycosylation correlates with the half life and activity of an EPO, such information, with its unparalleled quality, would be invaluable in developing a new EPO therapeutic. EPO is also O-glycosylated; the occupancy and heterogeneity of which could also be critical to demonstrate comparability among different drug substances. Using the ACQUITY UPLC Glycoprotein BEH Amide Column, we have outlined a simple sample preparation and subsequent HILIC separation that is capable of profiling these O-glycan attributes on intact rhEPO. In summary, we have demonstrated the use of two facile strategies that can be used to detail both the N and O-linked glycosylation of recombinant, human epoetin (rhEPO), a molecule which has been perceived to be challenging to characterize due to its relatively complicated glycosylation. Collectively, these tools could be used to accelerate the development of new biosimilars.

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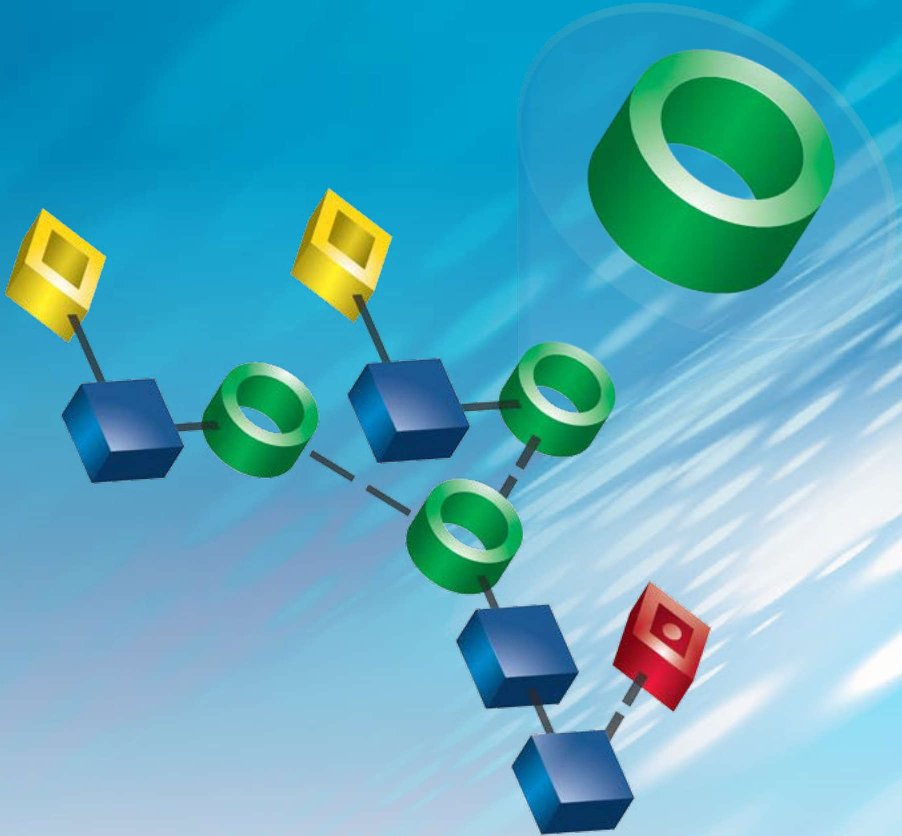

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Future Proofing the Biopharmaceutical QC Laboratory: Chromatographic Scaling of HPLC Monosaccharide Analyses Using the ACQUITY UPLC H-Class Bio System

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APPLICATION BENEFITS

- Geometric scaling of an HPLC method for determining monosaccharide composition in glycoprotein samples to UPLC®
- Application of scalable column chemistries for monosaccharide analysis
- Guidance for scaling HPLC gradient methods to UPLC
- A high sample throughput and high resolution UPLC approach for determining monosaccharide analysis

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

Empower® 3 Chromatography Data Software

XBridge® HPLC Columns

ACQUITY UPLC BEH Column

KEY WORDS

Glycosylation, glycoproteins therapeutics, N-linked glycans, monosaccharide, 2AA-derivatized monosaccharides, glycan composition, HPLC, UPLC

INTRODUCTION

Evidence for reliable and consistent glycosylation of glycoprotein therapeutics is typically obtained through LC-based analysis of N-linked glycans. On occasion, regulatory agencies request information of monosaccharide content as an orthogonal technique for confirming the glycan composition. Beyond profiling changes in total glycan composition, monosaccharide analysis can also be used as an exploratory technique for identifying various monosaccharide modifications, including phosphorylation and sulfation, which can be particularly challenging to discern when analyzing at the released glycan level in the absence of mass spectrometry.

Initial investigation of monosaccharide analyses resulted in a reliable and informative HPLC-based approach using 2-aminobenzoic acid (2AA)-derivatized glycan hydrolysates where each of the individual monosaccharides were successfully separated using reversed-phase chromatography. Since this time, the analytical demands placed on development and QC labs that are tasked with characterizing and monitoring glycoprotein therapies have increased dramatically. Assays used to monitor changes in glycosylation therefore need to be updated in order to maximize productivity through improved analytical efficiency.

In this application note, we illustrate the transfer of a robust HPLC monosaccharide method to the ACQUITY UPLC H-Class Bio System running as an HPLC, using monosaccharide standards as well as bovine fetuin and the commercially available monoclonal antibody cetuximab as analytes. Geometric scaling of the presented method is then performed in a stepwise manner to demonstrate the advantages of transferring legacy HPLC monosaccharide analyses to UPLC technology as a means of decreasing assay time.

EXPERIMENTAL

LC conditions

Waters ACQUITY UPLC H-Class Bio System, comprised of:
- ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM)
- ACQUITY UPLC H-Class Bio Sample Manager (FTN)
- ACQUITY UPLC H-Class Bio Column Heater (CH-A)
- ACQUITY UPLC FLR Detector
- Extension loop: 100 µL (p/n 430002625)

Waters columns:
- XBridge C_{18} 5 µm, 130 Å, 4.6 x 100 mm (p/n 186003115)
- XBridge C_{18} 3.5 µm, 130 Å, 2.1 x 100 mm (p/n 186003033)
- XBridge C_{18} XP 2.5 µm, 130 Å, 2.1 x 100 mm (p/n 186006031)
- ACQUITY UPLC BEH C_{18} 1.7 µm, 130 Å, 2.1 mm x 100 mm (p/n 186002352)

Mobile phase A: 0.2% N-butylamine, 0.5% phosphoric acid, and 1% THF in H_2O

Mobile phase B: 50% mobile phase A in acetonitrile

Excitation wavelength: 360 nm

Emission wavelength: 425 nm

Column temp.: 30 °C

Injection vol.: 4.6 mm x 100 mm format, 4.8 µL, 2.1 mm x 100 mm format, 1 µL

Following the described approach, typical run times for HPLC-based monosaccharide analysis are reduced from 45 minutes to just 17 minutes. Importantly, chromatographic resolution between measured critical peak pairs is observed to improve with migration to smaller column particle sizes. Selectivity is unaffected due to the availability of reversed-phase column chemistries in a number of particle sizes and dimensions. The data presented here indicate migration to UPLC technology offers significant advantages for improving monosaccharide chromatographic quality.

Sample preparation

Derivatization of monosaccharides was performed as previously described,[1,2] with a number of minor modifications as recommended by Stepan and Staudacher.[3] Monosaccharides from bovine fetuin were released by acid hydrolysis using 2 M TFA with hydrolysis occurring for 3 h at 100 °C. Resulting hydrolysates were then dried by centrifugal evaporation followed by reconstitution in 5 µL of 80 mg/mL sodium acetate trihydrate. A 2AA labeling solution was prepared by dissolving 30 mg of 2AA in 1 mL of 2% (w/v) boric acid in methanol. This suspension was then used to dissolve 30 mg of sodium cyanoborohydride. Of this preparation, 10 µL was added to each of the monosaccharide mixtures. Monosaccharides were labeled at 80 °C for 60 min. Upon completion of labeling, serial dilutions were performed to generate a 1000-fold dilution of the labeled material. For preparation of monosaccharide standards, labeling was performed as outlined above with the omission of acid hydrolysis.

Step	%B[1]	Method details (flow rate and time)							
		5 µm		3.5 µm		2.5 µm		1.7 µm	
		Flow (mL min^{-1})	Time (min)	Flow (mL min^{-1})	Time (min)	Flow (mL min^{-1})	Time (min)	Flow (mL min^{-1})	Time (min)
1	7	0.480	0.00	0.685	0.00	0.200	0.00	0.294	0.00
2	7	0.480	7.78	0.685	5.45	0.200	3.89	0.294	2.64
3	17	0.480	27.78	0.685	19.47	0.200	13.88	0.294	9.44
4	100	0.480	28.89	0.685	20.24	0.200	14.43	0.294	9.82
5	100	0.480	40.00	0.685	28.03	0.200	19.99	0.294	13.60
6	7	0.480	41.11	0.685	28.81	0.200	20.54	0.294	13.97
7	7	0.480	50.00	0.685	35.04	0.200	24.98	0.294	17.00

Calculations

Flow rate scaling: $F_2 = F_1\left(\frac{d_2^{\,2}}{d_1^{\,2}}\right)\left(\frac{d_{p1}}{d_{p2}}\right)$

F refers to flow rate, d refers to column I.D., and dp refers to particle diameter. In each case, 2 refers to the new column and 1 refers to the original column.

Injection volume scaling: $Vi_2 = Vi_1\left(\frac{r_2^{\,2}L_2}{r_1^{\,2}L_1}\right)$

Vi refers to injection volume, r refers to column internal radius, and L refers to column length. In each case, 2 refers to the new column and 1 refers to the original column.

RESULTS AND DISCUSSION

ACQUITY UPLC H-Class Bio System for HPLC monosaccharide analysis

To verify the ability of the ACQUITY UPLC H-Class Bio to run legacy analyses of 2AA-derivatized monosaccharides, we first established an HPLC separation using method conditions based on previously described chromatographic conditions[1,2] To evaluate the proposed method, a reference standard mix was prepared by combining individual monosaccharides into a common mix. This mix included the typical monosaccharides expected in biologically relevant samples, namely N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), glucose (Glc), mannose (Man), galactose (Gal), xylose (Xyl), and fucose (Fuc). In addition to these standards, two glycoprotein samples were also selected to determine the accuracy of this approach in determining monosaccharide composition. The first glycoprotein selected was bovine fetuin, a protein known to contain both N- and O-glycosylation sites. The second glycoprotein selected was the commercial monoclonal antibody cetumixab.

Following hydrolysis from the glycoprotein samples and 2AA derivatization, monosaccharides were separated using the aforementioned method. The resulting HPLC chromatogram (Figure 1) acquired on the ACQUITY UPLC H-Class Bio is consistent with previously published data.[2] In terms of chromatographic performance, peak capacity was measured together with selectivity and resolution between critical peak pairs. These data are summarized in Table 1. Consistent peak area was observed across separations using all particle sizes (Figure 2d). These data confirmed the separation of all relevant components and, therefore, established a suitable method for monosaccharide method scaling to UPLC technology.

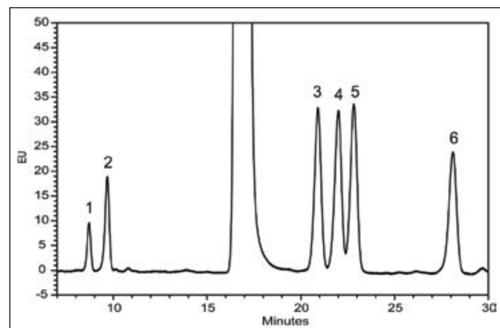

Figure 1. HPLC analysis of monosaccharides. A separation performed with a Waters XBridge 5 µm C_{18} Column using a previously described method1. Monosaccharides are identified as follows: 1) N-acetylglucosamine (GlcNAc), 2) N-acetylgalactosamine (GalNAc), 3) Galactose (Gal), 4) Mannose (Man), 5) Glucose (Glc), and 6) Fucose (Fuc).

Measurement		Particle size (µm)			
		5	3.5	2.5	1.7
Column ID (mm)		4.6	4.6	2.1	2.1
Column Length (mm)		100	100	100	100
Average $W_{1/2h}$		0.349	0.189	0.125	0.076
T_g		20.00	9.99	14.02	6.80
P_c		58	75	81	90
	GlcNAc	8.71	5.84	4.40	2.85
	GalNAc	9.69	6.49	4.87	3.15
	Gal	20.92	14.32	10.87	7.08
	Man	22.01	15.09	11.46	7.46
	Glc	22.83	15.66	11.92	7.78
	Fuc	28.12	19.39	14.76	9.74
	GlcNAc	3.65	3.83	3.69	3.67
	GalNAc	8.26	8.52	8.23	8.31
	Gal	22.17	22.48	22.45	22.06
	Man	23.04	23.07	23.05	22.72
	Glc	23.76	23.89	23.88	23.61
	Fuc	19.11	18.21	18.71	19.62
	GlcNAc, GalNAc	1.12	1.13	1.13	1.15
	GalNAc, Gal	2.26	2.38	2.47	2.67
	Gal, Man	1.05	1.06	1.06	1.06
	Man, Glc	1.04	1.04	1.04	1.05
	Glc, Fuc	1.24	1.25	1.26	1.28
	GlcNAc, GalNAc	2.52	3.10	3.16	3.32
	GalNAc, Gal	21.05	27.12	30.69	33.25
	Gal, Man	1.62	2.10	2.48	2.62
	Man, Glc	1.20	1.53	1.90	2.23
	Glc, Fuc	7.34	9.59	11.41	12.78

Table 1. Summary data for chromatographic analysis.

Migration of monosaccharide analysis from HPLC to UPLC improves resolution

In an effort to improve throughput of analyses and general chromatographic quality, the above described method was geometrically scaled in a stepwise manner to UPLC column technology. This involved scaling the flow rate to the new column dimensions and adjusting individual steps in the gradient method to deliver equivalent column volumes as itemized in the original recommended HPLC method. Details of the UPLC method can be found in the experimental section of this application note.

Several particle sizes of identical chemistry ranging from 1.7 to 5 µm were used in the scaling exercise. In the case of the 1.7 µm and 2.5 µm particles, a 2.1 mm x 100 mm column dimension was used while 4.6 mm x 100 mm column dimensions were used for the 3.5 µm and 5 µm particles. Flow rate and injection volume scaling calculations specific for individual column dimensions were determined using the appropriate equations defined in the experimental section of this application note. The duration for each step in the gradient table was subsequently modified based on the new flow rate and column volume to ensure consistent delivery of equivalent column volumes per change in organic composition when compared to the original method. The results of these calculations are summarized in the experimental section of this application note where flow rates and gradient step durations are itemized with respect to each column particle size.

To evaluate the results of method scaling, the monosaccharide mix was separated under the new gradient conditions for each particle size and column dimensions (Figure 2). Method scaling to UPLC column technology reduced the total required run time of the method from 50 min (in the case of the 5 µm column, Figure 2a) to just 17 min (in the case of the 1.7 µm column, Figure 2d), an improvement in efficiency of approximately 66%. Details of chromatographic performance are presented in Table 1.

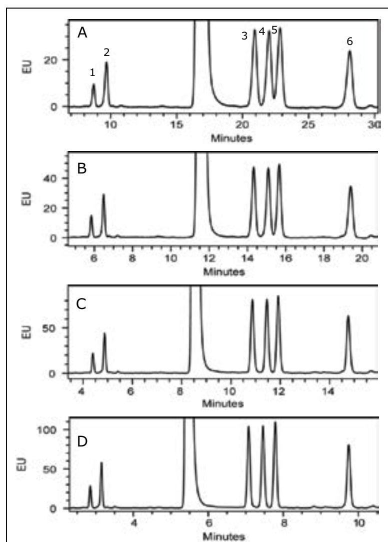

Figure 2. Geometric scaling of a monosaccharide separation. (A) 5 µm particle (B) 3.5 µm particle, (C) 2.5 µm particle, and (D) 1.7 µm particle. 1) GlcNAc, 2) GalNAc, 3) Gal, 4) Man, 5) Glc, and 6) Fuc.

In general, peak capacity was shown to increase with decreasing particle size (Figure 3a), an expected outcome based on the narrower peaks achieved with UPLC technology. The reduction in run time was not at the cost of resolution, where a general improvement was observed across all critical peak pairs as column particle size decreased (Figure 3b). Selectivity remained unaffected mainly due to the availability of identical column chemistry across multiple particle sizes (Figure 3c). Changes in column particle size did not impact relative peak area determination, evidenced by averaged peak areas for each monosaccharide across all column formats (Table 1 and Figure 3d). Taken together, scaling of the original monosaccharide method produced improved resolution in a shorter amount of time, with negligible impact to selectivity.

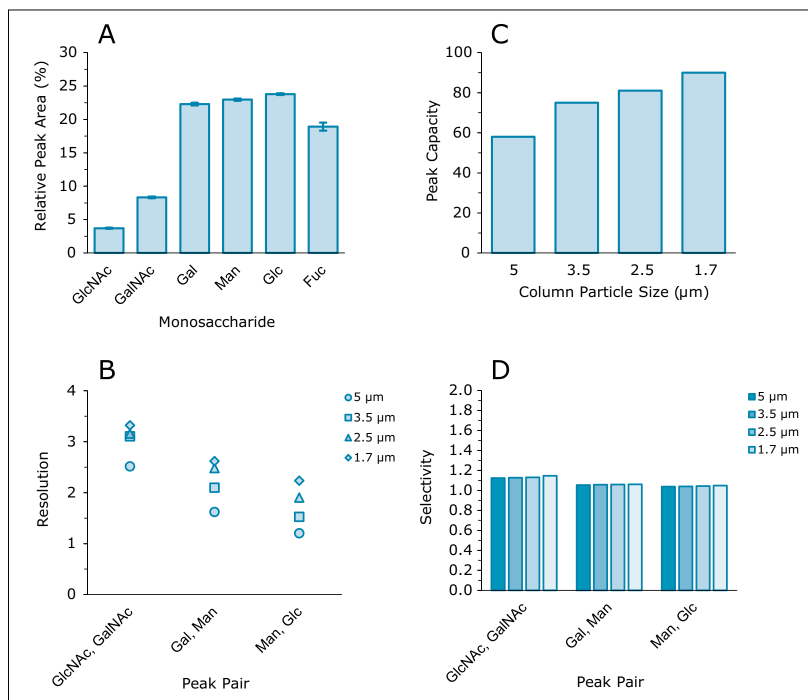

Figure 3. Quantitative analysis of geometric scaling. (A) Relative peak area measurements for individual monosaccharides, averaged across all columns with standard deviation denoted by error bars; (B) Resolution measurements from 3 monosaccharide peak pairs; (C) Peak capacity measurements of geometrically scaled methods across all column dimensions, and; (D) selectivity measured between 3 monosaccharide peak pairs.

Determination of monosaccharide content in glycoprotein samples

To verify this approach in determining monosaccharide composition, we analyzed both fetuin and cetuximab 2AA-labeled monosaccharides. Fetuin is known to contain O-glycosylation and therefore should demonstrate the presence of GalNAc within its profile. Cetuximab, on the other hand, contains no O-glycosylation and should therefore be absent of any GalNAc.

To perform the analysis, the 1.7-µm BEH C_{18} particle chemistry was used and results of the separations were compared to the monosaccharide standard (Figure 4a). GalNAc is clearly detected in the fetuin sample (Figure 4b), as evidenced by a chromatographic peak with similar retentivity to the GalNAc standard. Also consistent with literature is the absence of core fucosylation in fetuin, largely evidenced by the absence of a peak with similar retentivity to the fucose standard. This finding is consistent with previous reports of bovine fetuin N-glycosylation characterization.[4,5] Analysis of cetuximab reveals all individual monosaccharides with the exception of xylose, a monosaccharide not typically associated with mammalian N-linked glycosylation (Figure 4c). Mannose was found to be in higher quantity than galactose, a result consistent with previous literature indicating the presence of several high mannose structures in cetuximab.[6-8]

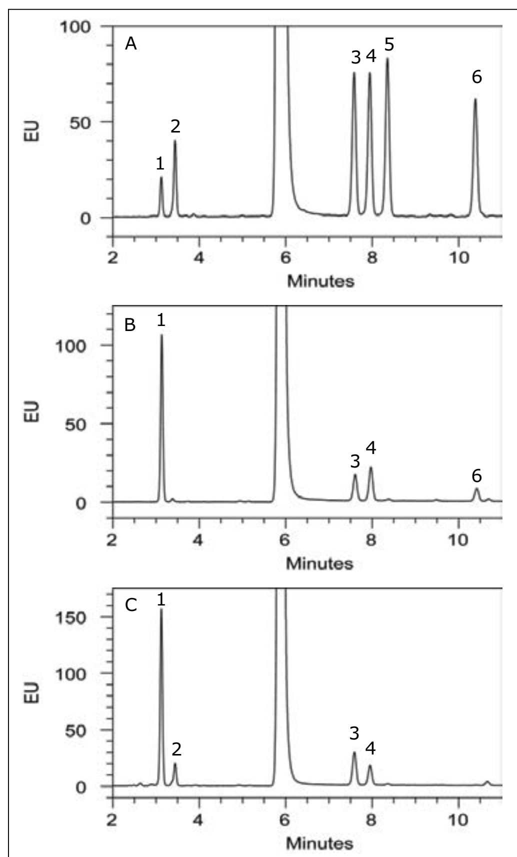

Figure 4. Monosaccharide analysis of fetuin and cetuximab using the developed UPLC separation. (A) monosaccharide standard, (B) cetuximab monosaccharides, (C) fetuin monosaccharides. In all chromatograms, monosaccharides are identified as: 1) GlcNAc, 2) GalNAc, 3) Gal, 4) Man, 5) Glc, and 6) Fuc.

CONCLUSIONS

As a complementary approach to released glycan analysis, monosaccharide profiling allows analysts to verify glycan composition determined in traditional HILIC-based separations. A well-established assay for monosaccharide analysis uses reversed-phase chromatography to separate 2AA-derivatized monosaccharides. In this application note, we have demonstrated the ability of the ACQUITY UPLC H-Class Bio System for running both HPLC and UPLC methods for monosaccharide analyses. This HPLC assay provided sufficient resolution of individual monosaccharides but was restricted in part by the time required to perform the separation. A significant reduction in runtime was obtained by transferring the legacy HPLC method to UPLC technology. With the modernized, UPLC-based separation, a higher throughput assay for monosaccharide analysis was thereby achieved.

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