

PLATFORM SOLUTION

BIOPHARMACEUTICAL

Key Applications

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Biopharmaceutical Platform Solution with UNIFI: Key Applications

Intact Protein MS Analysis | Peptide Mapping | Released Glycan Analysis | Bioseparations

Biotherapeutic proteins – such as monoclonal antibodies (mAbs), biosimilars, and antibody drug conjugates (ADCs) – are among the fastest growing category of therapeutics. Due to the complex nature of these molecules, biopharmaceutical companies and regulatory authorities are challenged to demonstrate comprehensive characterization, process robustness, and the ability to monitor product and critical quality attributes (PQA and CQAs) in development and for QC release.

The Waters Biopharmaceutical Platform Solution with UNIFI® combines the following into a robust, integrated, high-resolution analytics platform: high-resolution UPLC® separations, advanced optical and MS detection, and streamlined informatics. Each department of a biopharmaceutical organization can leverage this platform to produce analytical information, transfer successful methods, review data, generate reports, and manage historical results – all within a regulatory-compliant environment. The Waters Biopharmaceutical Platform Solution with UNIFI analyzes biotherapeutics at multiple levels of structure: intact protein, peptide digest, and released glycan.

Built for addressing analytical challenges, this platform solution provides an integrated set of tools to efficiently streamline laboratory workflows and improve productivity from discovery through QC.



CHAPTER 1

Intact Protein	4
Intact Protein Characterization	5
Structural Comparability Assessment of Innovator and Biosimilar Rituximab	7
Structural Comparison of Infliximab and a Biosimilar via Subunit Analysis.....	18
Streamlining Compliant and Non-compliant Intact Mass Analysis of Biotherapeutic mAbs	27
Routine LC-MS Analysis of Intact Antibodies.....	35
Development of Integrated Informatics Workflows for the Automated Assessment of Comparability for Antibody Drug Conjugates (ADCs) Using LC-UV and LC-UV/MS	43
Top-down Monoclonal Antibody (mAb) Analysis Using CID and ETD Fragmentation and the UNIFI Scientific Information System.....	53

CHAPTER 2

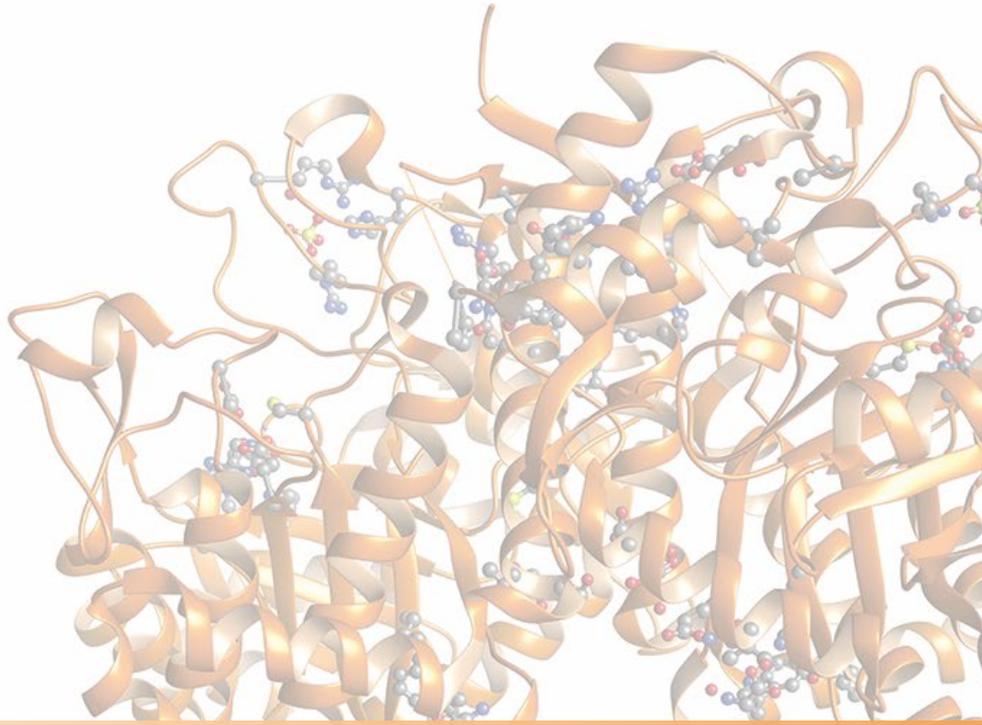
Peptide Mapping	62
Simplifying Verification of Peptide Mapping Results	63
Generating Automated and Efficient LC-MS Peptide Mapping Results	65
A Streamlined Data-dependent Acquisition (DDA) Peptide Mapping Workflow for Characterizing Therapeutic Proteins	72
Automated Quantitative Analysis of Antibody Drug Conjugates Using an Accurate Mass Screening Workflow in the UNIFI Scientific Information System	78
Automated Disulfide Bond Mapping, and Comparing Innovator and Biosimilar mAbs Using UNIFI	85

CHAPTER 3

Released Glycan Analysis	93
A Holistic Workflow for Acquiring, Processing, and Reporting Fluorescent-labeled Glycans.....	94
N-linked Glycan Characterization and Profiling: Combining the Power of Accurate Mass, Reference Glucose Units, and UNIFI Software for Confident Glycan Assignments.....	102
Optimization of GlycoWorks HILIC SPE for the Quantitative and Robust Recovery of N-linked Glycans from mAb-type Samples.....	112
Single-use and High-throughput HILIC SPE Device Formats and an IgG Control Standard for Facilitating N-glycan Analyses	121
Released N-linked Glycan Analysis Using the Glycan Application Solution with UNIFI.....	124
Applying a Novel Glycan Tagging Reagent, <i>RapiFluor</i> -MS, and an Integrated UPLC-FLR/QToF MS System for Low Abundant N-glycan Analysis	127

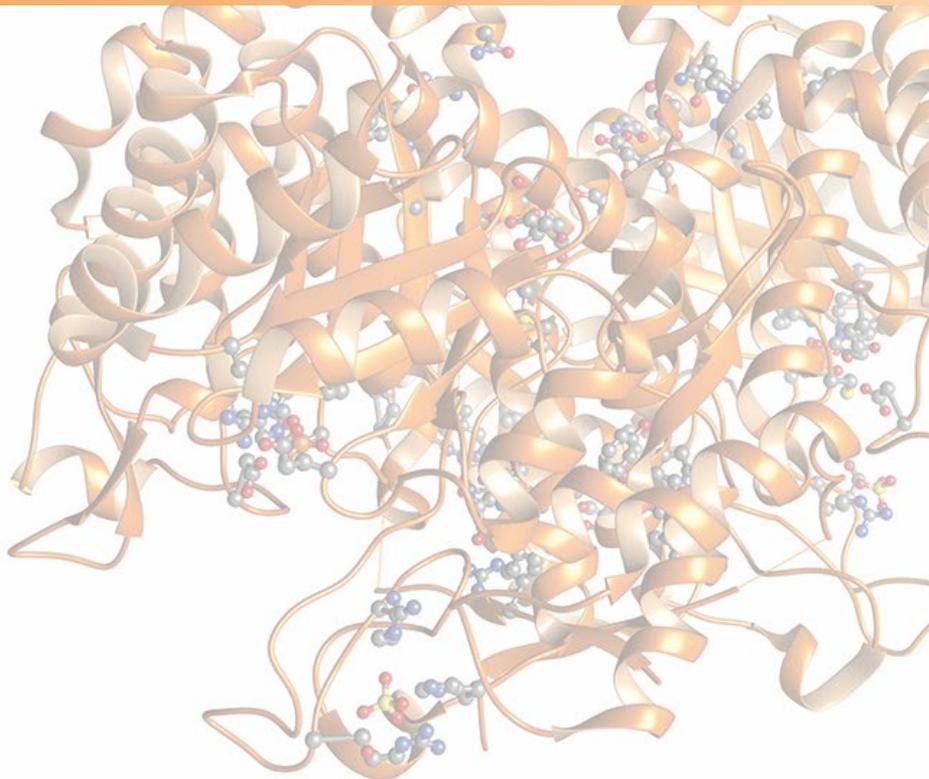
CHAPTER 4

Bioseparations	132
Developing Robust and Efficient IEX Methods for Charge Variant Analysis of Biotherapeutics Using ACQUITY UPLC H-Class System and Auto•Blend Plus	133
Charge Variant Analysis of Therapeutic Monoclonal Antibodies Using a pH Gradient Generated by Auto•Blend Plus	140
Simultaneous Determination of Molecular Size, Concentration, and Impurity Composition of Biotherapeutics with SEC	148



INTACT PROTEIN

Chapter 1



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.

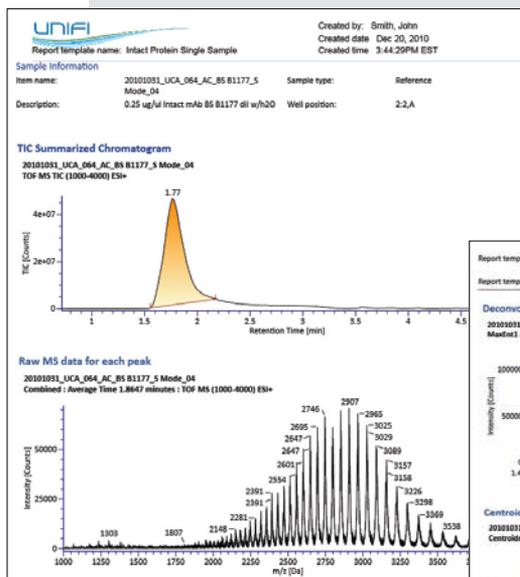


Figure 1. UNIFI's intact protein analysis report of an intact mAb, Trastuzumab. The report shows, from top to bottom, the integrated chromatographic peak, the charge state distribution, the continuum deconvoluted data, the integrated and processed data, and finally the summary table assigning major glycoforms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

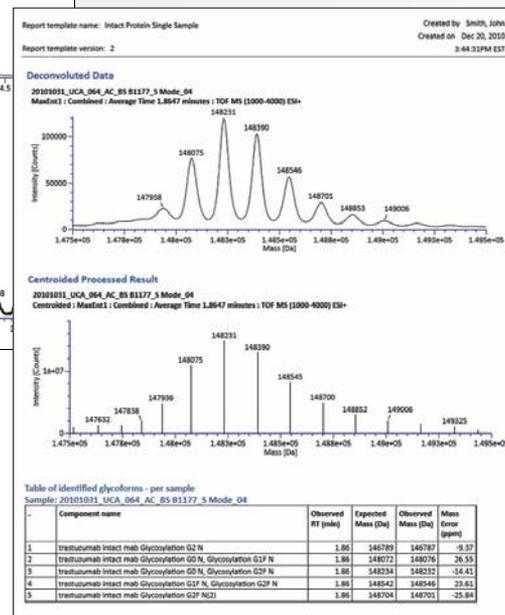


Table of identified glycoforms - per sample

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 G2 N, Glycosylation G1 F N	1.86	148072	148076	26.55
3. trastuzumab intact mAb Glycosylation G2 N, Glycosylation G2 F N	1.86	148234	148232	-34.41
4. trastuzumab intact mAb Glycosylation G1 F N, Glycosylation G2 F N	1.86	148942	148940	-23.81
5. trastuzumab intact mAb Glycosylation G2 F N	1.86	148704	148701	-25.84

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 C₄ 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_S 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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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Structural Comparability Assessment of Innovator and Biosimilar Rituximab Using the Biopharmaceutical System Solution with UNIFI

Vera B. Ivleva, Ying Qing Yu, Scott Berger, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

For comparability studies performed with biosimilars, the integration of a fit-for-purpose UPLC/Tof-MS system with GxP-friendly data management, available with the UNIFI™ Scientific Information System, facilitates the development of a biotherapeutic product. This system solution enables complex biosimilar development to be carried out using routine analytical methodologies that are streamlined by efficient, workflow-based data management and reporting.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

Xevo® G2 ToF Mass Spectrometer

Biopharmaceutical System Solution
with UNIFI

KEY WORDS

Biosimilars, mAb, biopharmaceutical,
rituximab

INTRODUCTION

Biopharmaceutical companies are challenged to design efficient analytical strategies for detailed assessment of structural comparability between biosimilar and innovator products. Extensive characterization increases confidence that a biosimilar product is safe and will meet regulatory compliance requirements for abbreviated approval pathways. Here, we demonstrate how an integrated biopharmaceutical LC/MS system utilizing the UNIFI Scientific Information System addresses these challenges by integrating and automating data acquisition, data processing, and result reporting into a seamless workflow for in-depth biotherapeutic structural characterization.

Comparability studies between an innovator, rituximab monoclonal antibody (mAb), and two biosimilar candidates were performed at the levels of intact protein, subunits (partially reduced antibody), and peptides using the Biopharmaceutical System Solution with UNIFI, shown in Figure 1. Differences in Critical Quality Attributes, such as primary structure (mutation), glycan fucosylation, and terminal amino acid heterogeneity were compared, quantified, and reported in a seamless workflow.

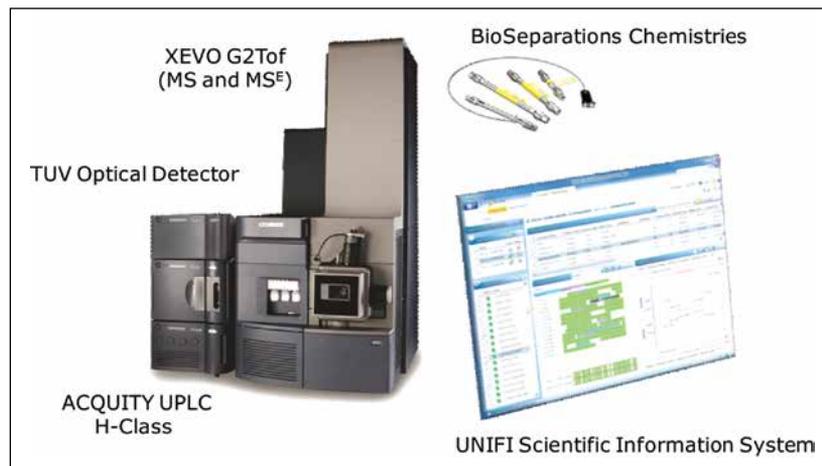


Figure 1. Biopharmaceutical System Solution with UNIFI.

EXPERIMENTAL**Sample Description**

Intact mass analysis: Innovator and both of the biosimilar mAb samples were diluted to 0.5 mg/mL using 25 mM ammonium bicarbonate, pH 7.9 for injection and analysis.

Reduced mAb analysis: The samples were diluted to 1 mg/mL in a reduction buffer (25 mM NaCl, 25 mM Tris, pH 7.5), and a concentrated DTT solution was added to the sample to obtain the final DTT concentration of 1.0 mM. The solution was then incubated at 37 °C for 20 min. The reduced samples were further diluted using a dilution buffer of 5% acetonitrile, 0.1% TFA to 0.2 mg/mL for LC/MS analysis.

Protein digestion: The samples were mixed with a denaturing buffer (8 M guanidine chloride, 1 M Tris, pH 7.5) to 1.0 mg/mL, reduced with 3 mM DTT, and alkylated with 7 mM iodoacetamide before buffer exchange over a NAP-5 column (GE Healthcare) to a digestion buffer of 100 mM Tris, pH 7.5. The samples were digested individually using either trypsin or chymotrypsin (S:E = 20:1) for 4 hrs. The digested samples were diluted with 3% acetonitrile, 0.1% TFA to 0.2 mg/mL for injection.

Method Conditions

Biopharmaceutical System

Solution with UNIFI: ACQUITY UPLC H-Class with Peptide Separation Technology (PST) and Protein Separation Technology (PrST) UPLC® Chemistries
Xevo G2 ToF,
ACQUITY UPLC TUV
Optical Detector
UNIFI Scientific
Information System

Intact Protein LC/MS Conditions

Column: ACQUITY UPLC BEH300 C4,
2.1 x 50 mm
Column temp.: 80 °C
Mobile phase A: water
Mobile phase B: acetonitrile
Mobile phase C: 1% formic acid (aqueous)
Detection: UV 280 nm

<u>Time</u> (min)	<u>Flow</u> (mL/min)	<u>%A</u>	<u>%B</u>	<u>%C</u>	<u>%D</u>	<u>Curve</u>
Initial	0.3	85	5	10	0	Initial
2.00	0.3	85	5	10	0	6
2.10	0.2	85	5	10	0	6
5.00	0.2	10	80	10	0	6
6.00	0.3	10	80	10	0	6
6.50	0.3	85	5	10	0	6
10.00	0.3	85	5	10	0	6

MS Conditions

Capillary: 2.5 kV
Sampling cone: 50 V
Extraction cone: 4 V
Source temp.: 150 °C
Desolvation temp.: 350 °C
Cone gas flow: 0 L/Hr
Desolvation gas flow: 800 L/Hr

Partially Reduced Protein LC/MS Conditions

Column: ACQUITY UPLC BEH300
C4, 2.1 x 50 mm

Column temp.: 80 °C

Mobile phase A: water

Mobile phase B: acetonitrile

Mobile phase C: 1% formic acid (aqueous)

Detection: UV 280 nm

Flow rate: 0.2 mL/min

Mobile phase A: water

Mobile phase B: acetonitrile

Mobile phase C: 1% formic acid (aqueous)

Detection: UV 214 nm

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.3	85	5	10	0	Initial
2.00	0.3	85	5	10	0	6
2.10	0.2	85	5	10	0	6
3.00	0.2	65	25	10	0	6
13.00	0.2	60	30	10	0	6
13.10	0.3	10	80	10	0	6
15.00	0.3	10	80	10	0	6
15.50	0.3	85	5	10	0	6
25.00	0.3	85	5	10	0	6

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.2	89	1	10	0	Initial
10.00	0.2	82	8	10	0	6
85.00	0.2	61	29	10	0	6
90.00	0.2	50	40	10	0	6
91.00	0.2	10	80	10	0	6
94.00	0.2	10	80	10	0	6
95.00	0.2	89	1	10	0	6
105.00	0.2	89	1	10	0	6

MS Conditions

Capillary: 3.0 kV

Sampling cone: 30 V

Extraction cone: 4 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 700 L/Hr

MS Conditions

Capillary: 3 kV

Sampling cone: 30 V

Extraction cone: 4 V

Source temp.: 100 °C

Desolvation temp.: 250 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 500 L/Hr

Tryptic Digest LC/MS Conditions

Column: ACQUITY UPLC BEH300
C₁₈, 2.1 x 150 mm

Column temp.: 65 °C

Chymotryptic Digest LC/MS Conditions

Column: ACQUITY UPLC BEH300
 C₁₈, 2.1 x 150 mm

Column temp.: 60 °C

Mobile phase A: 0.1% formic acid (aqueous)

Mobile phase B: 0.1% formic acid
 in acetonitrile

Detection: UV 214 nm

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.2	97	3	0	0	Initial
1.00	0.2	97	3	0	0	6
91.00	0.2	57	43	0	0	6
91.10	0.2	25	75	0	0	6
94.10	0.2	25	75	0	0	6
95.00	0.2	97	3	0	0	6
98.00	0.2	97	3	0	0	6

MS Conditions

Capillary: 3 kV

Sampling cone: 30 V

Extraction cone: 4 V

Source temp.: 120 °C

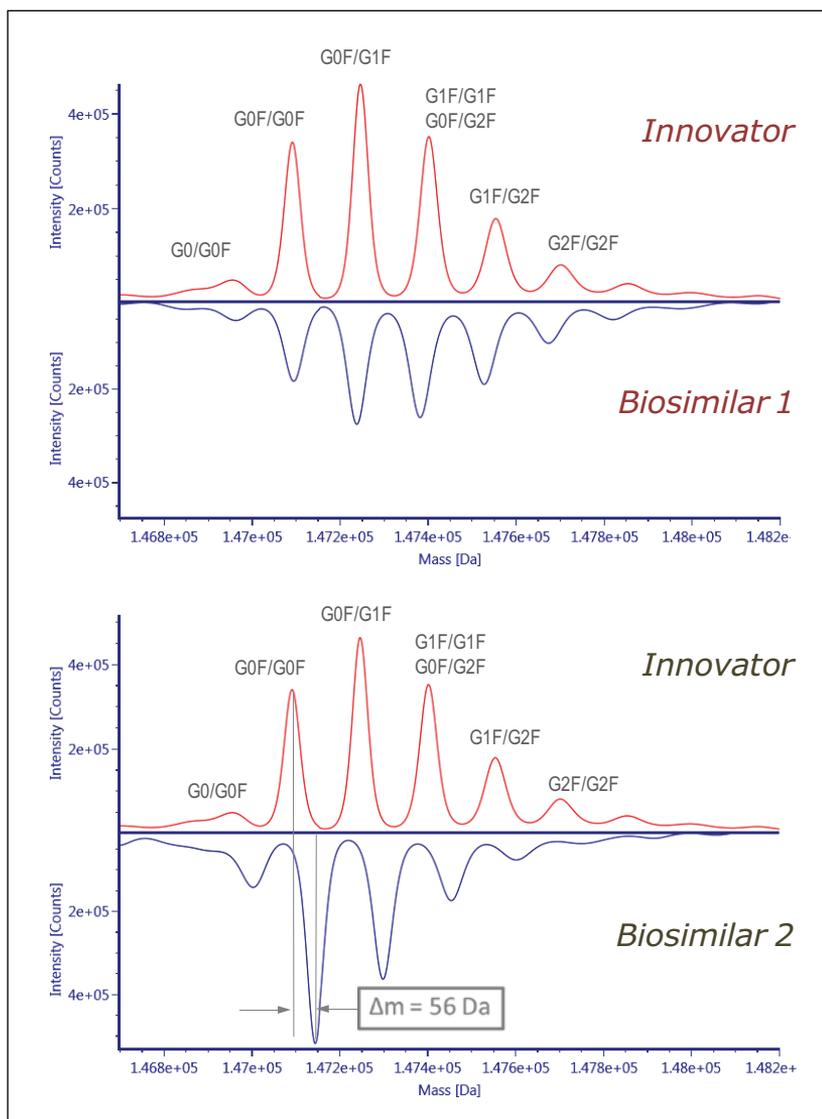
Desolvation temp.: 350 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 600 L/Hr

Workflow 1: Intact Protein MW Determination and Composition

For a quick assessment of the possible differences among the innovator, rituximab, and two biosimilar samples (Biosimilar 1 and Biosimilar 2), the intact protein mass analysis was performed. UNIFI has a built-in MaxEnt1 deconvolution capability for protein MW calculation and comparison. Figure 3 shows the distribution of glycoforms on the deconvoluted mAb spectra presented as the mirror plots. A systematic mass shift of 56 Da was observed in Biosimilar 2 glycoforms with respect to the innovator mAb; whereas, the Biosimilar 1 glycosylation profile displayed inconsistent mass difference (except G0F/G0F glycoform). The intact protein analysis data can also be viewed as raw, centroid spectra, or as a component summary, and can be used for the first-round evaluation of mAb sample heterogeneity.



Workflow 2: Reduction of mAb

A closer look at the reduced form of rituximab allowed users to confine the structure heterogeneity to the individual heavy or light protein chains. Partially reduced mAb analysis measured and compared PTM and glycosylation profile among the innovator and both biosimilar mAbs, as seen in Figure 4. Consistent with 56 Da mass shift observed from intact protein data, our data suggest that 28 Da mass difference, possibly an amino acid sequence variation, belongs to the heavy chain of Biosimilar 2. N-terminal pyroglutamination Q (PyrQ) levels were measured and reported for heavy and light chains. C-terminal Lys variants on the heavy chain as well as glycoform variants were automatically assigned in the UNIFI Review panel and plotted across all the samples of the innovator and biosimilars.

In comparison with the innovator, the obvious difference displayed in Biosimilar 1 in the deconvoluted HC spectrum is the higher degree of C-terminal Lys variation, which contributes to the inconsistent mass shift observed in the intact mass analysis. One of the remarkable differences between the two biosimilars was the relative abundance of G0 glycoform, which is known to correlate with antibody-dependent cellular cytotoxicity,¹ and is believed to affect drug safety and efficacy.

The summary plot tool allows users to select any observable data, such as response, mass error, retention time, etc., and trend it across all the injections, which is one of the UNIFI assets of the automatic and efficient data reviewing.

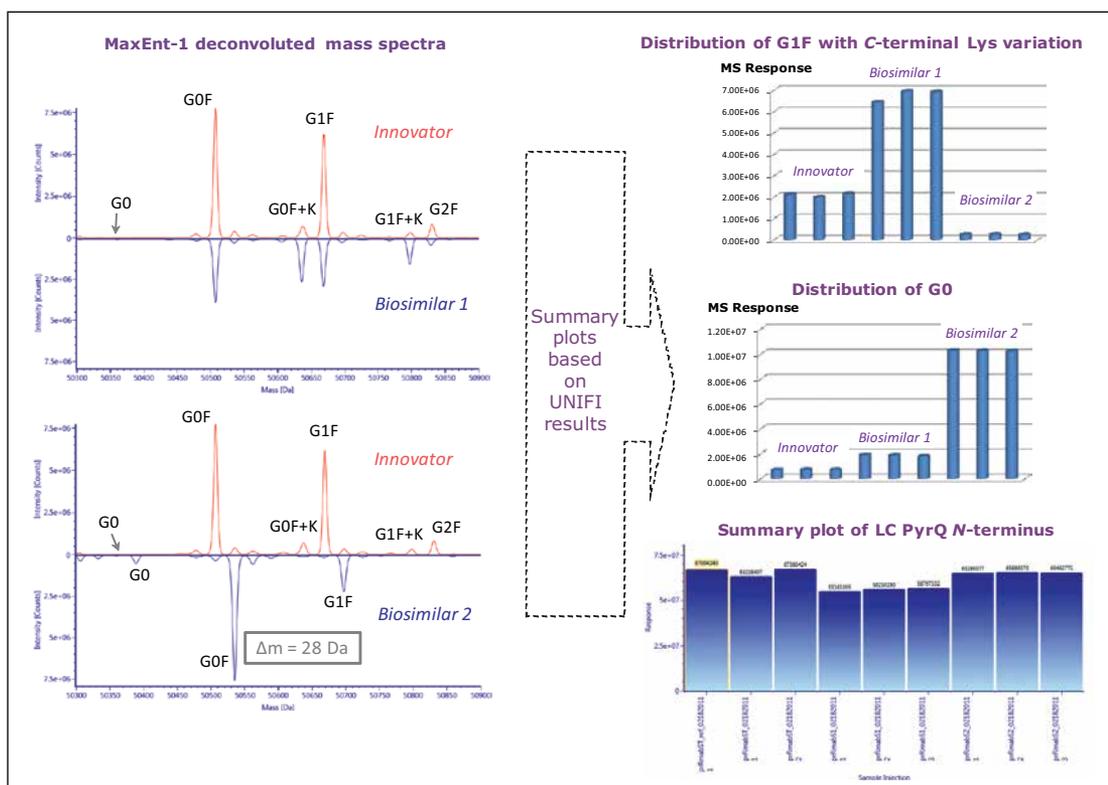


Figure 4. Partially reduced protein analysis. The following examples were measured and compared among three replicate injections of innovator and two biosimilar mAbs: C-terminal Lys variants on the HC, glycoform variant (G0) between biosimilars, amino acid sequence variant (+28 Da) on the heavy chain of Biosimilar 2, and N-terminal pyroglutamination Q (PyrQ) levels.

Workflow 3: Peptide Mapping

To localize the difference among the three mAb samples, peptide mapping data were collected. A mirror plot of the tryptic digest demonstrated C-terminal Lys variant exists only in the Biosimilar 1 peptide map, shown in Figure 5, which was consistent with the glycosylation profiling data at the reduced protein level.

The ultimate inquiry was localizing the amino acid mutation contributing to 28 Da mass shift of the Biosimilar 2. Based on published information,² an additional targeted sequence with Lys₂₁₈ → Arg₂₁₈ mutation was submitted to the method search. Compare mode view of the tryptic digest chromatogram, or component summary did not show a significant difference between the innovator and Biosimilar 2 mAb; therefore, no conclusion could be drawn about the primary sequence difference. The answer came with use of an alternative, non-specific enzyme, chymotrypsin. The chymotryptic map clearly showed the mass shift in a component view, as seen in Figure 6, and the peptide with a mutation site was automatically highlighted in the chromatogram, peptide map, and the component summary in the Review panel. Filtering the results in the Review by “showing unknown unique components” makes it easy to display the differences between the innovator (“reference”) and the biosimilar samples (“unknown”).

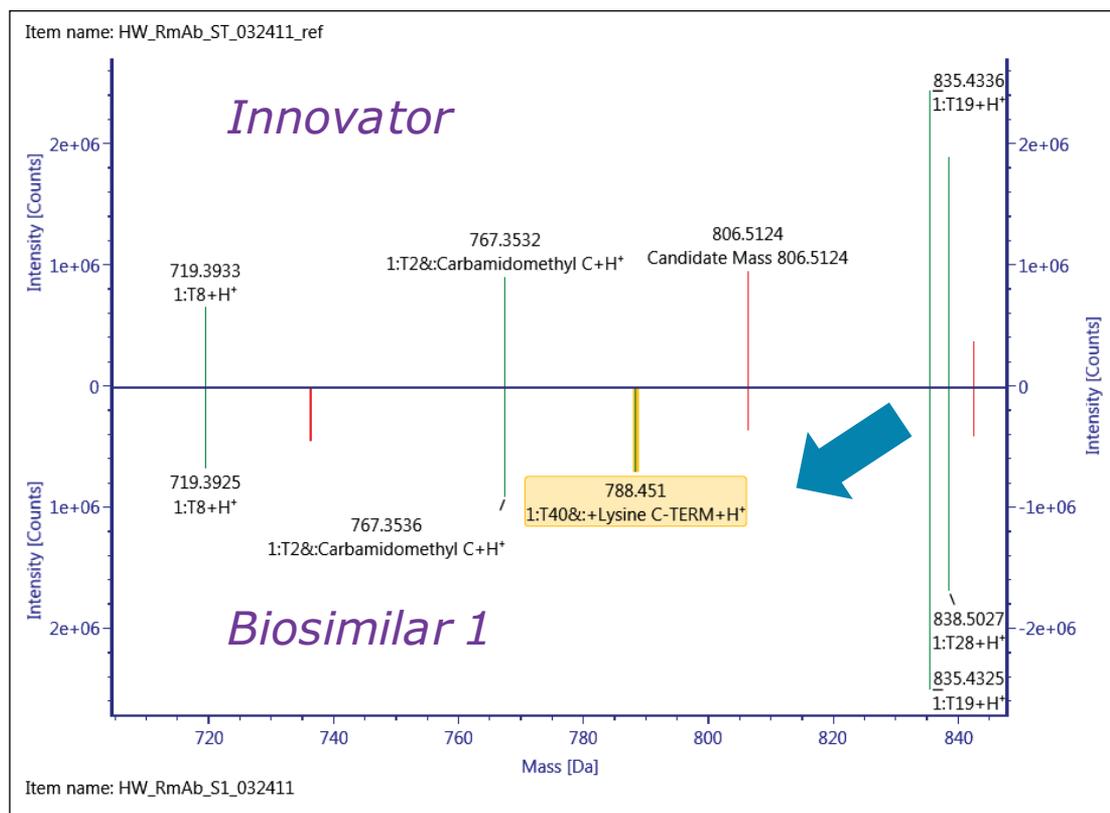


Figure 5. Tryptic digest comparison where components plot in compare mode. C-terminal Lys variant was observed only in the Biosimilar 1 peptide map.

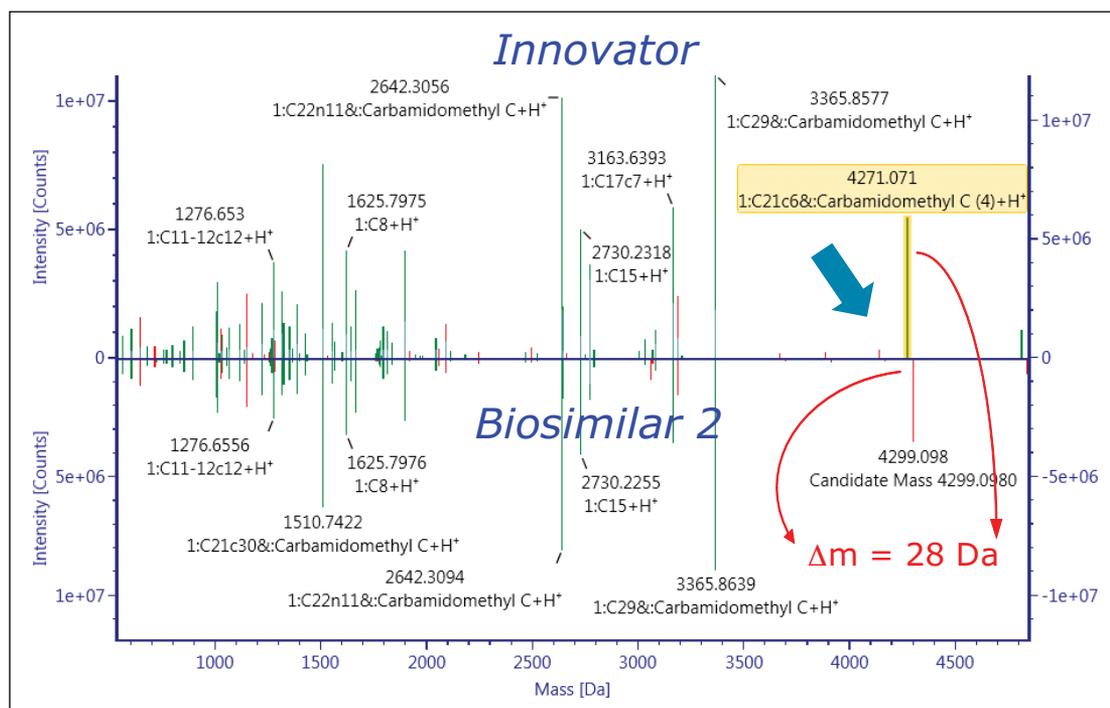


Figure 6. Chymotryptic peptide map analysis where components plot in compare mode, revealing 28 Da mass shift of the chymotryptic fragment in Biosimilar 2.

The reason that the tryptic map failed to pinpoint the amino acid substitution is that proteolytic cleavage occurs at Lys₂₁₇, Lys₂₁₈ or Arg₂₁₈. So, the very amino acid of question gets cleaved as a single amino acid entity. Chymotryptic digest, on the other hand, captures the mutation within a single peptide. Finally, Lys₂₁₈ → Arg₂₁₈ substitution was confirmed with MS^E data, as seen in Figure 7, which displayed a₁₆-ion fragment characteristic of Arg. UNIFI peptide map workflow proved the capability to confirm sequence mutation or other suspected PTMs.

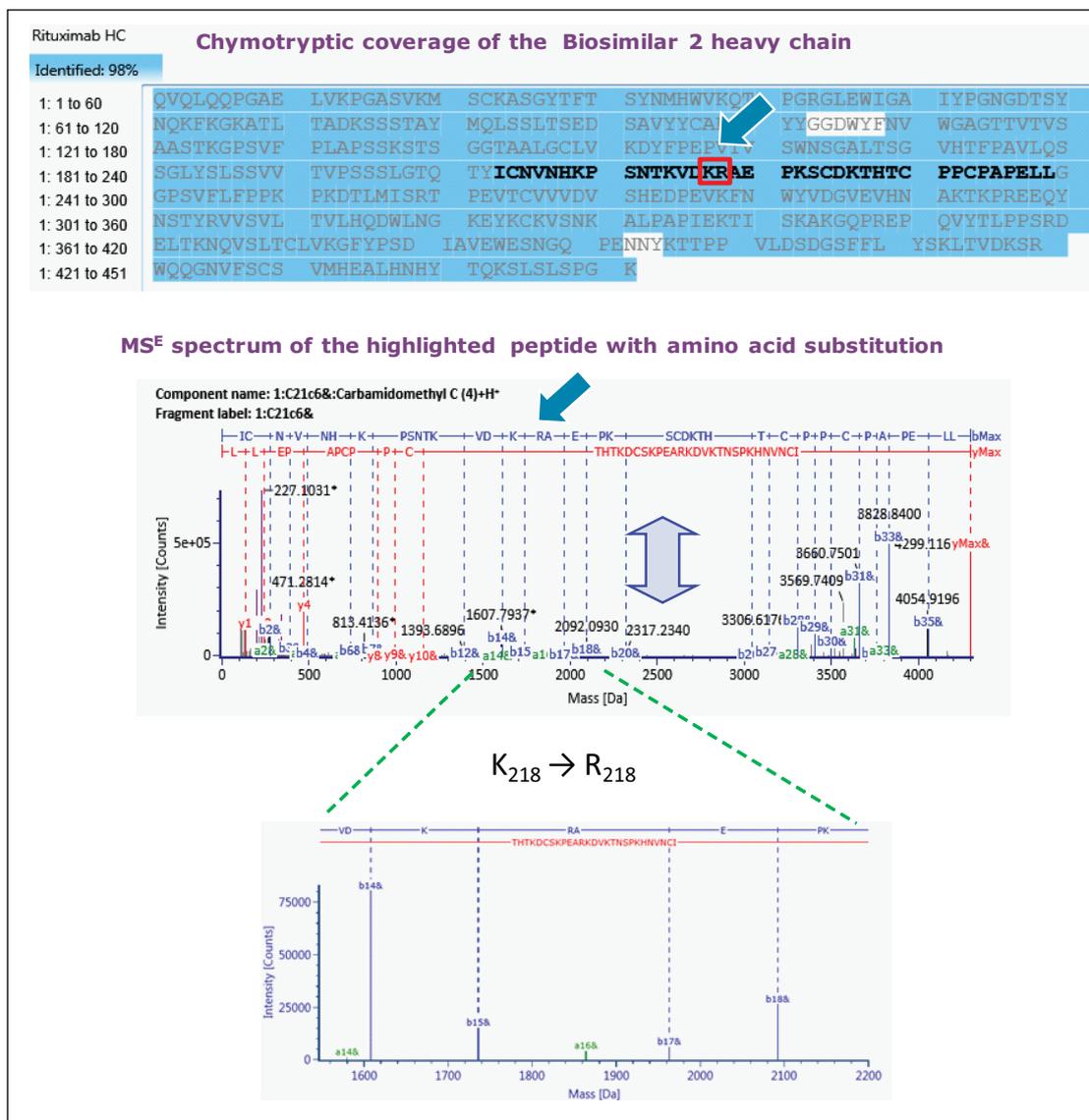


Figure 7. Chymotryptic digest analysis shows MS^E data confirm a single amino acid substitution (K → R) in Biosimilar 2.

CONCLUSIONS

UPLC/TOF MS analysis at intact mAb, reduced mAb, and peptide map levels enabled the detection of primary structural differences, and quantitative assessments of these variations. An integrated biopharmaceutical LC/MS system utilizing the UNIFI Scientific Information System with automated data acquisition, processing, and reporting for multiple analytical workflows enabled the efficient assessment of critical product attributes with minimal manual intervention.

The K → R mutation found in the Biosimilar 2 (of rituximab) study is not readily detectable under tryptic digest analysis. It demonstrates the need to routinely employ alternative digestion enzymes for product characterization. The integrated workflow of protein characterization at different levels, combined with intelligent methods and tools of UNIFI, will improve productivity and cut the cost of biosimilar drug development.

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2. Jefferis R, Lefranc MP. Human immunoglobulin allotypes. *mAbs*, 2009; 1 (4): 1-7.

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September 2012 720004445EN AG-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

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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Biopharmaceutical Platform Solution
with UNIFI®

ACQUITY UPLC® H-Class Bio System

Xevo® G2-S QTof

ACQUITY UPLC TUV Detector

UNIFI Scientific Information System

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 EMA⁵⁻⁸ 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. 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 TUV Detector
- UNIFI Scientific Information System

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

Intact protein LC/MS conditions

Column: ACQUITY UPLC BEH300 C4, 2.1 x 50 mm
 Column temperature: 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

Data acquisition and processing

MaxEnt1 for MS spectra deconvolution
 UNIFI Scientific Information System

Sample preparation

Three batches of innovator infliximab were acquired from Jenness Biotech, Inc. (Horsham, PA, USA). The batches were produced by the SP2/O 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 (21.0 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 BEH300 C4, 2.1 x 50 mm Column for 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 min. The chromatographic peaks eluting around 3.5 min 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.

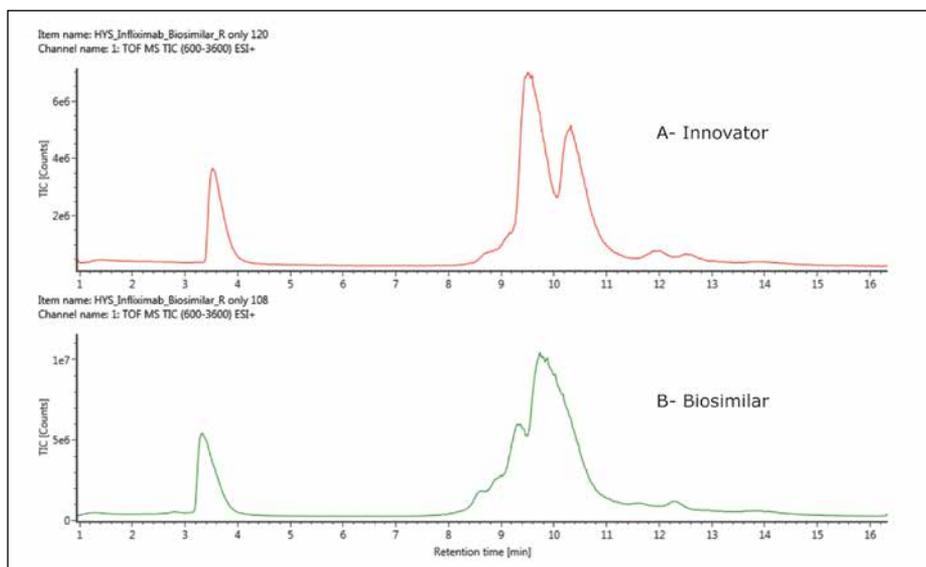


Figure 1. Reversed-phase (C4) chromatograms of the innovator infliximab (top) and a biosimilar infliximab (bottom). The signal trace is the Total Ion Current (TIC) from the mass spectrometer.

Figure 2 shows the comparison of the light chain spectra in a mirror plot using UNIFI Software, with the combined raw MS spectra shown on the left panel (as demonstrated by multiple charged spectrum envelopes) and the MaxEnt1 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 studies⁹ that show little or no post-translational modifications of LC subunits.

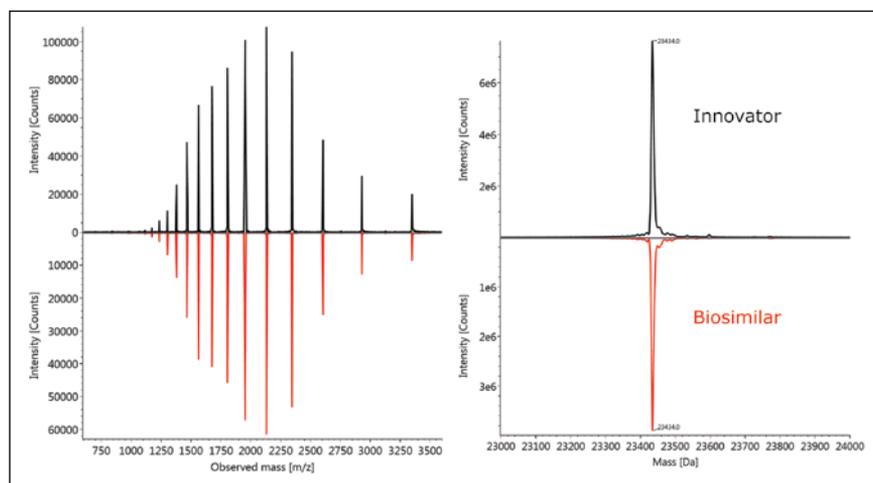


Figure 2. The combined raw MS spectra and deconvoluted spectra in mirror image plots. The MS spectra of the light chain (eluting around 3.5 min in Figure 1) from the innovator sample (top) and the biosimilar sample (bottom) are displayed.

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 min 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).

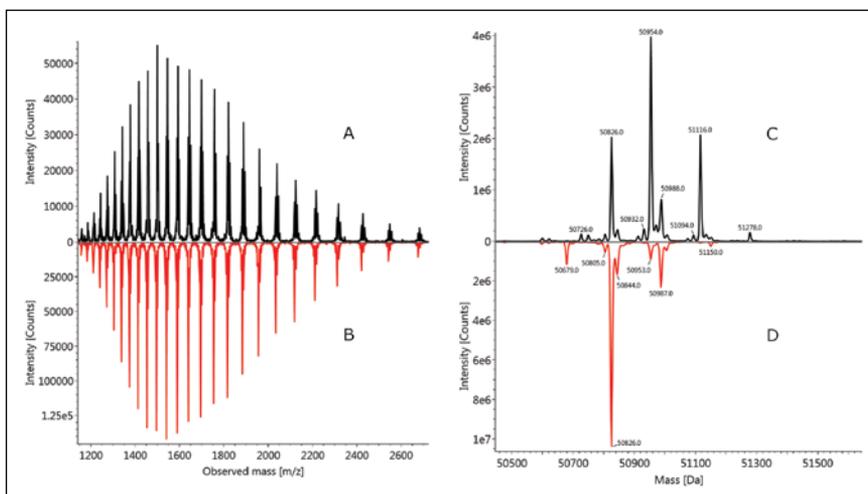


Figure 3. The combined raw MS spectra mirror image comparison between the heavy chains (eluted around 10 min in Figure 1) for the innovator and the biosimilar.

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 Software based on the mAb's reported sequence and the suspected PTM, and annotated, as displayed in Figure 4.

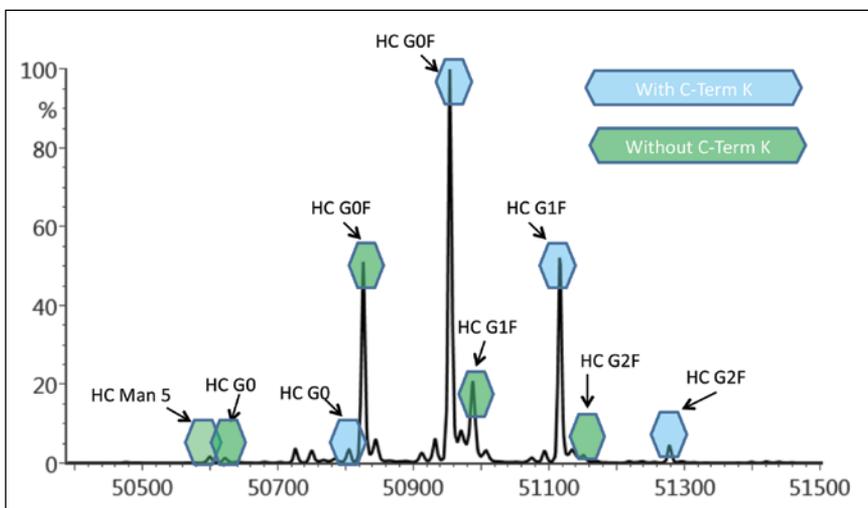


Figure 4. The innovator heavy chain deconvoluted spectra reveals the presence of glycoforms G0, G0F, G1F, G2F, and Man5 as well as lysine variations. Incomplete removal of lysine from the C-terminus of the HC is a known variant for IgG1.

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 MaxEnt1 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/O cells) as well as biosimilar products (infliximab 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 Waters 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 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 GO in percentage. This UNIFI plot offers a simple and direct view to demonstrate the variation in relative abundance of the GO 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 for other glycoforms identified in the analysis, such as GIF 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 GIF 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 GO glycoform compared to that of the innovator (SP2/O cell line) product. It is also observed that there are about twice as many GIF 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.

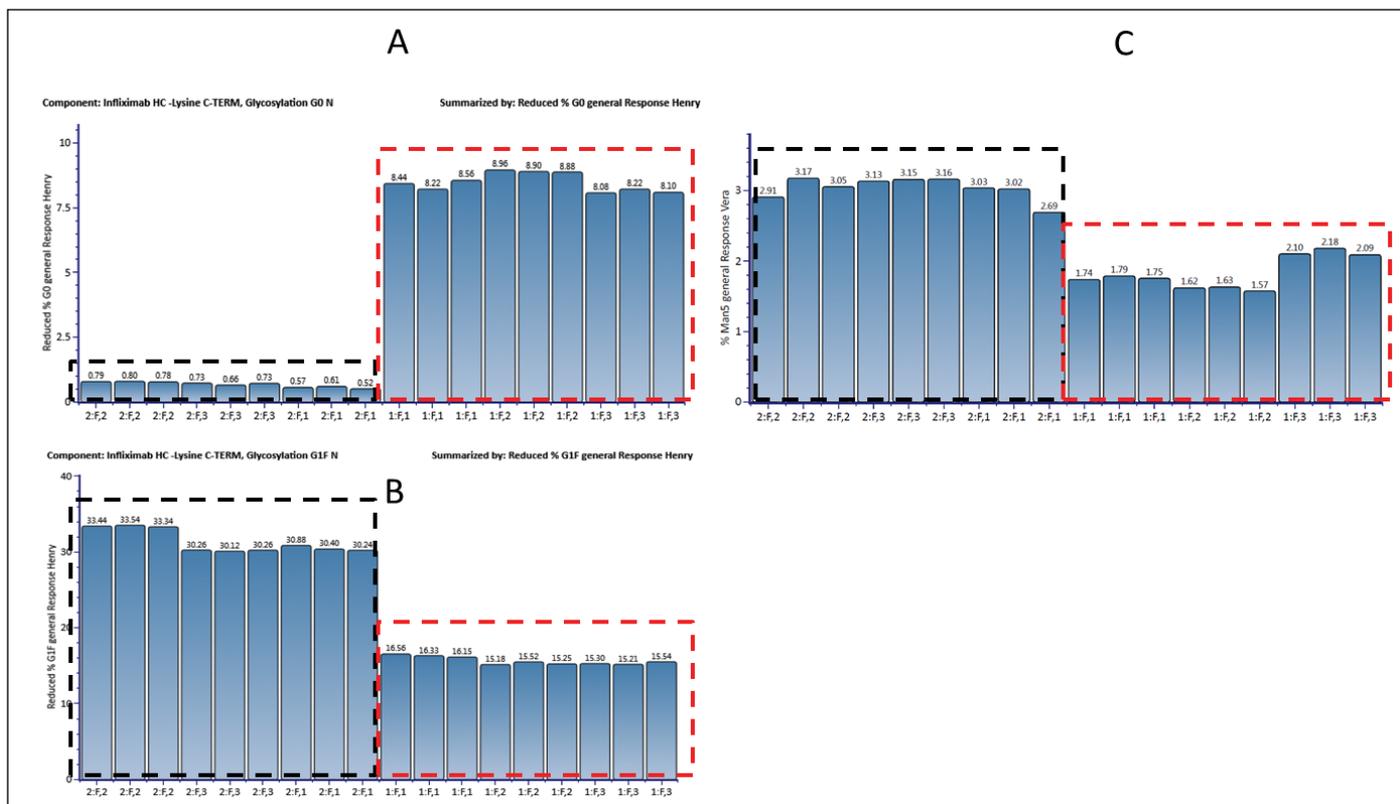


Figure 5. Relative abundance of the GO (A), GIF (B), and Man5 (C) glycoforms in infliximab HC, from all the injections of the innovator (left, blue) and biosimilar (right, Red) batches.

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 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 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/O 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.

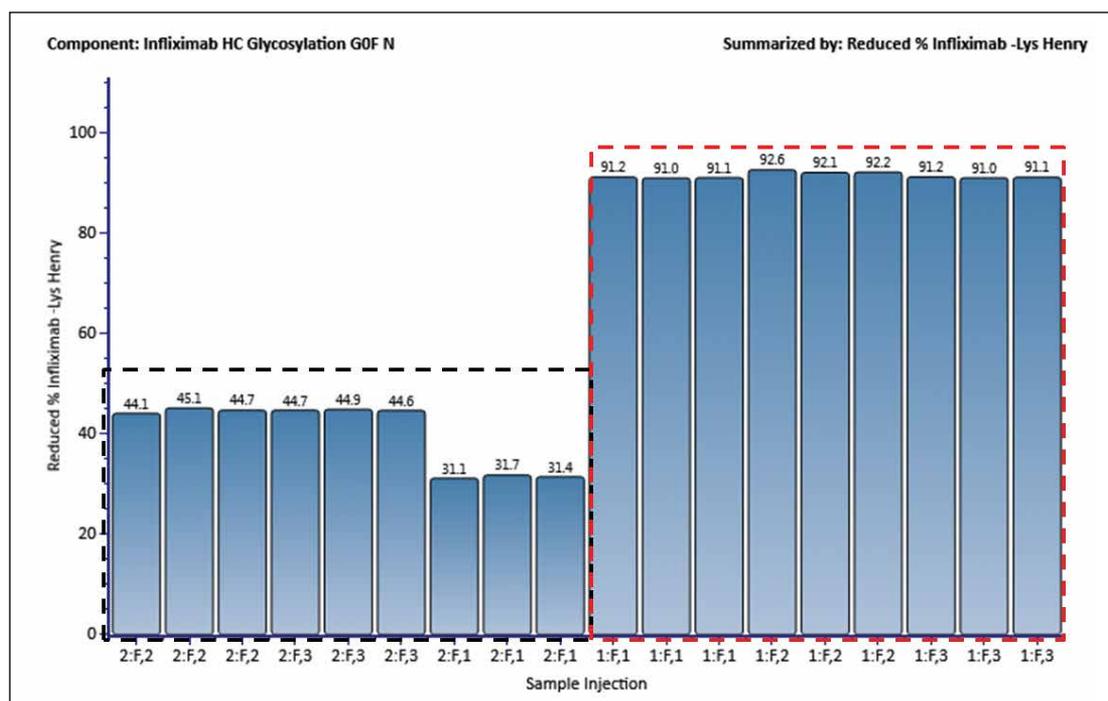


Figure 6. The percentage of clipped-lysine (OK) variants, automatically calculated in UNIFI, is shown for the innovator (left, blue) and biosimilar (right, red) batches.

CONCLUSION

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 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 GO, 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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October 2013 720004796EN AG-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

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.

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[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	Flow	%A	%B	%C	%D	Curve
(min)	(mL/min)					
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.

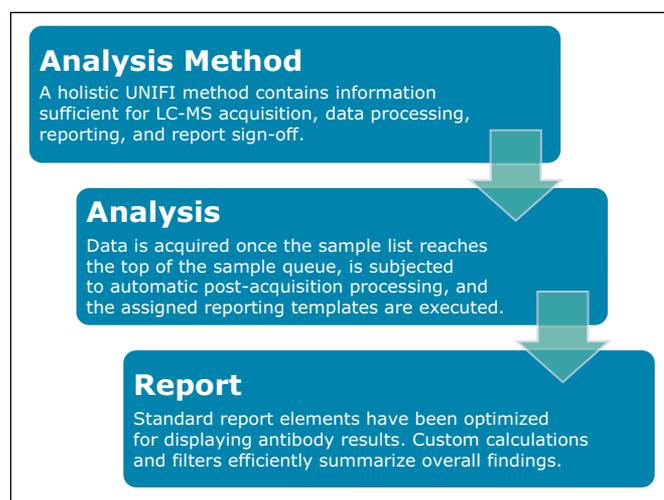


Figure 1. Intact mass analysis workflow with the UNIFI Scientific Information System.

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.

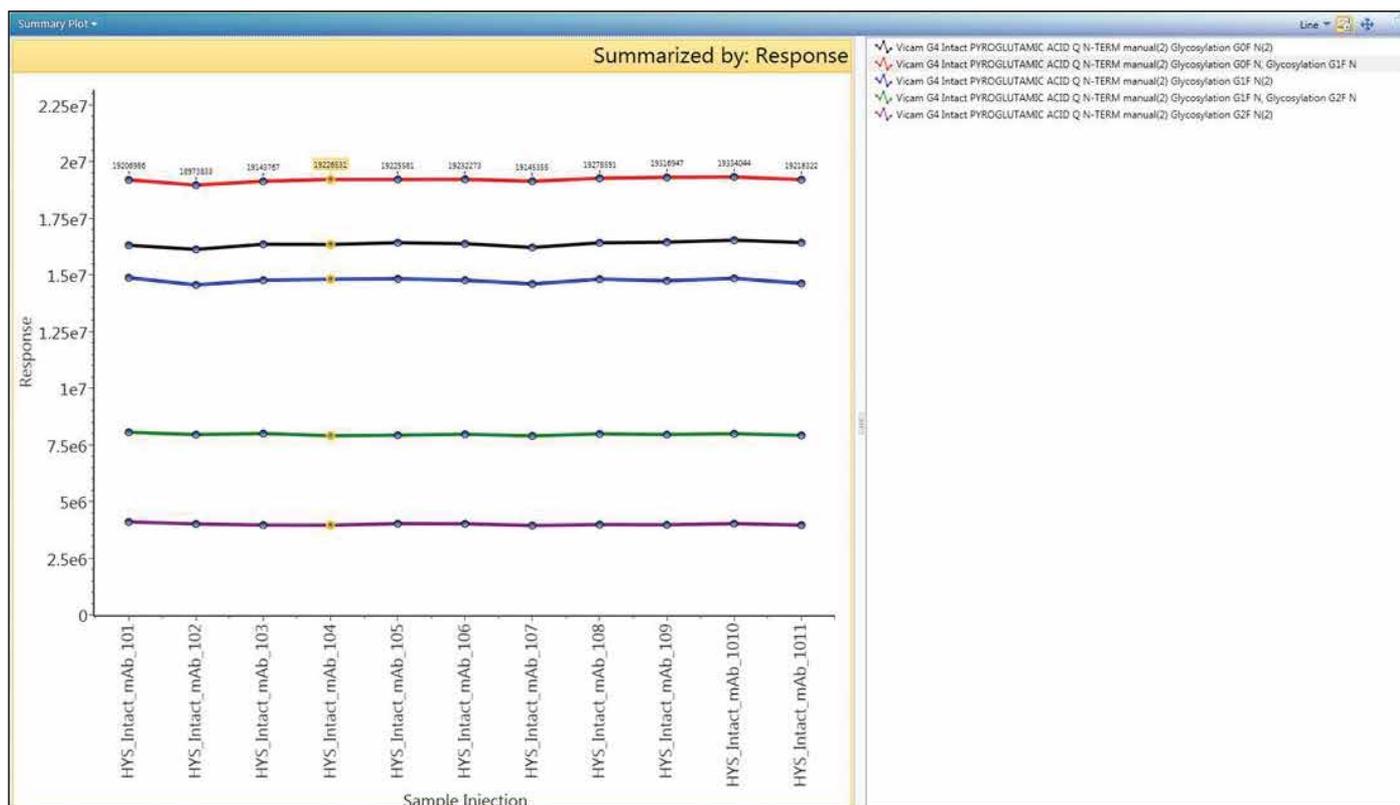


Figure 4. summary plot view of MS response for 5 mAb glycoforms (n = 11 injections).

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.

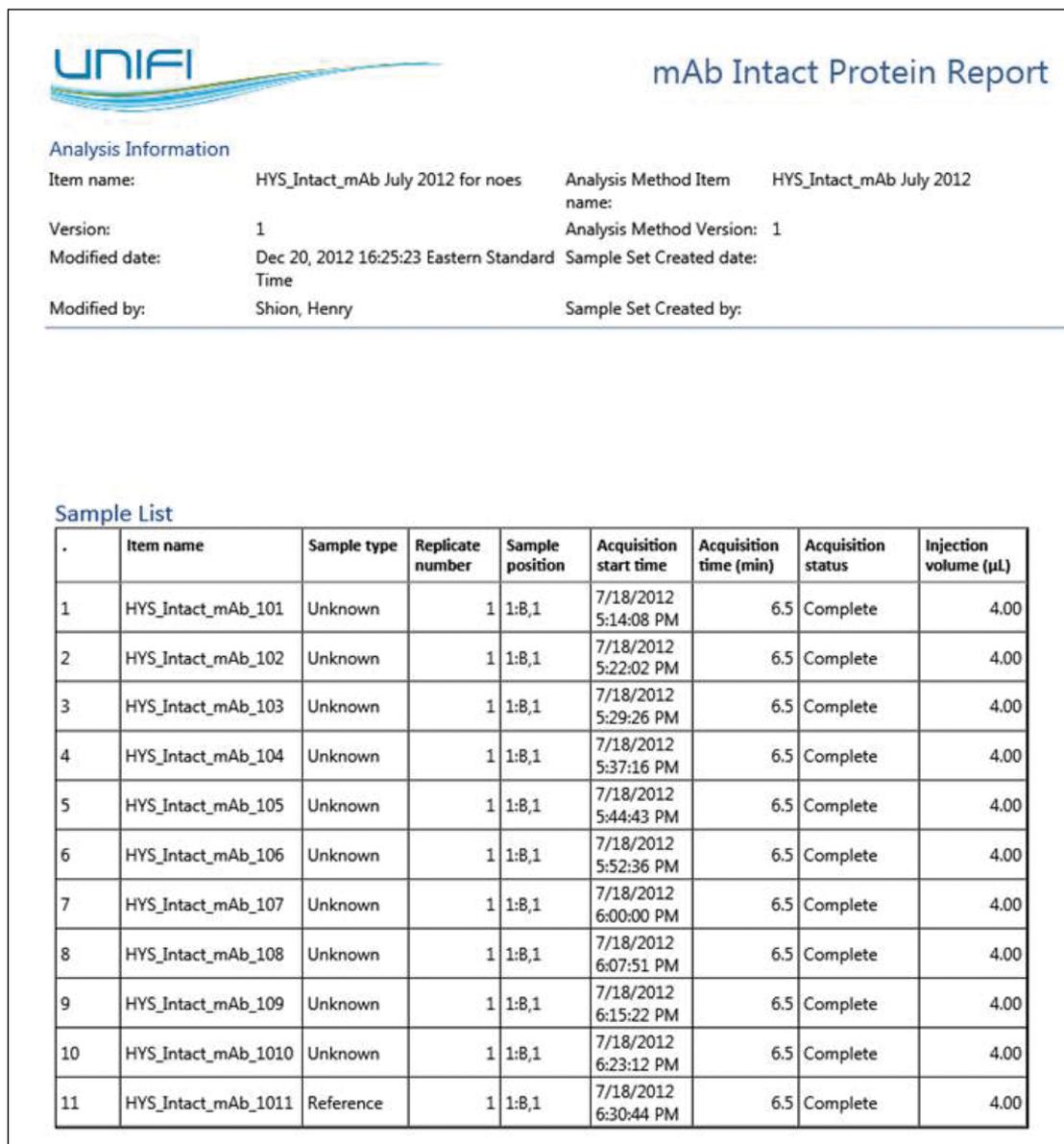


Figure 5. Typical first page of an intact protein LC-MS report in UNIFI Software that summarizes sample and acquisition details.

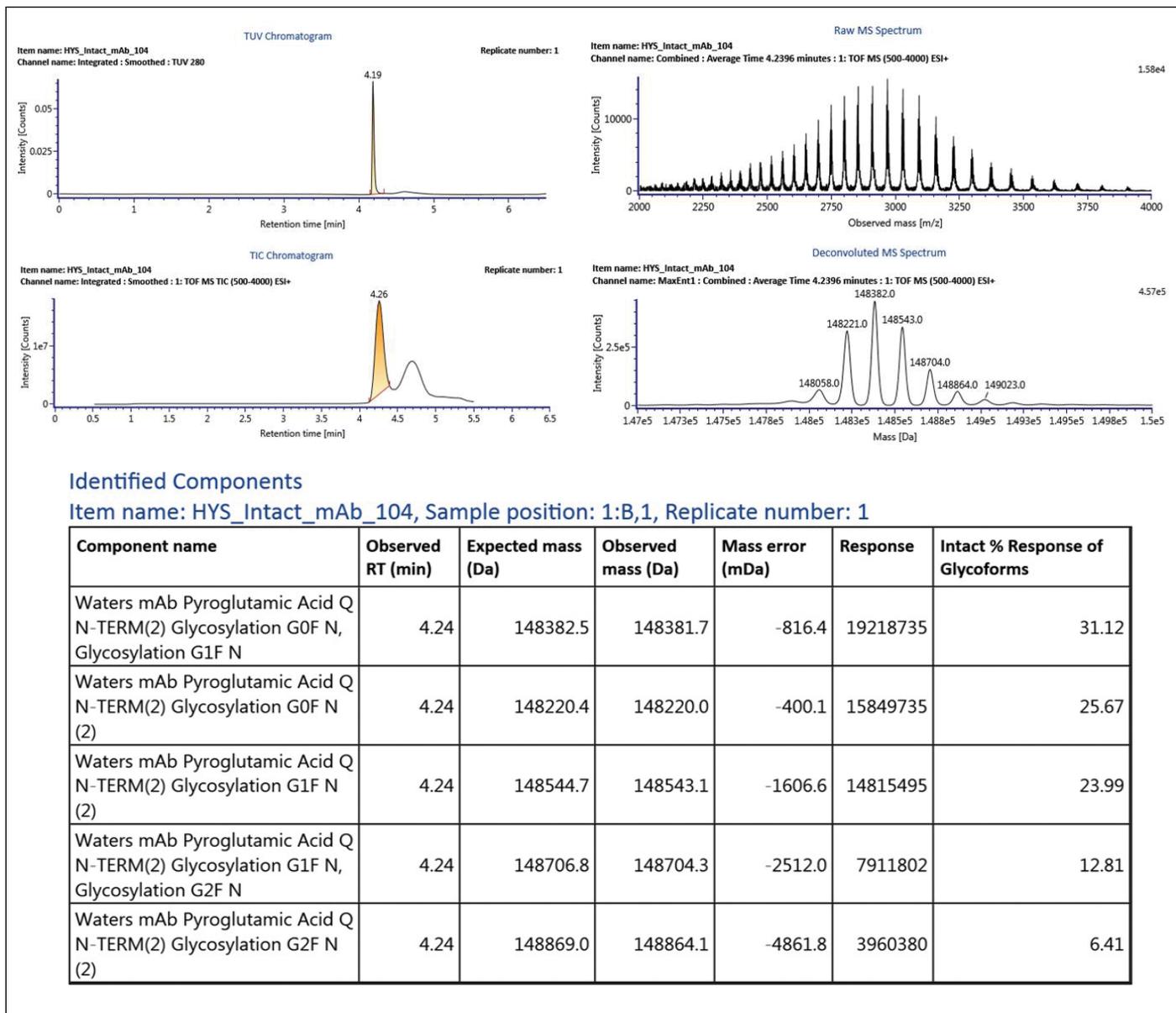


Figure 6. Example report object grouping (TUV and TIC chromatograms, raw and deconvoluted MS spectra, and identified component response summary table) from a single injection within the analysis.

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.

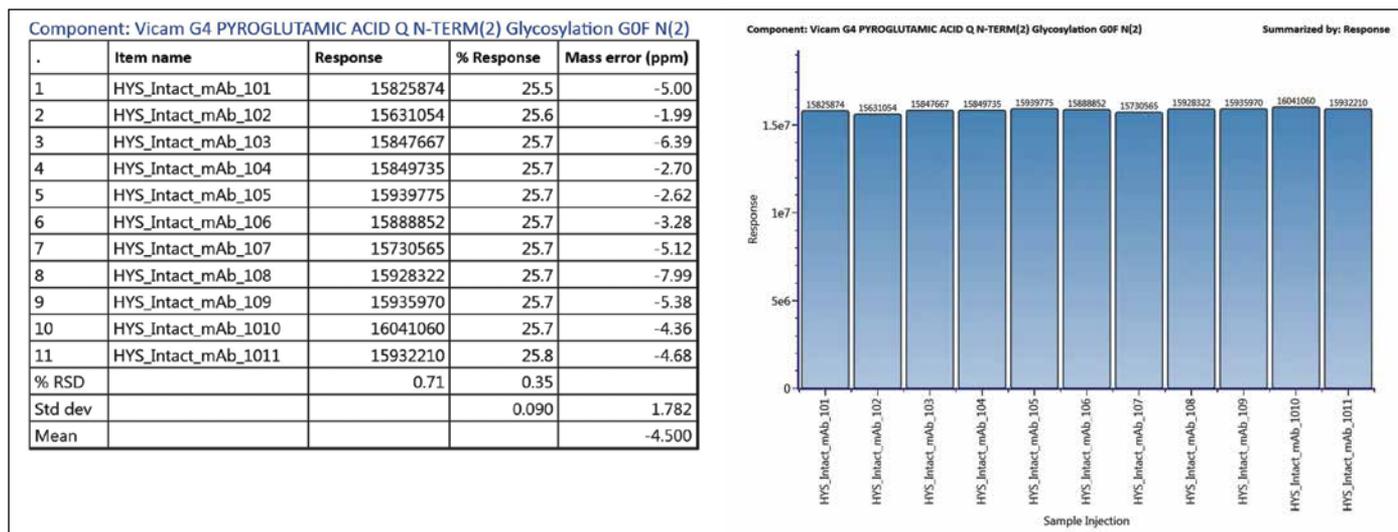


Figure 7. The report object summarizes MS response and mass accuracy/precision across all injections within the sample set in table and bar chart formats.

CONCLUSIONS

The intact mass analysis workflows within the Biopharmaceutical 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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Waters Corporation
 34 Maple Street
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Routine LC-MS Analysis of Intact Antibodies

Henry Shion
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

This application note describes in detail the routine LC-MS analysis of intact antibodies using the Waters® mAb Mass Check Standard. This document can serve as a reference for system qualification or as a troubleshooting guide for routine LC-MS intact mass analysis when using the mAb Mass Check Standard on the Xevo® G2-XS QToF Mass Spectrometer.

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[Intact mAb Mass Check Standard](#)

KEY WORDS

Antibodies, mAb, intact mass analysis, ACQUITY UPLC H-Class Bio, Xevo G2 QToF MS, Xevo G2-S QToF MS, Xevo G2-XS QToF MS, MaxEnt™ 1, deconvolution, Biopharmaceutical Platform Solution with UNIFI

INTRODUCTION

Intact mass analysis of monoclonal antibodies (mAbs) via LC-MS methods can be accomplished in minutes. It is typically used to confirm the mass of the target mAb, and can also be used to profile product-related variants such as glycosylations.

This application note offers experimental details for routine LC-MS analysis of mAbs. The Xevo G2-XS QToF System was used for data collection. An antibody reference compound, the Intact mAb Mass Check Standard was used as a test sample to illustrate the process.

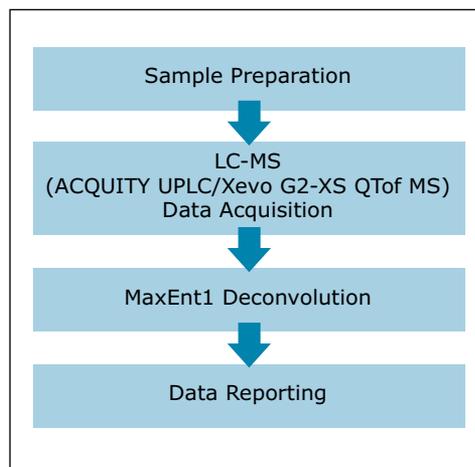


Figure 1. Workflow of Routine LC-MS Intact Mass Analysis.

EXPERIMENTAL

Sample preparation

Reference Standard: The Intact mAb Mass Check Standard was used for this application.

- Xevo G2 QTof MS

Add 100 μL of DI water to the Max Recovery Vial that contains 1 mg of the mAb standard. Sonicate for 5 minutes. The mAb concentration of this solution is 10 mg/mL or 67 μM . Dilute the solution 20 fold with Eluent A* of the LC mobile phase to prepare a solution containing 3.3 μM (0.5 $\mu\text{g}/\mu\text{L}$) mAb.

- Xevo G2-S QTof MS and Xevo G2-XS QTof MS

Add 100 μL of DI water to the Max Recovery Vial that contains 1 mg of the mAb standard. Sonicate for 5 minutes. The mAb concentration of this solution will be 10 mg/mL or 67 μM . Dilute this solution 100 fold with Eluent A* of the LC mobile phase to prepare a solution containing 0.67 μM mAb (0.1 $\mu\text{g}/\mu\text{L}$) mAb.

- 50 mM ammonium bicarbonate or ammonium acetate can be used as an alternative diluent instead of DI water.

LC-MS system

ACQUITY UPLC H-Class Variants

TUV optical detector*

Xevo G2 QTof, Xevo G2-S QTof, or Xevo G2-XS QTof, MassLynx,[®] MaxEnt1, and or UNIFI Scientific Information System

**It is not recommended to have a PDA optical detector inline for intact mass analysis. For optical detection needs, a TUV detector is recommended.*

UPLC conditions

Column:	ACQUITY UPLC BEH C4, 300Å, 2.1 mm x 50 mm Column
Column temp.:	80 °C
Mobile phase A:	0.1% formic acid in water*
Mobile phase B:	0.1% formic acid in acetonitrile*
Detection:	UV 280 nm

Gradient table:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.40	95.0	5.0	Initial
1.00	0.40	95.0	5.0	6
1.01	0.20	95.0	5.0	6
3.50	0.20	5.0	95.0	6
3.70	0.40	5.0	95.0	6
4.00	0.40	95.0	5.0	6
4.50	0.40	5.0	95.0	6
5.00	0.40	95.0	5.0	6
5.50	0.40	95.0	5.0	6

Total run time: 6.5 min, injection volume 5 μL *

**LC-MS grade water, acetonitrile and formic acid are highly recommended for mobile phase preparations. Injection volume could be 10 μL as well.*

**Recommended run time is 6.5 min.*

MS conditions*

Capillary:	3 kV
Sampling cone:	150V
Source offset:	80 V
Source temp.:	150 °C
Desolvation temp.:	500 °C
Cone gas flow:	0 L/Hr
Desolvation gas flow:	800 L/Hr
Data acquisition mass range:	$m/z = 500$ to 4000 amu
Suitable lockmass profile:	Glu Fibrinopeptide B (or other, if preferred)
Vacuum read back:	ToF vacuum is very important to intact mass analysis. A better vacuum will help to generate higher quality data.

**Please note that for different proteins (either varied by structure, or molecular weight), MS parameters may be changed to get better MS spectra quality. Here are the MS settings that can be adjusted within:*

Capillary: 1.5 to 3.5 KV, Sampling cone: 120 to 195V, source offset: 80 to 120 V, source temperature: 135 to 150 °C, desolvation temperature: 350 to 550 °C, cone gas flow: 0 to 100 L/Hr, and desolvation gas flow: 600 to 1000 L/Hr.

RESULTS AND DISCUSSION

Routine LC-MS analysis for intact antibodies was conducted using a fast and simple desalting method described above. This section shows typical experiment results and discusses practices for achieving optimal results.

TIC and TUV Chromatograms

Figure 2 shows the typical TUV and TIC chromatograms for the Intact Mass Check Standard.

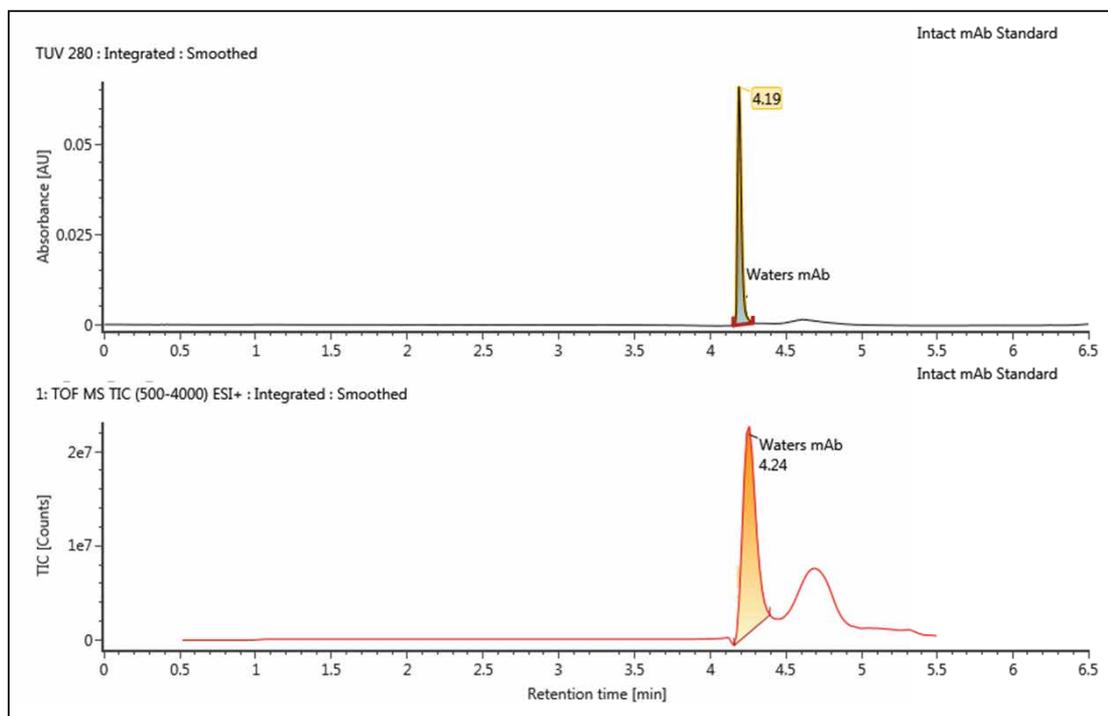


Figure 2. Typical TIC and TUV chromatograms (screen capture from UNIFI) from a routine LC-MS intact mass analysis.

Chemical background signals

Less than 1,000 ion counts (1 second scan with the mass range of $m/z = 500$ to 4000) should be expected for background ion signal intensity, as shown in Figure 3.

A high chemical background signal will affect the raw spectrum quality (spectrum resolution and mass accuracy) in intact mass analysis. A high chemical background signal is very often attributed to contaminations from the LC system. The most commonly observed contaminants are polymers (e.g. polyethylene glycol (PEG) or PEG related materials). Residual TFA used as mobile phase additive from previous analysis could also interfere with new analysis that uses different mobile phase additive (such as formic acid).

Figure 4 shows what PEG polymer peaks look like in the mass spectrum. Figure 5 shows an example of the TFA adduct ion series (TFA+Na with 136 Da mass differences among the peaks) as the background contamination peaks.

If the LC system is deemed contaminated, the LC system must be cleaned. A cleanup procedure is included in Appendix A.

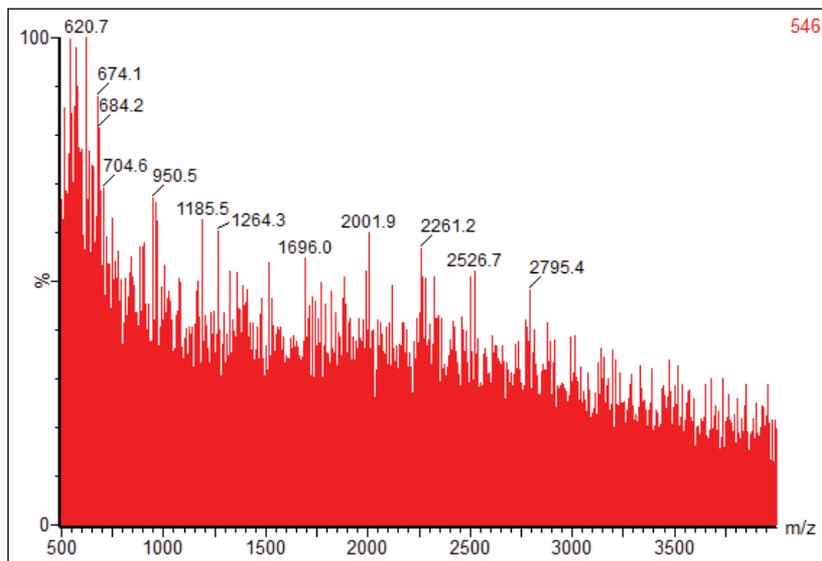


Figure 3. Normal single scan MS spectrum chemical background signal level.

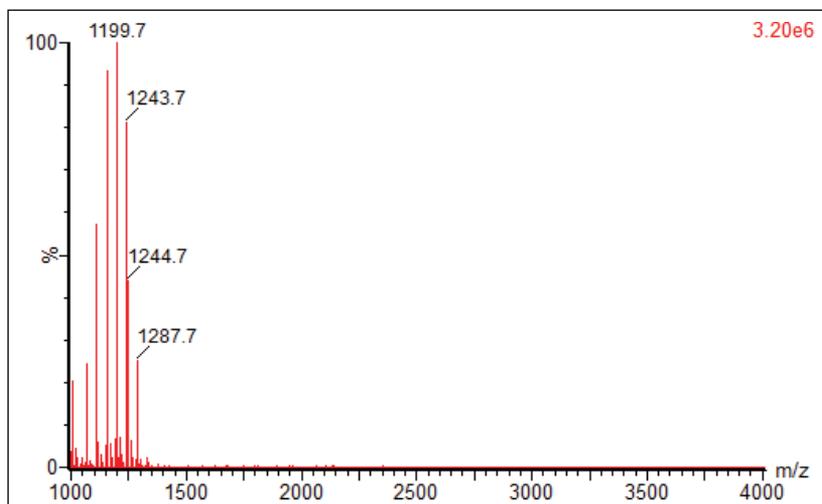


Figure 4. Example of PEG (+44 Da mass differences between the peaks) contaminations.

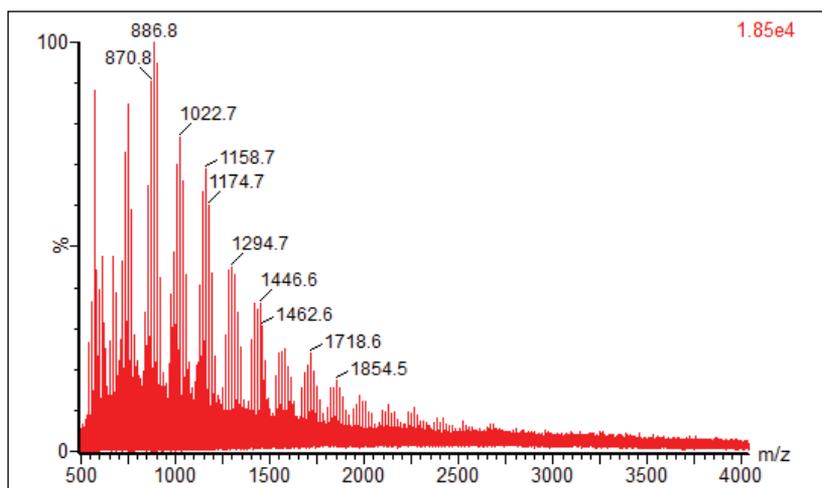


Figure 5. Example of TFA (+136 Da mass differences between the peaks) contaminations.

Raw spectrum

Figure 6 shows a typical example of a high quality raw spectrum (combined data, showing m/z range from 1900 to 4000 amu). The peak valley (as shown in Figure 7) between the first and the second glycoforms should be roughly 30% of the base peak height. Figure 7 shows a spectrum with the zoomed mass region, which shows the valley between each glycoform is approximately 27% of the base peak. This meets the criteria of the quality standard. The quality of the deconvoluted spectra, the measured mass accuracy of the glycoforms as well as their peak intensity is greatly improved by having a low valley between glycoforms in the raw spectrum.

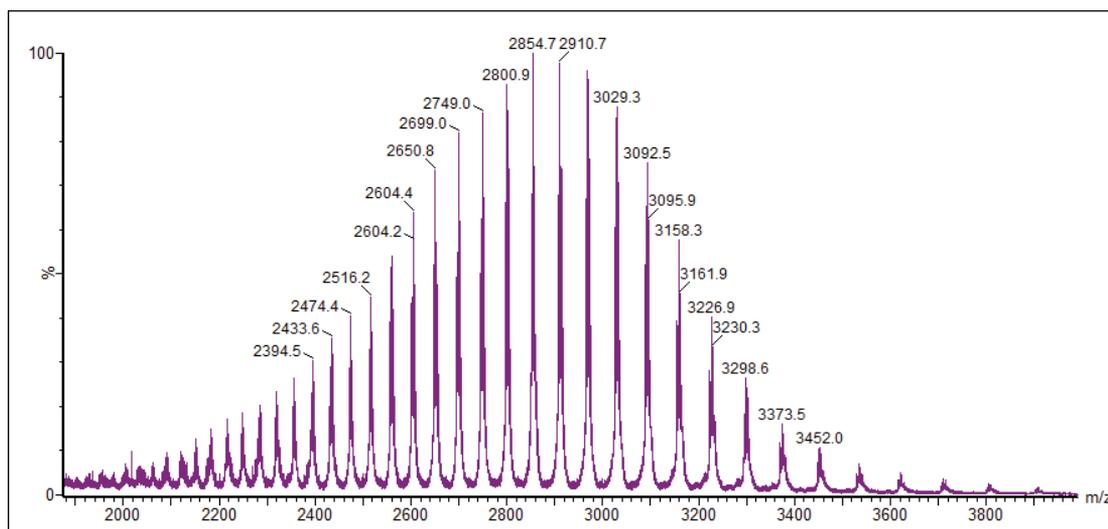


Figure 6. Waters mAb standard combined raw spectrum.

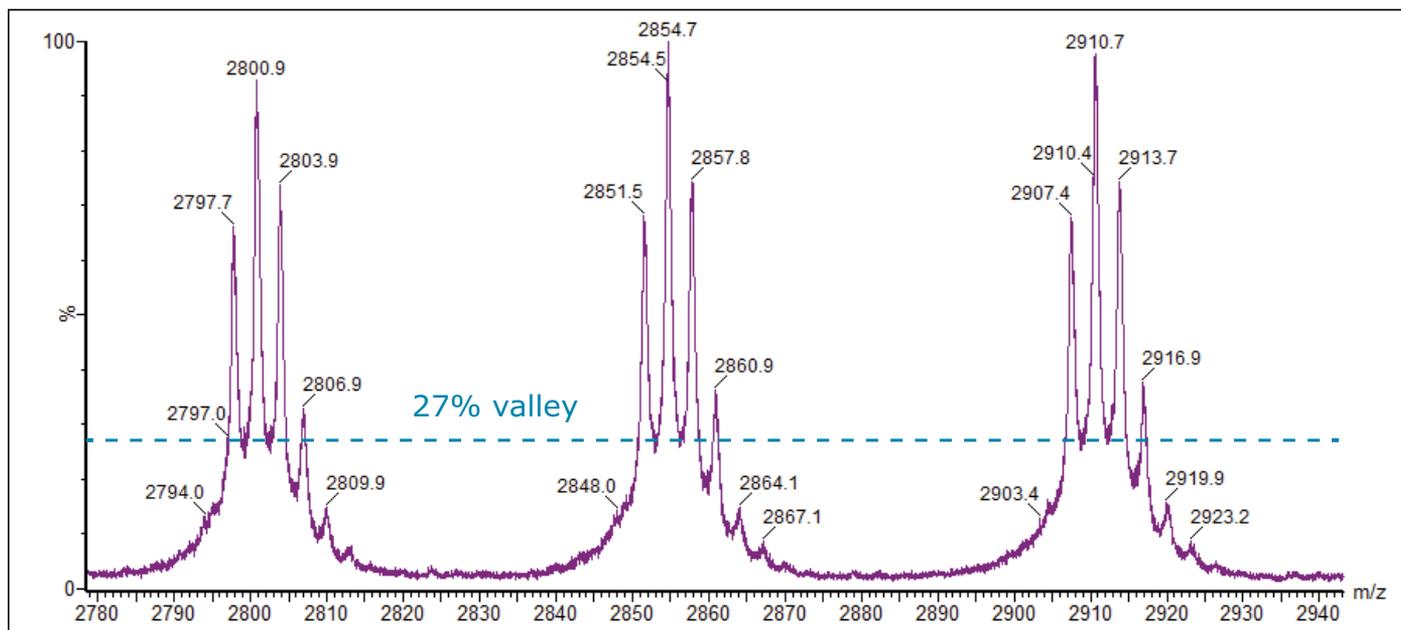


Figure 7. Waters mAb standard combined raw spectrum, zoomed in region.

Deconvolute combined raw spectrum using MaxEnt1

Figure 8 shows the MaxEnt1 settings in MassLynx to deconvolute combined raw spectrum (similar settings can be used in UNIFI software for deconvolution).

When deconvoluting raw intact mass data by MaxEnt1, the peak width setting at half height and the number of iterations have the greatest impact on the final quality of the deconvoluted spectrum. Figure 9 shows the deconvolution spectrum from the raw spectrum in Figure 6.

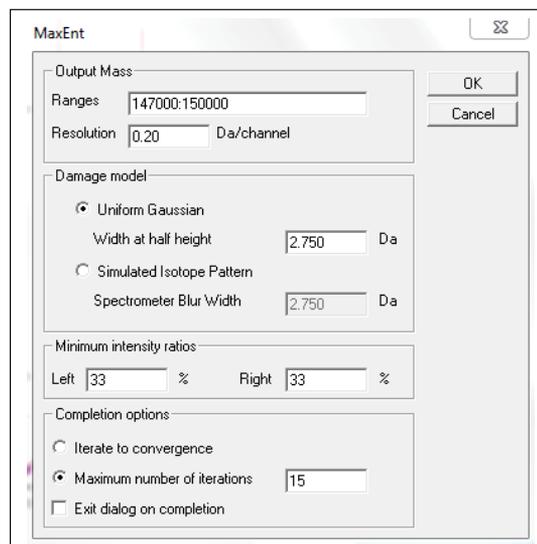


Figure 8. Recommended MaxEnt1 parameter settings were used to deconvolute combined raw spectrum in MassLynx (similar settings can be used in UNIFI Software).

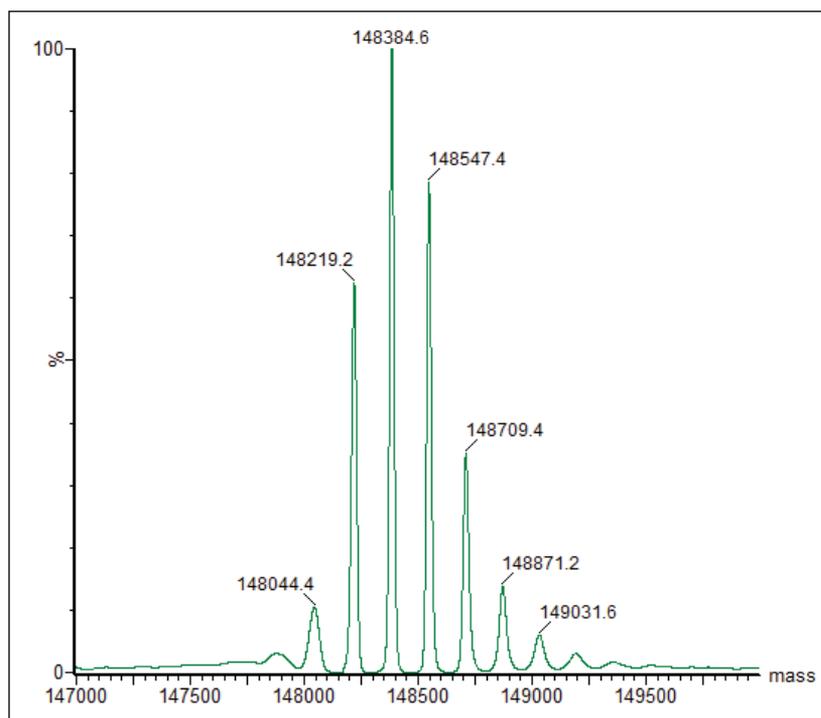


Figure 9. Deconvoluted spectrum, resulted from MaxEnt1 parameter settings as displayed in Figure 8.

The expected masses for the five major glycoforms are listed in Table 1.

The mass accuracy for the 5 major glycoforms is typically less than 50 ppm. Better mass accuracy (5 to 20 ppm) can be achieved with high quality mass spectrum, which is achievable following the recommendations given in this application note.

Calculated MW of the Major Glycoforms of the Waters mAb		
Peak number	mAb Glycoform	Expected mass (Da)*
1	M+GOF+GOF	148220.4
2	M+GOF+G1F	148382.5
3	M+G1F+G1F	148544.7
4	M+G1F+G2F	148706.8
5	M+G2F+G2F	148869.0

*expected mass (Da) was calculated in UNIFI.

Table 1. Expected average masses of the five major glycoforms (from the Mass Calculator tool in UNIFI).

Relative abundance of the major glycoforms

Deconvoluted MS ion counts are widely used for glycoprofiling for intact mAbs. The relative abundance for this particular sample batch is shown in Table 2 (calculated automatically in UNIFI):

Relative Abundance of the Major Glycoforms of the Waters mAb		
Peak Number	mAb Glycoform	Relative Abundance %
1	M+G0F+G0F	25.65
2	M+G0F+G1F	30.99
3	M+G1F+G1F	23.92
4	M+G1F+G2F	12.98
5	M+G2F+G2F	6.45

Table 2. Calculated relative abundance of the five major glycoforms (processed and calculated in UNIFI).

Beyond the information that is provided in this application note, additional information on LC-MS analysis for intact proteins using the Biopharmaceutical Platform Solution with UNIFI can be found in the Waters application note listed under References.

CONCLUSIONS

LC-MS intact mass analysis of antibodies becomes a routine and high throughput analysis in light of the advancements made in LC-MS technologies and informatics. Using the provided LC-MS experimental conditions as well as data processing settings, high quality intact mAb data can be obtained routinely for mAb identification and relative glycoform quantitation.

References

1. Shion, H. and Chen, W. Streamlining Compliant and Non-Compliant Intact Mass Analysis of Biotherapeutic mAbs with the Biopharmaceutical Platform Solution with UNIFI. 2013 Waters Application Note. 2013 ([P/N 720004617EN](#)).
2. Intact mAb Mass Check Standard Product Solution Brochure. Waters Brochure. 2013 ([P/N 720004610EN](#)).
3. Intact mAb Mass Check Standard Care and Use Manual. Waters Brochure. 2013 ([P/N 720004420EN](#)).

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Appendix A

The ACQUITY UPLC System wash protocol*

1. Replace column (if there was one) with a clean union.
2. Place all of the mobile phase channel tubing into wash solvent** bottle number one (Bottle 1), which should contain 50/50, H₂O/Methanol (wash solution number one). Run blank (H₂O) injection every 3 min, with LC flow change (step or linear flow gradient) from 0.1 mL/min to 0.5 mL/min, for 4 hours.
3. Move all of the mobile phase channel tubing into wash solvent bottle number two (Bottle 2), which should contain 100% IPA (wash solution number two). Run blank (H₂O) injection every 20 min, with LC flow change (step or linear flow gradient) from 0.1 mL/min to 0.5 mL/min, for 4 hours.
4. Move all of the mobile phase channel tubing into wash solvent bottle number one (Bottle 1), which should contain 50/50 H₂O/Methanol (wash solution number one). Run blank (H₂O) injection every 20 min., with LC flow change (step or linear flow gradient) from 0.1 mL/min to 0.5 mL/min, for 2 hours.
5. Move all of the mobile phase channel tubing into wash solvent bottle number three (Bottle 3), which should contain 100% H₂O (wash solution number three). Run blank (H₂O) injection every 10 min, with LC flow change (step or linear flow gradient) from 0.1 mL/min to 0.5 mL/min, for 4 hours.

Solvent Bottle Table		
Bottle 1	Bottle 2	Bottle 3
50/50=H ₂ O/MeoH	100% IPA	100% H ₂ O

Appendix Table 1. Solvent bottle contents.

*This wash protocol can be used to clean up contaminants, such as TFA and PEGs, in Waters ACQUITY UPLC systems.

**Please notice all the solvents used in the protocol should be LC-MS compatible.

Development of Integrated Informatics Workflows for the Automated Assessment of Comparability for Antibody Drug Conjugates (ADCs) Using LC-UV and LC-UV/MS

Henry Shion, Robert Birdsall, Steve Cubbedge, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

We demonstrate an integrated informatics workflow using UNIFI® Software that streamlines data acquisition, processing, and reporting for ADC analysis by LC-UV or LC-MS, so that CQAs information, such as average DAR and drug load distribution for multiple batches of ADCs, from multiple analytical techniques can be effectively compiled for rapid quantitative assessment.

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC TUV Detector

ACQUITY UPLC Protein BEH SEC Column

ACQUITY UPLC BEH C4 Column

Protein-Pak™ Hi Res HIC Column

Xevo® G2-S QToF MS

KEY WORDS

Antibody drug conjugates, ADCs, cysteine-conjugated ADCs, lysine-conjugated ADCs, native intact mass analysis, hydrophobic interaction chromatography, HIC, SEC, antibody-to-drug ratio, DAR, drug payload, drug distribution

INTRODUCTION

Antibody Drug Conjugates (ADCs) have been emerging as a new class of anti-cancer therapeutics, combining the targeted delivery capability of a monoclonal antibody (mAb) with the potency of conjugated cytotoxic agents. By releasing the cytotoxic drugs inside target tumor cells, ADCs minimize exposure of non-target cells to toxic drug payloads and thereby reduce potential side effects of the treatment.

Liquid chromatography (LC) and mass spectrometry (MS) are among the most commonly used analytical techniques for characterizing ADCs.^{1,2} Very often, a suite of techniques, such as hydrophobic interaction chromatography (HIC) LC-UV, SEC native LC-MS, and /or RP LC-MS, can be applied to achieve a more comprehensive characterization profile of an ADC.

Structural information that is derived from different analytical methodologies, especially when used to determine critical quality attributes (CQAs) such as drug-to-antibody ratio (DAR) and drug load distribution (which measures the homogeneity of the ADC population), is challenging to assemble to provide effective assessment.³ Different informatics tools and manual calculations are usually needed for processing the complex datasets, leading to losses in productivity and time. With more than 60 ADCs currently in development,⁴ more efficient informatics approaches, such as automated data acquisition, data processing and data reporting, are needed to support pharmaceutical researchers who are charged with characterizing and quantifying ADCs.

In this work, we illustrate the benefits of applying an integrated informatics workflow that has been developed for UNIFI Software to streamline data acquisition, processing, and reporting for ADC analysis by LC-UV or LC-MS.

This workflow brings together information from multiple analytical techniques, so that it can be effectively used for rapid and quantitative assessment of the comparability of ADCs. DAR values and drug load distribution for multiple batches of ADCs are calculated automatically. We also perform a comparability study across analytical techniques based on the DAR values for different ADC samples.

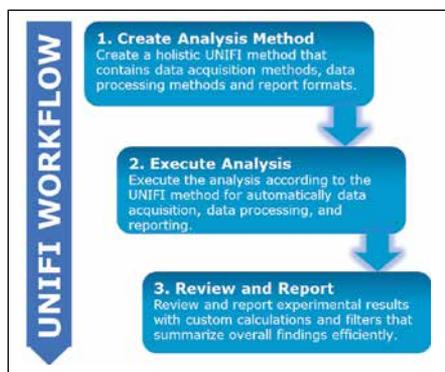


Figure 1. ADC analysis workflow with the UNIFI Scientific Information System.

EXPERIMENTAL

LC (HIC) UV

The Waters Protein-Pak Hi Res HIC Column (4.6 x 100 mm, 2.5 mm) was conditioned prior to use as per the column care and use manual. Analysis samples were diluted to a concentration of 2 mg/mL in 1 M ammonium sulfate before injections.

LC conditions

LC system:	ACQUITY UPLC H-Class				
Detector:	ACQUITY UPLC TUV				
Absorption wavelength:	280 nm				
Vials:	Total recovery vial, 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)				
Column:	Protein-Pak Hi Res HIC, 2.5 µm, 4.6 mm x 100 mm, (p/n 176003576)				
Column temp.:	25 °C				
Sample temp.:	4 °C				
Injection vol.:	10 µL				
Mobile phase A:	125 mM phosphate buffer, pH 6.7 with 2.5 M (NH ₄) ₂ SO ₄				
Mobile phase B:	125 mM phosphate buffer, pH 6.7				
Mobile phase C:	Isopropyl alcohol				
Mobile phase D:	Water				
Gradient table:					
Time	Flow	%A	%B	%C	%D
	(mL/min)				
Initial	0.700	50	0	5	45
10.00	0.700	0	50	5	45
10.01	0.700	50	0	5	45
30.00	0.700	50	0	5	45

Native SEC QToF MS

Samples were diluted to a concentration of 0.5 mg/mL in 50 mM ammonium acetate before injection.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Column:	ACQUITY UPLC Protein BEH SEC, 200Å, 1.7 µm, 4.6 mm x 150 mm (p/n 186005225)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
Mobile phase:	50, 100, or 200 mM ammonium acetate in H ₂ O
Gradient:	Isocratic at 0.3 and 0.1 mL/min with total run time of 20 min

Time (min)	Flow (mL/min)	Comp. %A	Comp. %B	Comp. %C	Comp. %D	Curve
0.00	0.300	0.0	0.0	0.0	100.0	Initial
2.75	0.300	0.0	0.0	0.0	100.0	6
2.76	0.100	0.0	0.0	0.0	100.0	6
7.50	0.100	0.0	0.0	0.0	100.0	6
7.60	0.300	0.0	0.0	0.0	100.0	6
20.00	0.300	0.0	0.0	0.0	100.0	6

Total run time: 20 min

MS conditions

MS system:	Xevo G2-S QTof
Mode:	ESI+ sensitivity
Capillary:	3.0 kV
Sampling cone:	150 V
Source offset:	80 V
Source temp.:	500 °C
Desolvation temp.:	500 °C
Cone gas flow:	300 L/h
Desolvation gas flow:	800 L/h

RPLC QTof MS

Samples were diluted to a concentration of 1 mg/mL in 50 mM ammonium acetate before injection.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Column:	ACQUITY UPLC Protein BEH C4, 300Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186004495)
Column temp.:	80 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	1% TFA (in water)
Mobile phase D:	1% FA (in water)

Gradient:

Time (min)	Flow (mL/min)	Comp. %A	Comp. %B	Comp. %C	Comp. %D	Curve
0.00	0.400	85.0	5.0	10.0	0.0	Initial
1.00	0.400	85.0	5.0	10.0	0.0	6
2.00	0.400	60.0	40.0	0.0	0.0	6
2.10	0.200	60.0	40.0	0.0	0.0	6
8.00	0.200	25.0	75.0	0.0	0.0	6
9.00	0.400	5.0	85.0	10.0	0.0	6
10.00	0.400	5.0	85.0	10.0	0.0	6
11.00	0.400	85.0	5.0	10.0	0.0	6
15.00	0.400	85.0	5.0	10.0	0.0	6

Total run time: 15.0 min

MS conditions

MS system:	Xevo G2-S QTof
Mode:	ESI+ sensitivity
Capillary:	3.0 kV
Sampling cone:	150 V
Source offset:	80 V
Source temp.:	500 °C
Desolvation temp.:	500 °C
Cone gas flow:	0 L/h
Desolvation gas flow:	800 L/h

Data management**UNIFI Scientific Information System v1.7 for data acquisition, processing and reporting**

UNIFI v1.7 was used for data collection and processing for all experiments, configured using an intact protein analysis type that defines the automated processing.

- Data processing first deconvolutes the mass spectra and searches for the theoretical mass values for the various antibody (protein) and drug entities that can occur within the samples
- It then matches them based upon specified tolerance values as a means to identify the components
- The components are identified using a syntax that includes the identification of the modifier, which, in this experiment, includes the antibody (protein) drug, and also includes the number of drug modifiers, which is the drug load
- Next, these identifiers are used in custom calculations to group components containing the drug by antibody and drug load
- Finally, the responses of the component and load values are utilized to calculate the DAR value. Custom fields containing the necessary custom calculations are included in the method such that they are automatically calculated, saved in the results, and available for viewing, trending, and reporting
- Both custom fields and analytical methods are protected by an administration and security framework via configurable user access controls, based upon roles that facilitate their use in routine analyses, particularly in regulated laboratories

Methods

Analyses of ADC samples were performed using LC-UV and LC-UV/QToF-MS. Samples were analyzed at the intact protein level by LC(HIC)-UV and by LC-MS under either native (SEC-MS) or denatured conditions (reversed-phase LC-MS) with the control of a common informatics platform. The total run time for both HIC-LC and LC-MS experiments was 20 min.

CQAs, such as the DAR value and drug load distribution, were automatically calculated based on either the HIC/UV chromatographic peak areas or the deconvoluted mass spectra using the informatics tool following automated data acquisition.

Comparability assessment for multiple batches of ADC samples were undertaken based on the DAR values and relative drug distribution. Both cysteine-conjugated and lysine-conjugated ADC mimics were prepared by a collaborator at a concentration of 10 mg/mL in formulation buffer.

RESULTS AND DISCUSSION

Analysis of cysteine-conjugated ADCs for determination of DAR value based on HIC LC-UV

HIC is a leading technique for the characterization of CQAs of ADCs, including DAR values and drug loading distribution. As a non-denaturing technique, HIC is often used to characterize cysteine-conjugated ADCs due to the nature of the conjugation chemistry. The intra-chain disulfide bonds that normally are present to maintain the linkages between the heavy and light chains of the mAb are occupied by drug conjugates. The cysteine-conjugated ADCs, when exposed to standard reversed-phase conditions (e.g., acetonitrile), would be reduced to sub-units that are dictated by which cysteine disulfide bridges remain intact after conjugation; characterization information such as drug distribution would be lost. However, non-covalent interactions such as hydrogen bonding and ionic pairing are sufficiently strong enough to maintain the ADC's tertiary structure when separated in non-denaturing conditions such as a salt gradient. This makes HIC ideal for determining CQAs such as drug distribution and DAR values for cysteine-conjugated ADCs.

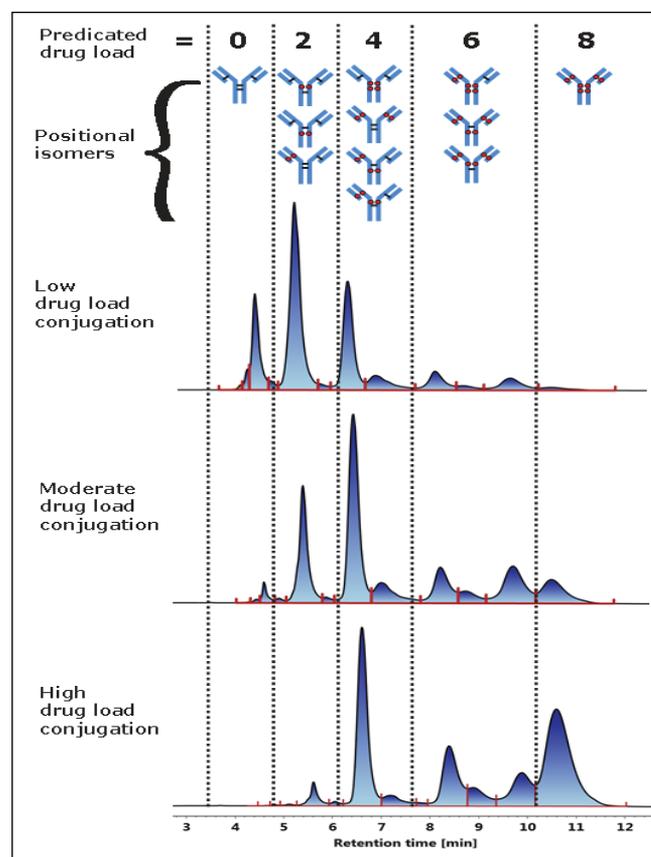


Figure 2. Cysteine conjugated ADC analysis using HIC. Drug distribution was determined for three different cysteine-conjugated ADC samples with increasing drug load.

In HIC chromatography, the separation mechanism applied is to increase the hydrophobicity of the protein by starting at a concentration of high salt to ion-pair with charge sites on the protein. With the charge sites masked, the hydrophobicity of the protein is increased, which allows the protein to bind to the low-retention surface of the HIC stationary phase (n-butyl surface). From the gradient listed, the salt concentration is decreased, thus exposing the charge sites of the protein, which increase the protein's hydrophilicity or affinity to the mobile phase, allowing it to elute. The drug conjugate, which is hydrophobic, increases the retention time of the ADC based upon its drug load. This behavior results in a HIC separation profile where the peaks are grouped by drug distribution (e.g., 0, 2, 4, 6, and 8 drug conjugates). DAR values can be readily calculated from the peak area of the drug profile distribution, as shown in Figure 3.

Waters Biopharmaceutical Platform Solution with UNIFI offers the ability to automatically calculate the DAR values of the cysteine-conjugated ADCs. This is achieved by using a custom field option when setting up the processing method. As shown in the component summary pane of Figure 3, custom fields were programmed to calculate the individual DAR values for each drug distribution (e.g., 0, 2, 4, 6, and 8). A custom field was also designed to automatically calculate the total DAR value of the sample as seen in the last column of the component summary pane.

The Biopharmaceutical Platform Solution with UNIFI also features strong reporting functionality, with a workflow that gathers meaningful analytical measurements that can be presented as automated custom reports for the efficient communication and cataloging of analytical results. Report templates can be readily constructed and customized for the assessment of analysis results.

Figure 4 is an example of a report template designed for HIC characterization of biotherapeutics such as ADCs.

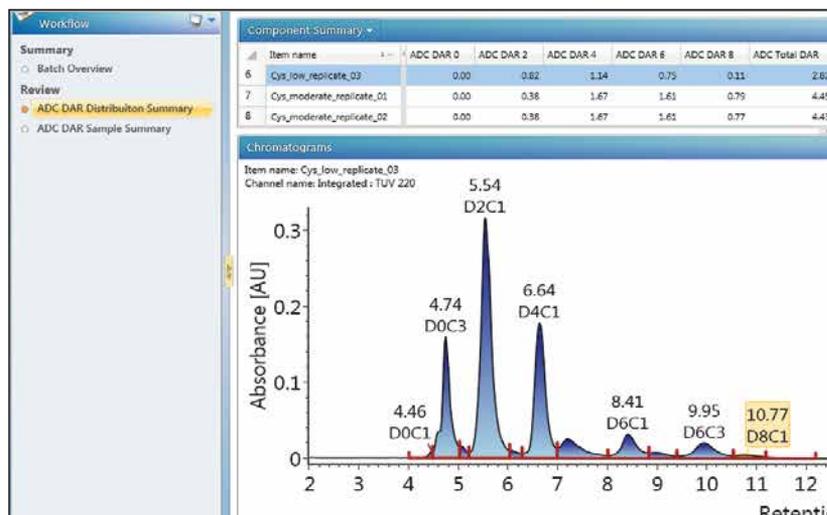


Figure 3. Integrated informatics for ADC analysis. Custom fields are used to calculate the DAR value using peak area and display the results in the component summary pane.

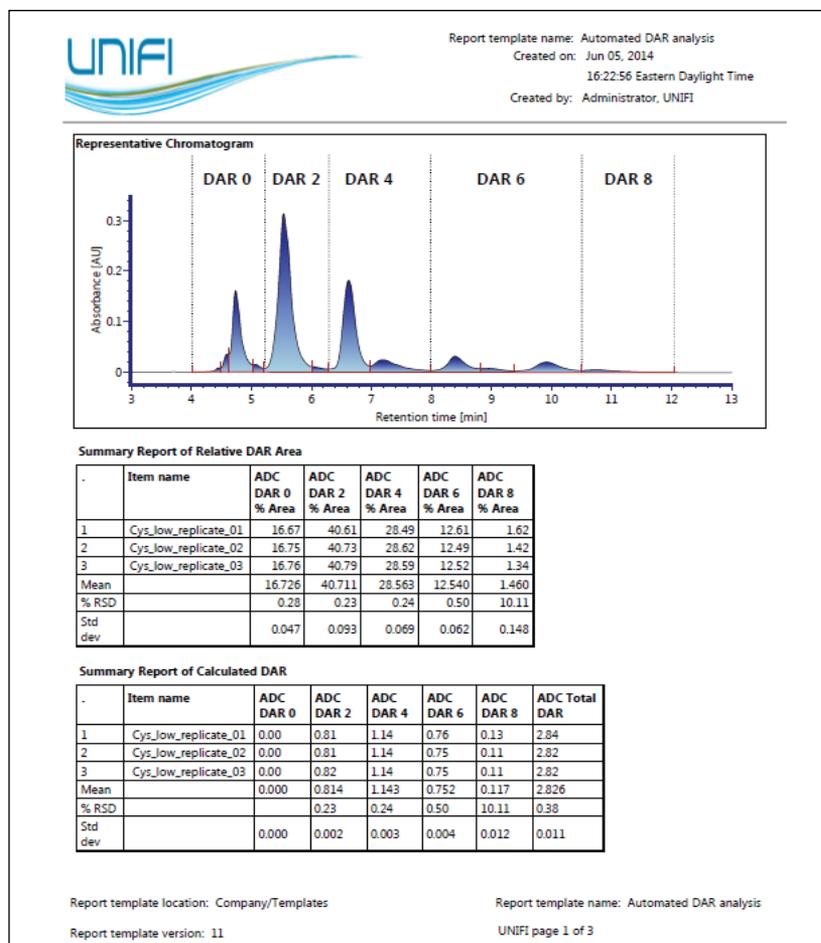


Figure 4. Automated reporting using UNIFI informatics. A report of peak area, DAR value, and associated statistics was automatically generated after data acquisition and analysis.

Using the results for the purified IgG sample from Figure 3, a summary report of the relative area and calculated DAR value based on that area, as well as the corresponding statistical evaluation (mean and % RSD) are generated after data acquisition and processing. With this flexibility to design custom report templates based on analytical needs, the Biopharmaceutical Platform Solution with UNIFI serves as a powerful integrated system for the acquisition, processing, and reporting of results for ADC biotherapeutics.

Analysis of cysteine-conjugated ADCs for the determination of DAR values based on native intact mass analysis (SEC LC-MS)

In a reversed-phase LC-MS experiment, the cysteine-conjugated ADCs will dissociate into the light chain and heavy chain subunits because of the acidic mobile phase conditions. Therefore, intact native mass analysis is performed using the non-denaturing conditions to keep the protein in its near-native state in the gas phase.

The commonly used buffer that maintains a protein in the native state is not suitable to ionization of the protein in mass spectrometry. Ideal buffers for MS intact analysis are ones that allow proteins to remain in their folded state, yet are volatile enough to enable sufficient ionization. Aqueous solutions of ammonium acetate (NH_4OAc) are usually chosen for this purpose.

For our native SEC LC-MS experiments, we used a concentration of 50 mM NH_4OAc . Because the protein is close to its native state in NH_4OAc solution, the surface area is smaller than that of its denatured counterpart in the reversed-phase acidic mobile phases (usually with half organic and half water). Thus, proteins ionized in intact native mass analysis will have fewer charges than that in a reversed-phase experiment.

As shown in Figure 5, the narrower charge envelope distributions are centered around an m/z range of 5,200 with a 30+ charge state, rather than wider charge envelope centered around 2500 m/z with a 60+ charge state for a reversed-phase denatured experiment.

Figure 5 displays the native SEC LC-MS raw spectra of the cysteine-conjugated ADC samples. The charge envelope distributions were compared for three different cysteine-conjugated ADC samples with increasing drug load, as well as with the unconjugated mAb as a control.

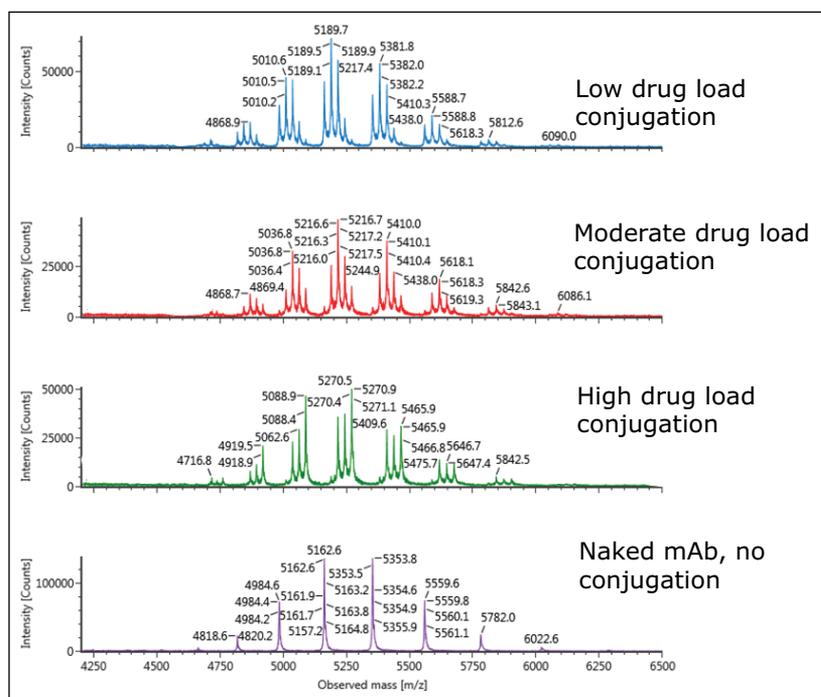


Figure 5. Native SEC LC-MS raw spectra of cysteine-conjugated ADC samples from different drug loading.

Figure 6 shows the deconvoluted intact mass spectra from the raw mass spectra for deglycosylated cysteine-conjugated ADCs from the native SEC LC-MS analysis shown in Figure 5. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load, as well as with the unconjugated mAb.

As described in the previous section on HIC analysis, as well as in details about the informatics used in the Experimental section, the Biopharmaceutical Platform Solution with UNIFI can seamlessly and automatically acquire, process, and report data. Figure 7 is a screen capture of the UNIFI review pane that displays the processed and calculated data for DAR values from the native SEC LC-MS experiments.

In this integrated workflow, the summed raw spectrum for each sample (as shown in Figure 5) obtained by combining the total ion chromatogram (TIC) peak (as shown in Figure 7) was deconvoluted using MaxEnt1 (as shown in Figure 6). The identified peak areas or the intensities in the deconvoluted spectrum were used to calculate the individual drug payload DAR and the total DAR for the sample, with the assistance of custom fields performing calculations in the background.

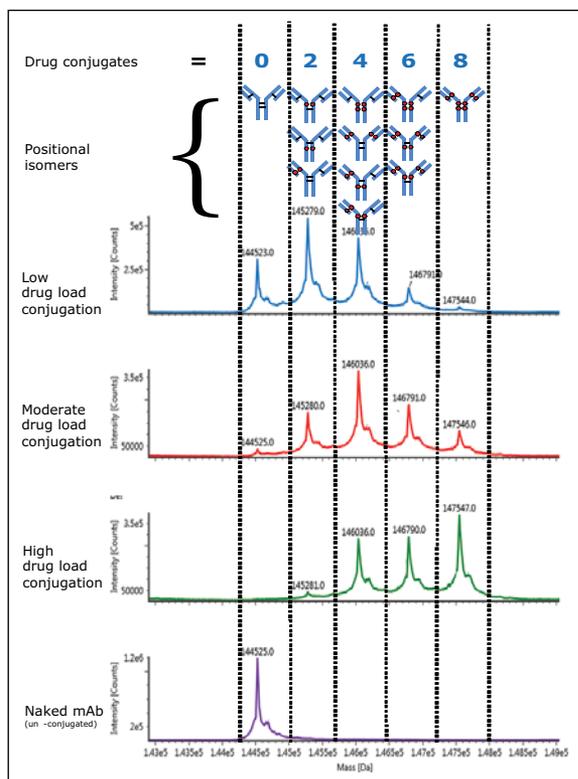


Figure 6. Deconvoluted intact mass spectra for cysteine-conjugated ADCs from native SEC-LC-MS after deglycosylation. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load.



Figure 7. UNIFI review pane shows the automatically process data and calculated DARs from the native SEC-LC-MS experiments.

Figure 8 shows the CQAs of DAR and drug load distribution for three batches of ADCs, with a comparison between the HIC and the native SEC LC-MS experiments in table (top) and 3D graph format (bottom). The experimental results from the two orthogonal methods show excellent agreement between the two methods for both the individual DARs, as well as the total average DARs for all three drug loading levels.

For instance, the individual DARs with six drug payloads for the three samples from the HIC method were 0.75, 1.61, and 1.72; while for the native LC-MS method they are 0.60, 1.45, and 1.72 respectively. The total DAR values from the HIC method were 2.83, 4.44, and 5.97; while for the native SEC LC-MS method, they are 2.72, 4.40, and 5.97 respectively. This is very important data for lot-to-lot and batch-to-batch comparison studies.

Considering that all of the information was obtained automatically, we believe that this integrated informatics workflow in UNIFI will be an enabling tool for increasing productivity during the ADC product development processes.

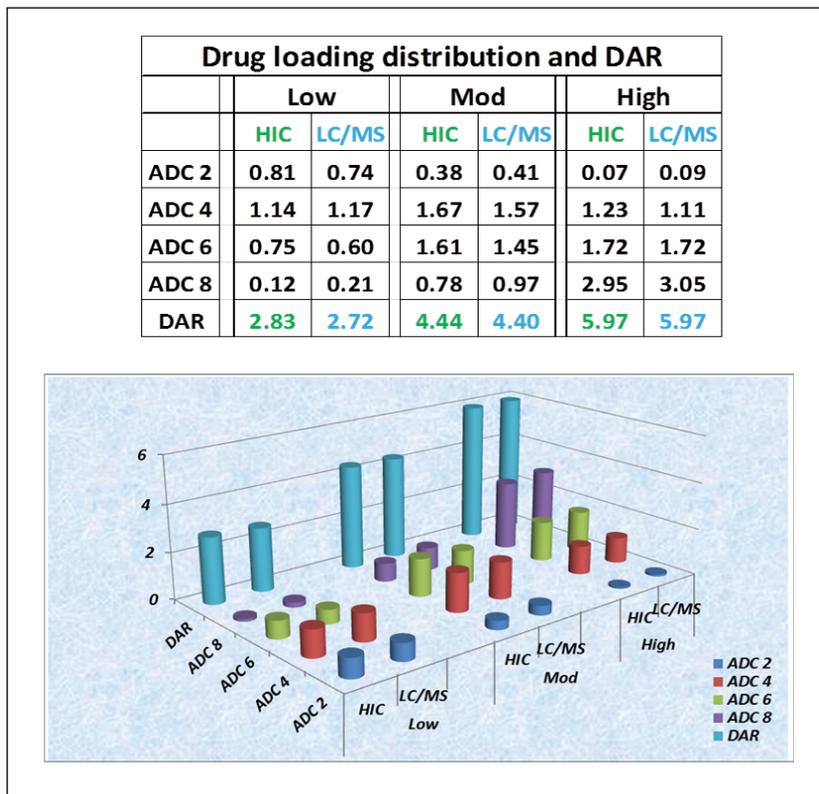


Figure 8. DARs comparison between HIC and native SEC-LC-MS experiments in table (top) and 3-D graph format (bottom) shown excellent agreement between the two methods for all three drug loading levels.

Analysis of Lysine-Conjugated ADCs for the Determination of DAR Values Based on RP-LC/MS Intact Mass Analysis

Unlike cysteine-conjugated ADCs, the intra-chain disulfide bonds that maintain linkages between the heavy and light chains of the mAb for the lysine-conjugated ADCs are intact. Therefore, reversed-phase chromatography is suitable for the analysis of lysine-conjugated ADCs when linker chemistry is not labile at acidic pH.

Figure 9 is a screen capture of the UNIFI review pane that shows the experimental results of the reversed-phase LC-MS analysis for three batches of lysine-conjugated ADCs with increasing drug payload. The component summary table displays the identified drug load distribution based on the deconvoluted spectra peaks (Figure 10), with automatically calculated DARs, integrated chromatogram, and DAR values bar chart for the three level drug loading lysine-conjugated samples. The DARs determined here can be used for lot-to-lot comparison of lysine conjugates. However, calculated absolute DAR values need to be verified by an orthogonal technique.

The spectra in Figure 10 compare the difference in drug distribution for three batches of lysine-conjugated ADC preparation at various levels of drug loadings and can be automatically generated in the report. It was observed that as many as up to 12 drugs were bonded to the mAb of interest.

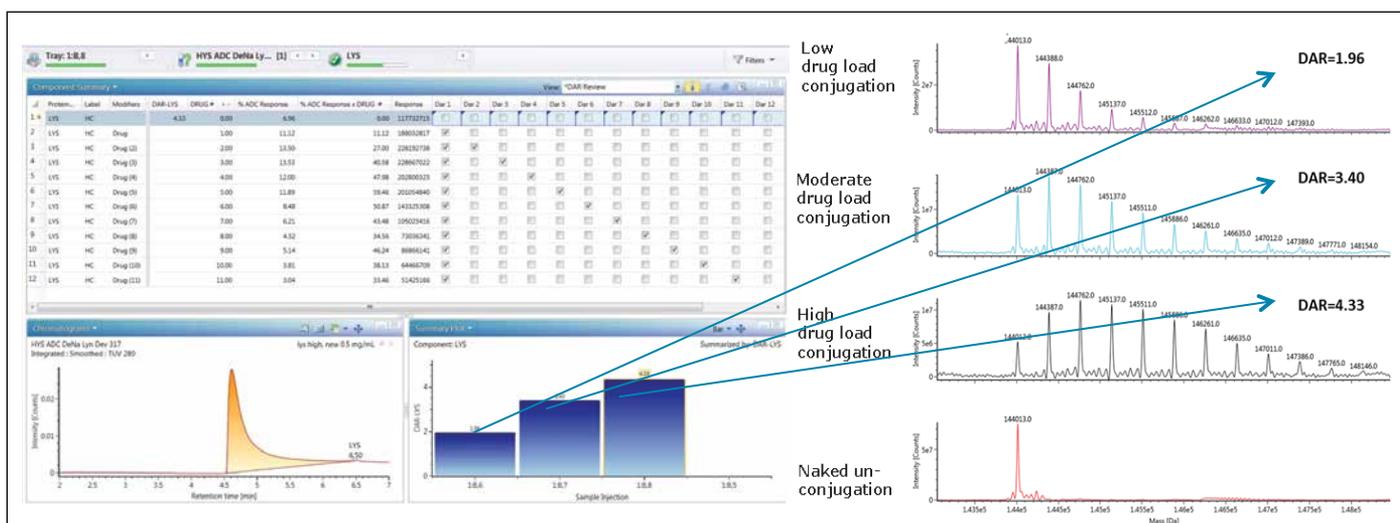


Figure 9. UNIFI review panel displays the experiment results from the automated analysis of lysine-conjugated ADCs to determine the DAR values by RP-LC-MS intact mass analysis.

Figure 10. The deconvoluted MS spectra for lysine-conjugated ADCs.

CONCLUSIONS

In this application note, we have demonstrated the use of an integrated informatics workflow in UNIFI Software that streamlines data acquisition, processing and reporting for ADC analysis by LC-UV or LC-MS, so that information from multiple analytical techniques can be effectively integrated for rapid and quantitative assessment of the comparability of ADCs.

DAR values and drug loading distributions for cysteine-conjugated ADCs are automatically acquired from HIC LC-UV analysis and from native SEC LC-MS analysis, and the results show excellent agreement between these two methods. The workflow automatically produces DARs and drug loading distributions for lysine-conjugated ADCs from RP LC-MS analysis.

This automated workflow removes the necessity of manual data processing and reduces the associated human errors. The workflows in UNIFI can be saved and shared, enabling consistent high-quality data generation, processing, and reporting for complex ADC datasets with an unparalleled capability to aggregate and manage data.

Collectively, we believe this integrated informatics workflow will be an enabling tool for organizations working in the discovery or development of ADC biotherapeutics, providing increased confidence in results, productivity, and financial gains.

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T: 1 508 478 2000
F: 1 508 872 1990
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Top-down Monoclonal Antibody (mAb) Analysis Using CID and ETD Fragmentation and the UNIFI Scientific Information System

Min Du,¹ Martin DeCecco,² Henry Shion,¹ Laetitia Denbigh²

¹Waters Corporation, Milford, MA, USA, ²Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

The UNIFI® Scientific Information System streamlines data processing, reviewing, and reporting for monoclonal antibody fragmentation data, and enables the confirmation of protein sequences and post-translational modifications.

WATERS SOLUTIONS

[SYNAPT® G2-Si HDMS® System](#)

[MassLynx® Software](#)

[UNIFI Scientific Information System](#)

KEY WORDS

collision induced dissociation (CID), electron transfer dissociation (ETD), monoclonal antibody (mAb), antibody-drug conjugate (ADC), trastuzumab (T-mab), intact mass analysis, top-down analysis, MS-MS fragmentation, UNIFI

INTRODUCTION

Direct fragmentation of proteins (top-down) provides an orthogonal approach to verify terminal sequence confirmation and localize protein modifications. Protein fragmentation approaches are primarily qualitative and have limited dynamic range to assess variation. Additionally, these approaches have no simple mechanism to determine modification abundance, making them a complementary technology to peptide mapping for mAbs and other biotherapeutics. Top-down protein fragmentation generates diverse fragment ion types and a multitude of charge states with overlapping spectral patterns. This data complexity complicates the process of producing primary structural assignments – usually through manual annotation – leading to the necessary development of software tools that automatically deconvolute raw spectral MS-MS data, annotate fragmentation patterns to protein sequences, and generate reports. These challenges have impeded broader usage of top-down protein analysis for routine biotherapeutic mAb development.

This application note demonstrates the acquisition of protein top-down analysis data by collision induced dissociation (CID) or electron transfer dissociation (ETD) MS-MS fragmentation from the SYNAPT G2-Si HDMS Mass Spectrometer (Figure 1). The data from these analyses was imported into UNIFI for processing, review, and report generation. This automated data processing workflow enabled efficient sequence verification, along with identification of modifications for a mAb and its subunits.

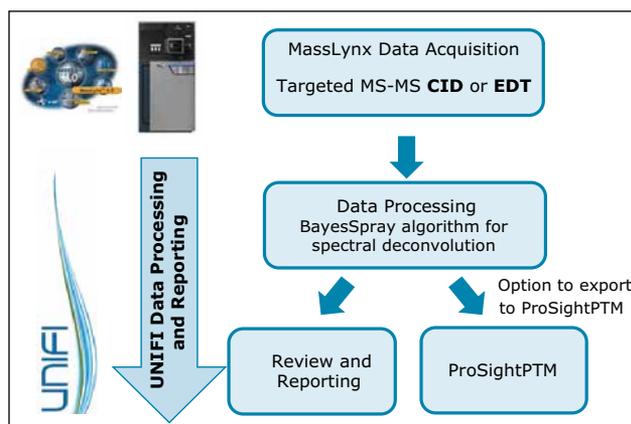


Figure 1. UNIFI top-down analysis workflow automates processing and reporting for targeted MS-MS data acquired using MassLynx.

EXPERIMENTAL

Methods

Trastuzumab was treated by IdeS (Genovis) protease and reduced by DTT (SigmaAldrich) to produce light chain (LC), Fd, and Fc/2 fragments. Intact mass and the subsequent top-down analyses were performed on an ACQUITY UPLC H-Class Bio System connected to a SYNAPT G2-Si HDMS Mass Spectrometer. The analyses were processed in UNIFI using the dedicated protein intact mass and top-down workflows. Data acquisition for the CID or ETD MS-MS fragmentation was performed on selected charge states.

LC conditions

LC system:	ACQUITY UPLC® H-Class Bio System
Detector:	ACQUITY UPLC Tunable UV (TUV), 280 nm
Column:	ACQUITY UPLC Protein BEH C ₄ , 1.7 µm, 2.1 mm x 50 mm (P/N 186004495)
Column temp.:	80 °C
Sample temp.:	4 °C
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient table:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.4	95	5	Initial
1.00	0.4	95	5	6
1.10	0.1	95	5	6
2.50	0.1	77	23	6
12.50	0.1	71	29	6
13.00	0.1	5	95	6
13.10	0.4	5	95	6
14.00	0.4	5	95	6
14.50	0.4	95	5	6
17.00	0.4	95	5	6

Targeted MS-MS analysis

MS system:	SYNAPT G2-Si HDMS
Mode:	ESI+ sensitivity mode
Capillary:	2 kV
Sample cone voltage:	40 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	800 L/h
Full scan MS:	Scan rate=0.5 sec Mass range=500–2000 <i>m/z</i>

Targeted MS-MS conditions

Scan rate=1 Hz; mass range=50–2000 *m/z*

For CID fragmentation, the collision energy level was ramped from 20 to 40 eV

For ETD fragmentation, MS-MS data was acquired over a one second scan (signal accumulation) period with an anion refill time of 100 ms between scans

Informatics

MassLynx v4.1 for SYNAPT instrument control and data acquisition

UNIFI Scientific Information System v1.8 for data processing, reviewing, and reporting¹

ProSight PTM 2.0 as an alternative informatics annotation engine using data exported from UNIFI in the .PUF file format²

RESULTS AND DISCUSSION

Intact mass analysis of trastuzumab subunit

Trastuzumab subunit LC-MS analysis data was acquired on an ACQUITY UPLC H-Class Bio System coupled with a SYNAPT G2-Si HDMS Mass Spectrometer under MassLynx control. The data was then imported into UNIFI for processing, review, and report generation. The UNIFI review panel (Figure 2) includes: (A) A component summary table displaying the trastuzumab subunit and assigned glycoforms; (B) Total ion count (TIC) and base peak ion (BPI) chromatograms; (C) Summed raw MS spectra and MaxEnt1™ deconvoluted spectra; (D) Component plot.

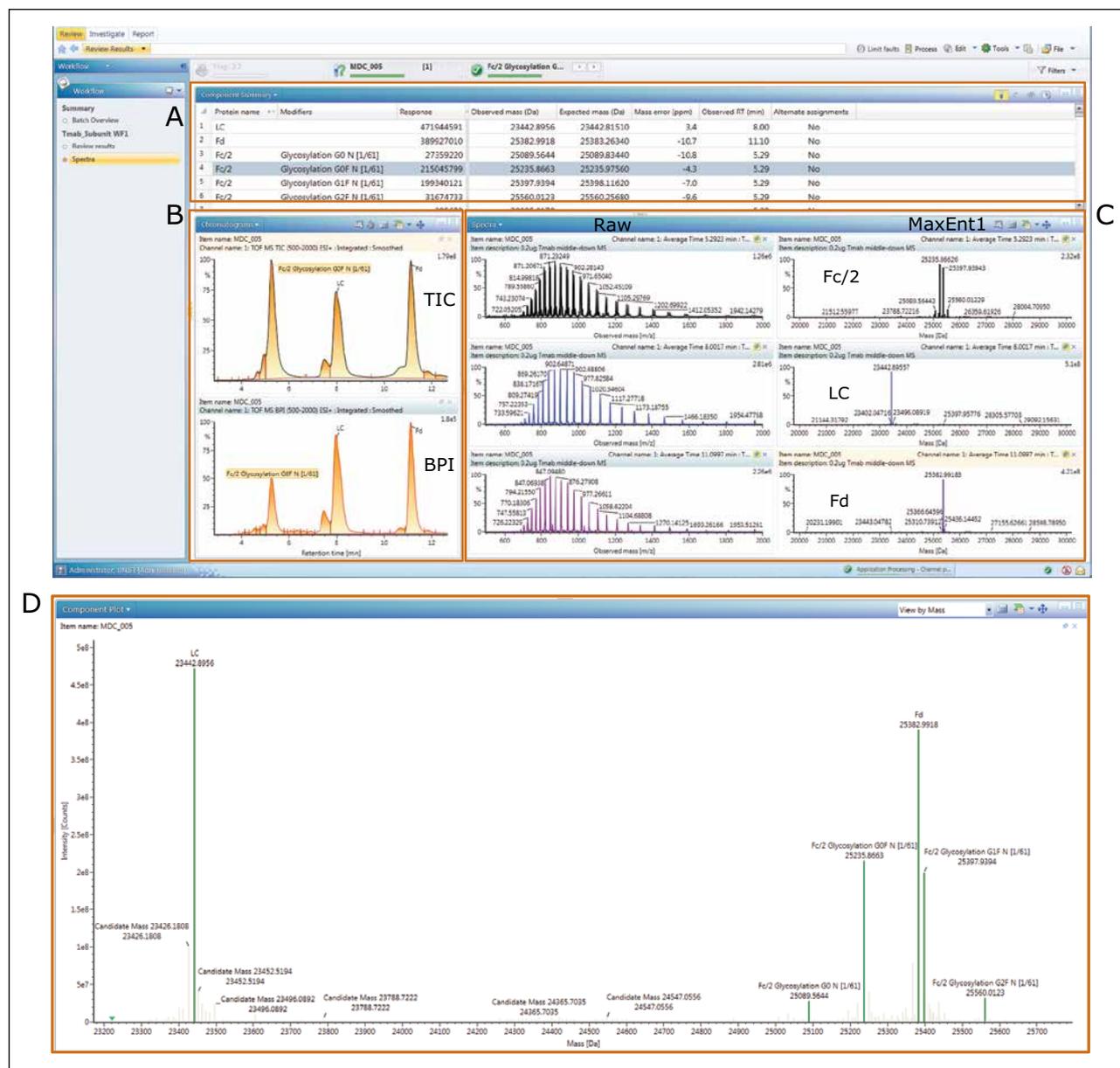


Figure 2. Trastuzumab (T-mab) subunit LC-MS analysis with MaxEnt1 deconvolution processing and UNIFI intact MS analysis workflow.

The T-mab subunits and their modified forms were identified. The light chain (LC), Fd, and Fc/2 glycoforms (GO, GOF, GIF, and G2F) were displayed with the deconvoluted average mass in the component table (Figure 2A) and the component plot (Figure 2D). The TIC chromatogram exhibited good separation of the subunit peaks with identity annotation (Figure 2B). Easy access to the raw and deconvoluted spectra facilitated data inspection and reviewing (Figure 2C).

Top-down analysis of trastuzumab subunit with ETD fragmentation

Following the subunit analysis, a highly-charged precursor ion was chosen for efficient ETD fragmentation with automated data processing and reporting.

UNIFI provides a new protein top-down analysis workflow (Figure 3) featuring a novel Bayesian inference algorithm – BayesSpray – for deconvolution of both isotope resolved (peptide) and non-resolved (protein) data. It can automate the processing and reporting of collision induced dissociation (CID) as well as electron transfer dissociation (ETD) MS-MS fragmentation, enabling streamlined sequence verification and identification of modification sites.

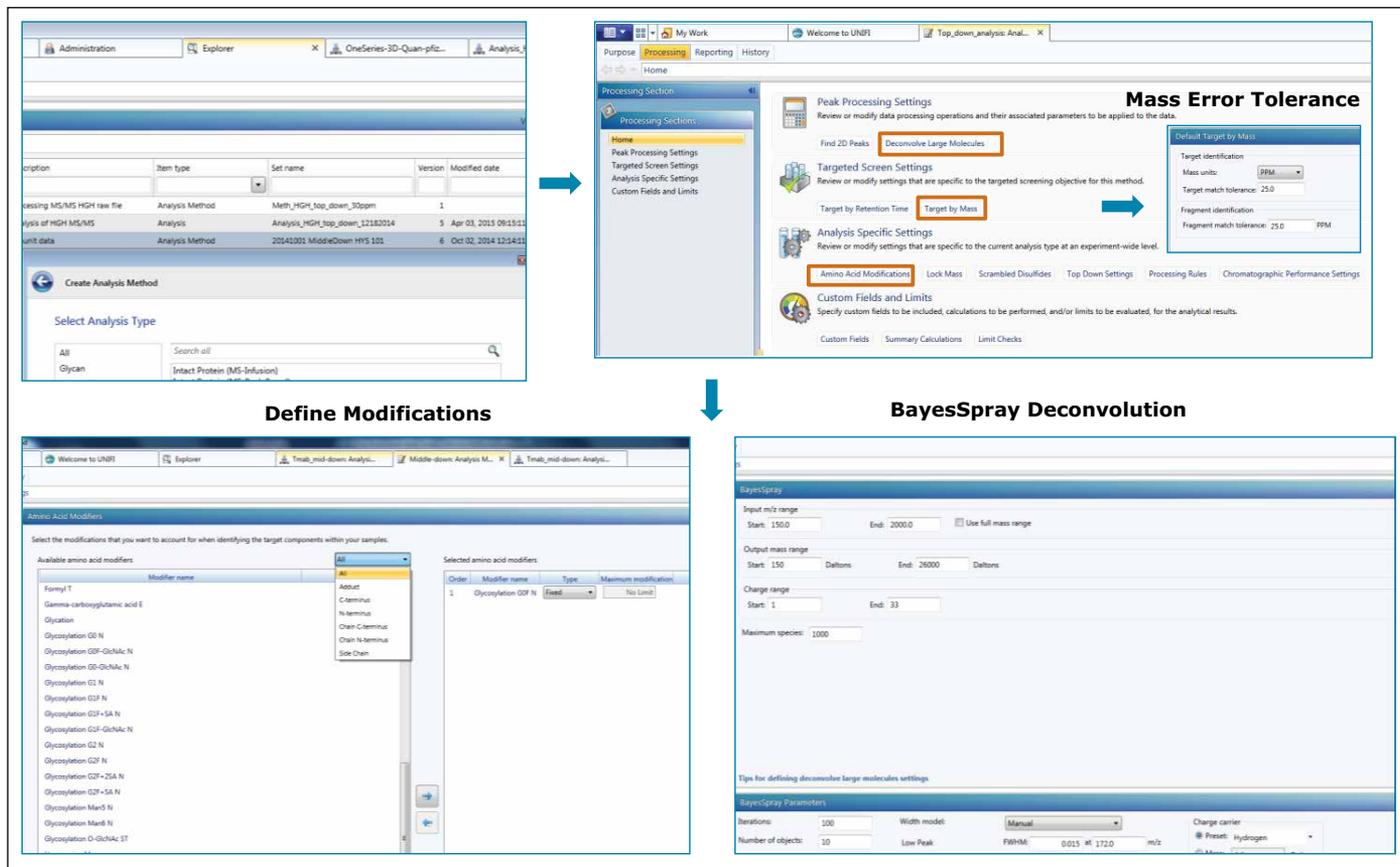


Figure 3. Create analysis method for top-down analysis.

An example data of infusion top-down analysis with ETD fragmentation on Fc/2 subunit is shown in Figure 4. One precursor ion (765.8 m/z , 33+) corresponding to the Fc/2 GOF glycoform was selected for ETD fragmentation. The MassLynx raw data was then imported into UNIFI for automated processing. The review panel display can be easily customized and configured to interactively investigate the processed data. As an example, an intuitive data review panel is displayed in Figure 4: (A) Component summary table to display the identified trastuzumab subunit; (B) Sequence coverage map; (C) Summed raw, BayesSpray mocked, and deconvoluted CID fragmentation spectra; (D) Annotated deconvoluted spectra and fragmentation ion table. 42 c- and 41 z-ions were assigned, which is roughly 38.1% backbone fragment ion coverage for the Fc/2 subunit.

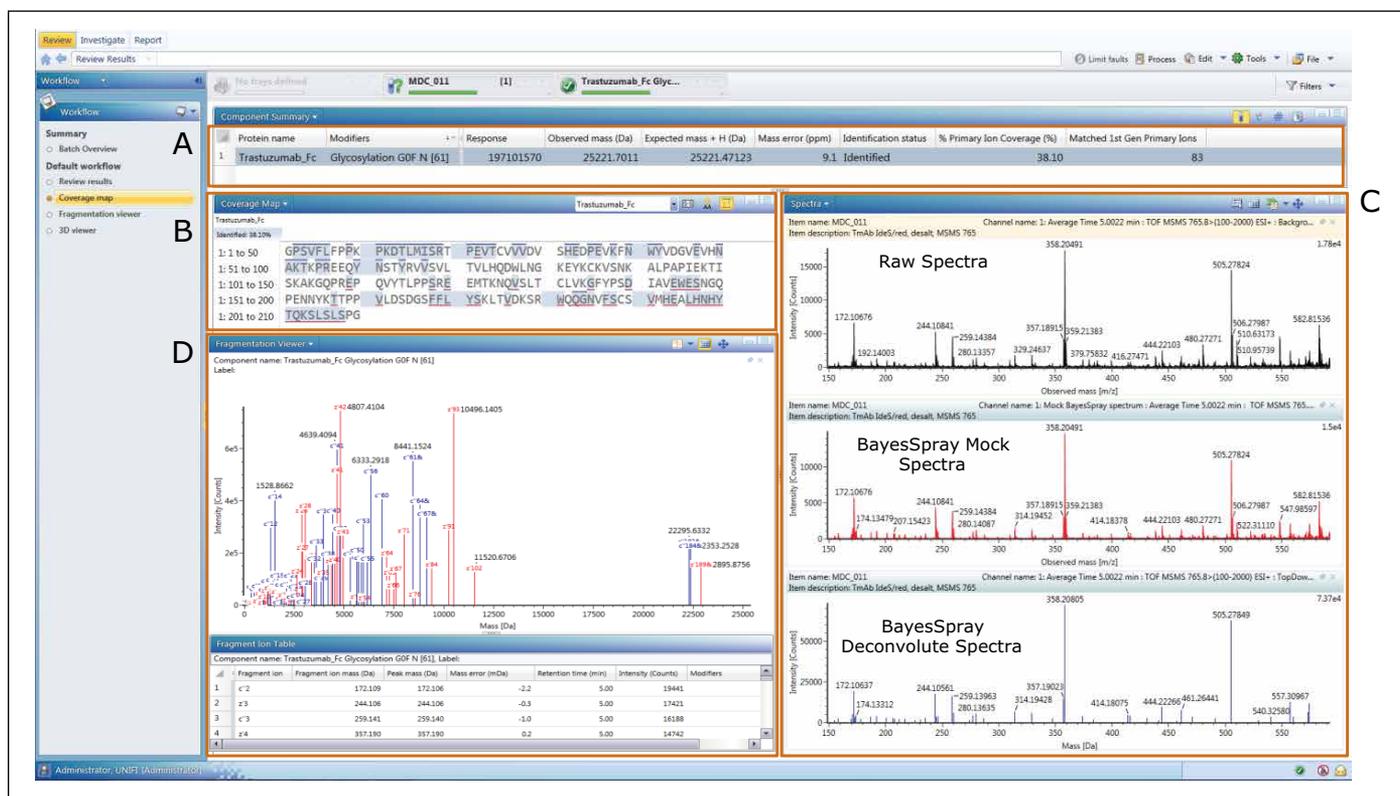


Figure 4. Example of infusion top-down analysis of the trastuzumab Fc/2 subunits with targeted MS-MS data acquisition using ETD fragmentation.

The UNIFI top-down workflow offers an option to export the processed MS-MS data (BayesSpray deconvoluted fragment ions/intensity list) in a .PUF format file that can be processed further with ProSight. ProSight was designed by the Proteomics Center of Excellence at Northeastern University for protein top-down analysis in order to identify fragments and obtain sequence coverage information.

The example result from ProSight PTM 2.0 assigned the same fragment ions and had the same backbone fragment ion coverage as the UNIFI results (Figure 5). However, the amino acid modifications must be assigned manually to be considered when using ProSight, whereas a UNIFI analysis method can include multiple modifications. In this case, ProSight PTM highlighted N in orange, which was assigned with a custom modification by manually adding the mass of GOF.

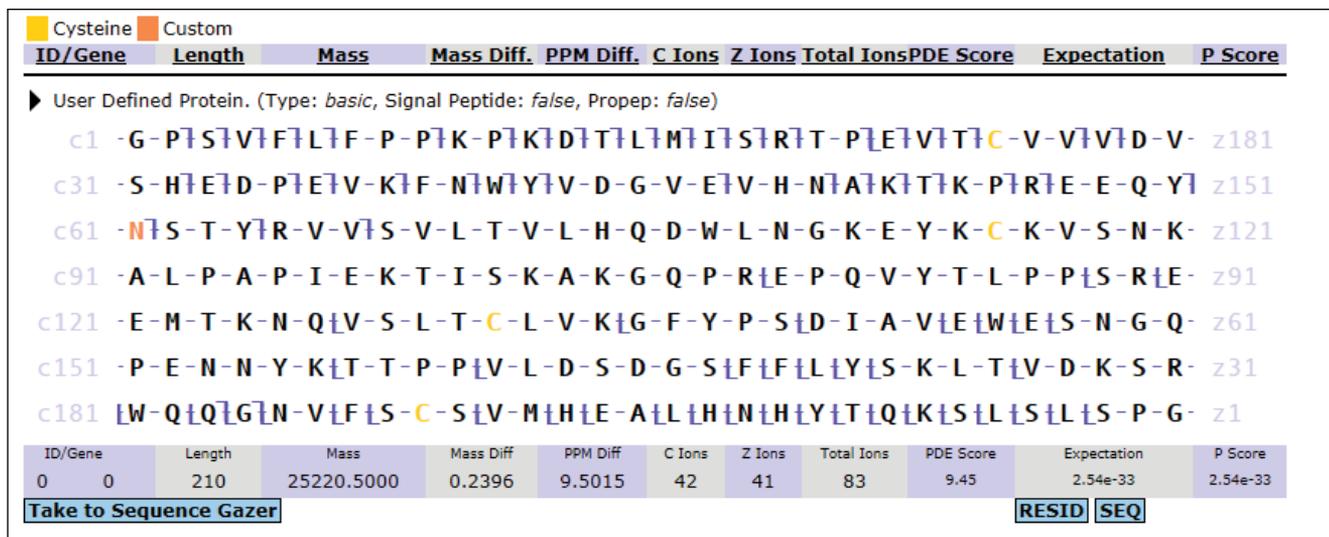


Figure 5. ProSight PTM2.0 analysis results of the previously described experiment provide the same backbone fragment ion coverage as UNIFI.

Top-down CID fragmentation followed by IMS separation

Beyond the conventional top-down methodology, the SYNAPT G2-Si HDMS Mass Spectrometer provides a unique capability for post-fragmentation ion mobility separation. This additional gas phase separation of the fragment ions is based on the charge and collision cross sectional differences (Figure 6).

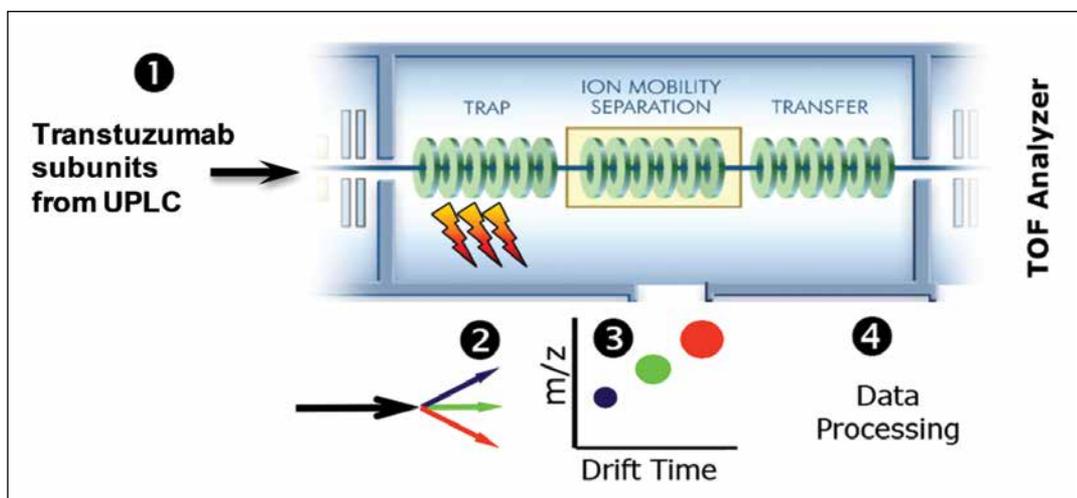


Figure 6. Schematic of ion mobility-based fragment separations provided by SYNAPT G2-Si HDMS. (1) Select one charge state of the trastuzumab subunit using the quadrupole. (2) Fragment using CID or ETD in the TRAP cell. (3) Separate CID fragment ions based on charge, size, and mass by ion mobility. (4) Process to simplify the fragmentation data.

The same sample was separately analyzed by LC-MS/MS (CID-IMS) on SYNAPT G2-Si HDMS. Singly and multiply charged fragment ions were extracted into separate raw files by DriftScope™ prior to the data being imported into UNIFI and processed. Figure 7 shows the Fc/2 GOF (precursor m/z 902.2, 28+) top-down analysis data with CID fragmentation followed by ion mobility separation. Figure 7B and 7C display the results derived from isolated, singly charged and multiply charged fragments. The raw spectra with only the 1+ charged ions facilitated the manual data interpretation. The fragments were assigned exclusively to the N- and C-terminal sequences. In total, 19 b- and 31 y-ions were assigned, which is roughly 23.81% fragment ion coverage for the Fc/2 subunit. This additional separation produced more intuitive spectra, and simplified data review and terminal sequence confirmation.

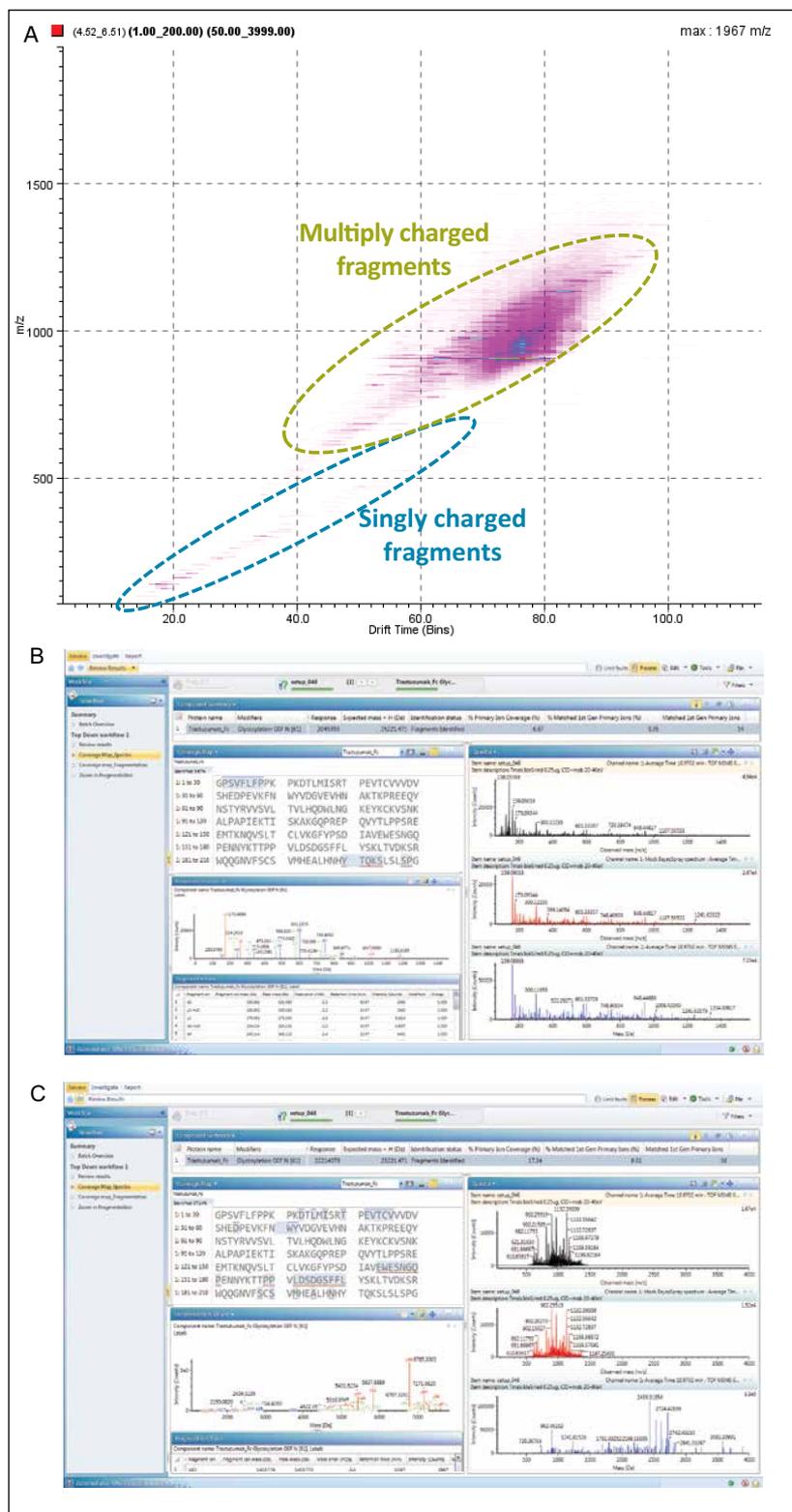


Figure 7. Example of LC-MS/MS top-down analysis of the trastuzumab Fc/2 subunits with targeted MS-MS data acquisition using CID fragmentation followed by ion mobility fragment separation. (A) Extracted ions using DriftScope. (B) Review panel of multiply charged fragment ions data processing result. (C) Review panel of singly charged fragment ions data processing result.

Report generation of top-down analysis

Report templates can be created and customized to meet specific requirements, and then saved to be applied to future analyses. Figure 8 provides a snapshot of the trastuzumab Fc/2 CID-IMS top-down analysis report of extracted singly and multiply charged fragment ions. This report includes acquisition and sample information, sequence coverage map, fragmentation viewer/table, and 3D illustration of IMS data – all of which can be organized within a single report format. The object properties and report templates are user-configurable. Multiple reports can be executed for one analysis to answer all scientific questions in an efficient format.

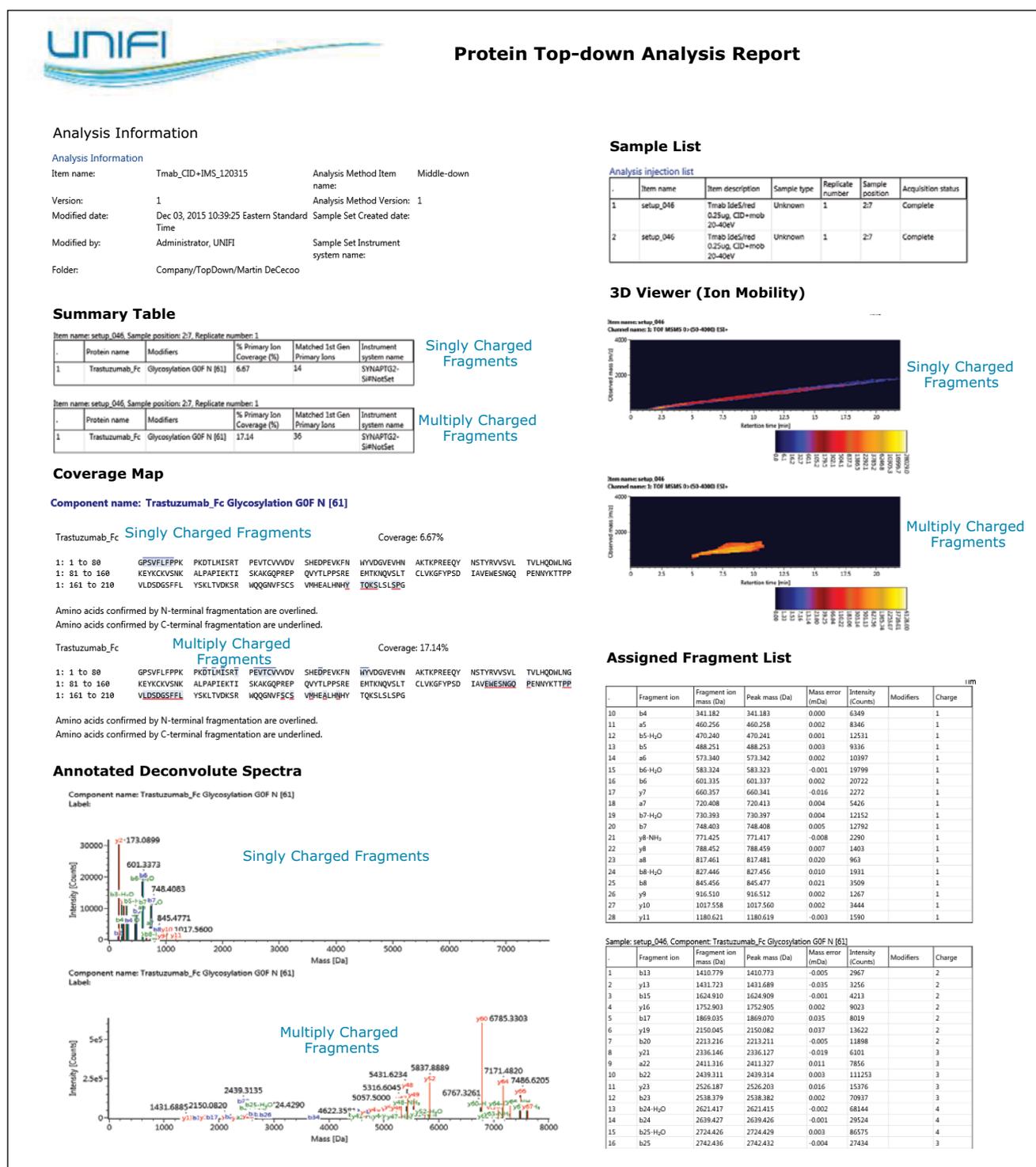


Figure 8. Example of trastuzumab Fc/2 CID-IMS top-down analysis report.

CONCLUSIONS

The described automated workflows for intact mass and top-down protein analysis facilitated the confirmation of protein terminal sequences and localization of modifications suggested by the intact mass results. This data processing workflow supported MS-MS data acquired with either CID or ETD fragmentation, overcoming the challenges of manually interpreting and reporting complex protein top-down results. The SYNAPT G2-Si HDMS System provided the unique capability to separate fragment ions by gas-phase ion mobility, generating simplified spectra for inspection and reviewing. Extracting and analyzing singly charged fragment ions from the top-down experiment enabled the terminal sequence to be readily confirmed, while multiply charged fragments provided data of the internal regions of the sequences within the mAb subunit. This ability to automate processing, fragment assignment, and organization of results in a templated UNIFI report facilitates the efficient communication of top-down data within and across biopharmaceutical organizations.

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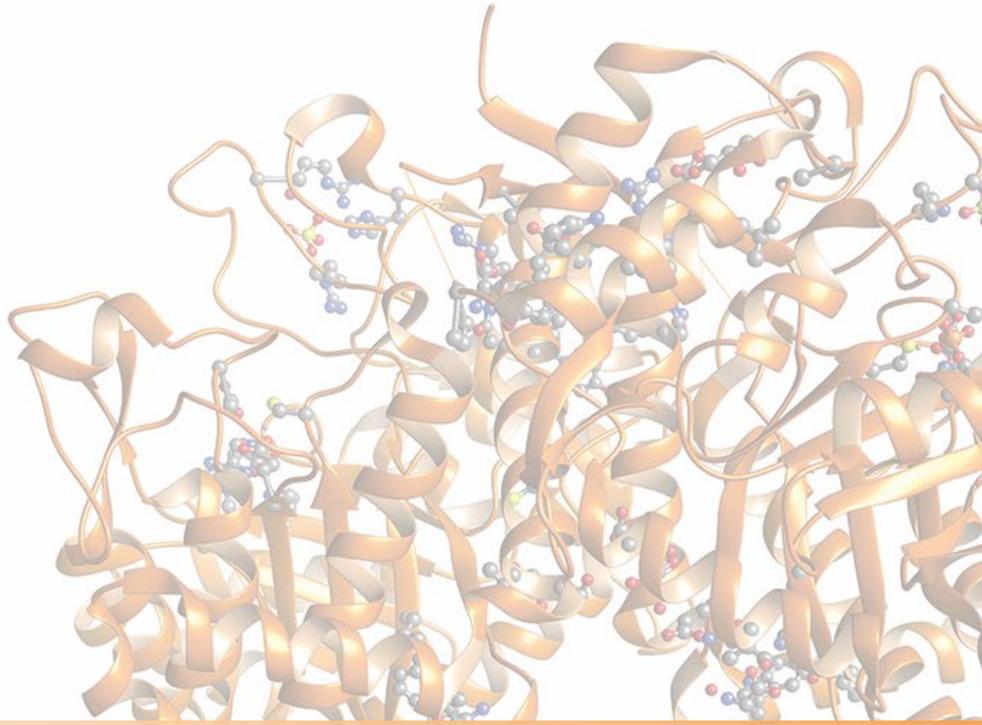
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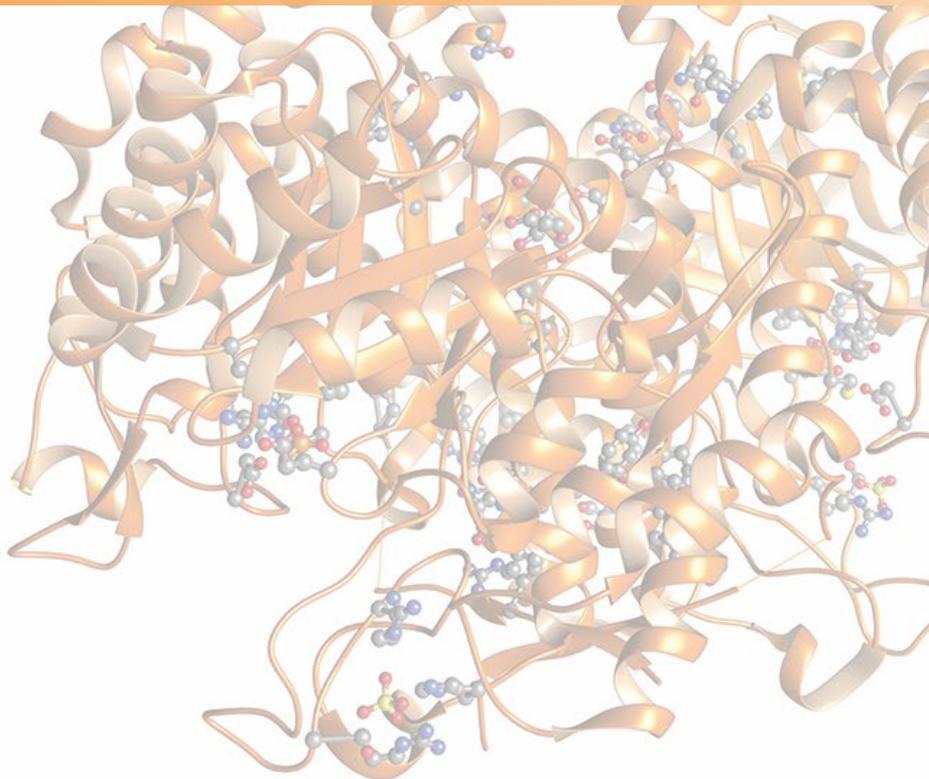
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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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PEPTIDE MAPPING

Chapter 2



Simplifying verification of peptide mapping results: A fundamental capability of the Waters Biopharmaceutical System Solution with UNIFI

GOAL

To demonstrate the enhanced capabilities of the UNIFI™ Scientific Information System to facilitate a user's review of raw LC/MS data from UPLC®/MS or UPLC/MS^E peptide mapping experiments.

BACKGROUND

Peptide mapping is a fundamental tool for the initial characterization of a biotherapeutic protein, and for monitoring that protein for new structural variants and altered levels of known product variants. The execution of such studies with high-resolution MS detection creates an information-rich pool of molecular data that can be challenging to mine for key results.

Software workflows designed for UPLC/MS peptide map analysis, such as BiopharmaLynx™ for MassLynx™ Software and the UPLC/MS peptide mapping workflow within Waters' Biopharmaceutical System Solution with UNIFI, can dramatically reduce data processing time and prevent manual review of data sets.

However, laboratory SOPs and the desire for user verification of key findings often result in the need to go beyond the use of the processed results generated by these tools. Analysts are often required to delve into the raw chromatographic and spectral data to verify information. The UNIFI Scientific Information System has been developed to manage this biopharmaceutical workflow need, and includes enhanced functionality to support such user verification workflows.

User verification of results is simplified, laboratory practices can be standardized, and time between data collection and development decisions will be reduced.

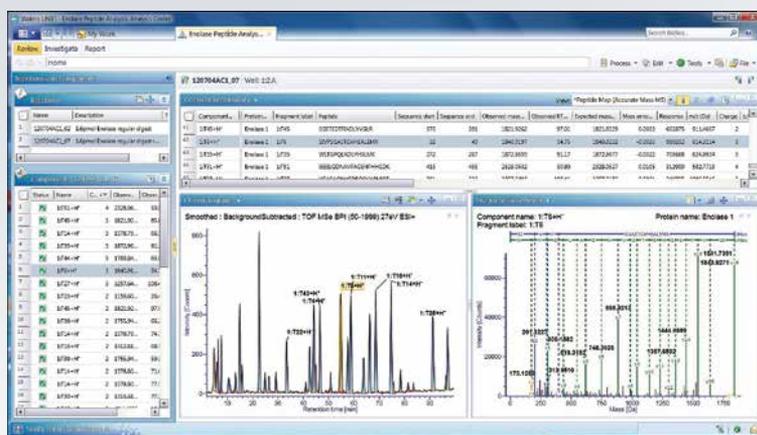


Figure 1. The Review tab in UNIFI facilitates component-driven examination of processed results for UPLC/MS^E peptide mapping data.

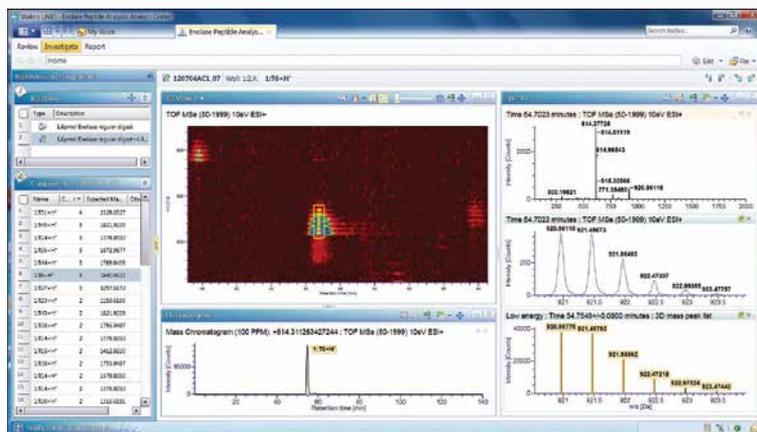


Figure 2. The Investigate tab in UNIFI permits both component-driven and open-ended analysis of raw UPLC/MS^E data.

THE SOLUTION

A UPLC/MS^E peptide map (120 min gradient) was acquired for the Enolase I protein from *S. cerevisiae*. Data were processed using the UNIFI UPLC/MS peptide mapping workflow using typical parameters (< 10 ppm mass error, assignment of tryptic, semi-tryptic, and single missed cleavage peptides, with the possibility of Asn deamidation, and Met oxidation).

A subset of the processed results for the map is displayed as a screen-captured image of UNIFI's Review tab for this data set (Figure 1). The Review tab provides interactive navigation of processed results between the main data table and various graphical display windows. Here, processed data for the selected T6 tryptic peptide is highlighted on the base peak intensity (BPI) chromatogram (~ 54.75 min) in the Chromatograms window, and the associated MS^E spectral data is displayed in the Fragmentation Viewer window. Along with tabular results, such information can be used for rapid confirmation that automated peak assignments were correctly generated.

For unexpected results, the user can dive deeper into the raw data to verify qualitative and quantitative findings in the processed data. Opening the Investigate tab (Figure 2) reveals areas for graphical and tabular display of chromatographic and mass spectral results directly from the raw UPLC/MS data.

These views support open-ended investigations driven by the user, e.g., selecting masses for data extraction, summing spectra, applying background corrections, etc. Conversely, processed components are available to enable component-driven analyses of the raw data. For simplification of this figure, the same T6 peptide was selected for further investigation.

- The 3D graphical viewer tool in Figure 2 (top left, 2D view, smart-zoom of the 2+ ions) reveals the absence of any interference within the spectral data for this component, and highlights the series of isotopic detections within the 2+ charge state cluster.
- More importantly, T6 related extracted ion chromatograms (bottom left), summed spectra (top right, mid right), and processed spectral identification data (bottom right) were automatically generated using selectable commands associated with the component window (Figure 3).
- This component-driven analysis of raw UPLC/MS data not only reduces the amount of time for a user to access this additional map verification data, but standardizes the way in which this is accomplished across a single map, or across all mapping analyses conducted by a laboratory.

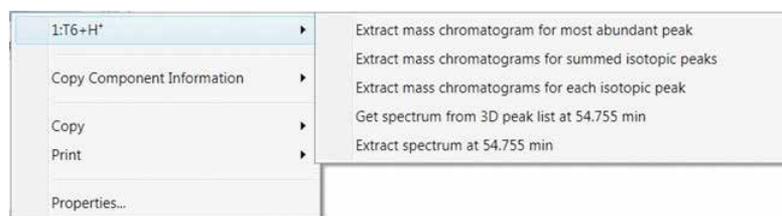


Figure 3. For each component detected, the extracted mass chromatograms, summed MS spectra, and associated processed spectral features can be automatically generated.

SUMMARY

The ability of a scientist to have free reign for the review of raw data underlying a processed data set is a fundamental requirement for software applied for the analysis of complex biotherapeutic UPLC/MS data. The capabilities of the UNIFI Scientific Information System go beyond this basic requirement, and facilitate the intelligent generation of meaningful raw chromatographic and mass spectral data views, using values derived from detected map components. User verification of results is simplified, laboratory practices can be standardized, and the time between data collection and better development decisions will be reduced.

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Generating Automated and Efficient LC/MS Peptide Mapping Results with the Biopharmaceutical Platform Solution with UNIFI

Vera B. Ivleva, Ying Qing Yu, Scott Berger, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The ability to acquire, process, and report LC/MS peptide mapping data has been integrated and automated using the Biopharmaceutical Platform Solution with the UNIFI. This enables laboratories to generate communicable information directly following sample analysis or data review. The UNIFI Scientific Information System organizes data and information and simplifies the review of large amounts of complex peptide mapping results, limiting opportunities for human error, and saving untold hours of formerly manual effort that go into producing the results and reports needed to advance molecules through the development process.

WATERS SOLUTIONS

Biopharmaceutical Platform Solution
with UNIFI®

ACQUITY UPLC® H-Class System

Xevo® G2 QTof MS

KEY WORDS

Peptide mapping, mAb, automation,
reporting

INTRODUCTION

Characterization of protein biopharmaceuticals requires the analysis of primary structure and thorough investigation of sequence variants. Confirmation of protein sequence, quantification of minute changes in the protein covalent structure, and assessment of disulfide bond patterns are routinely performed using LC/UV/MS with the assistance of specialized informatics tools. Most of these informatics tools for the biopharmaceutical laboratory are designed for facilitating intense characterization of novel samples, not for higher throughput data processing and results management for large sets of routine samples.

Increases in peptide mapping samples from increasingly complex quality by design (QbD) methods, formulations, stability, and clone screening workflows have created the need for larger batch processing and reporting of routine analytical studies within a biopharmaceutical laboratory. The Biopharmaceutical Platform Solution with UNIFI merges robust UPLC®/UV/Tof-MS characterization technologies with automated data acquisition, processing, and reporting workflows. The platform is designed to address the challenges of routine analysis with large sample sets in either regulatory compliant or non-compliant laboratory organizations.

This application note details the automated analysis of a set of peptide mapping samples that have been spiked with varying levels of a peptide impurity, using the Biopharmaceutical Platform Solution with UNIFI. Such controlled studies are useful for verifying system performance, and for establishing expectations for subsequent studies conducted with unknown samples. In addition to describing the results from this spiking study, we focus on demonstrating the tools that enable efficient review and reporting of peptide mapping data within UNIFI.

EXPERIMENTAL**LC conditions**

System:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC BEH130 C ₁₈ , 2.1 x 100 mm, 1.7 μm
Column temp.:	65 °C
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	1% formic acid
Detection:	UV 214 nm
Injection vol.:	10 μL

LC gradient table:

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.2	89	1	10	0	Initial
90.00	0.2	35	55	10	0	6
91.00	0.2	10	80	10	0	6
94.00	0.2	10	80	10	0	6
95.00	0.2	89	80	10	0	6
115.00	0.2	89	1	10	0	6

MS conditions

Capillary:	3.0 kV
Sampling cone:	25 V
Extraction cone:	2.0 V
Source temp.:	100 °C
Desolvation temp.:	250 °C
Cone gas flow:	0 L/Hr
Desolvation gas flow:	500 L/Hr
Scan time:	0.5 sec
Low energy:	4 V
High energy ramp:	25 V to 40 V
Mass range acquisition:	100 to 2000 <i>m/z</i>
LockMass compound:	GFP 500 fm/μL, 10 sec interval, 0.5 sec scan time

Informatics

UNIFI Scientific Information System

Biopharmaceutical Platform Solution with UNIFI

- ACQUITY UPLC H-Class System
- ACQUITY UPLC Peptide Separation Technology (PST) Column Chemistry
- ACQUITY UPLC TUV Optical Detector
- Xevo G2 QTof MS
- UNIFI Scientific Information System

Sample preparation**Protein digestion**

Denatured trastuzumab (2.1 mg/mL, 8 M guanidine chloride, 1 M Tris, pH 7.5) was reduced with 0.5 M DTT and alkylated with 0.5 M iodoacetamide. Buffer exchange (100 mM Tris, pH 7.5) over a NAP-5 column (GE Healthcare) enabled efficient tryptic digestion (Sample: Enzyme = 42:1, 4 hrs at 37 °C) for subsequent analysis by peptide mapping.

GFP-spiked digest preparation

[Glu1]-Fibrinopeptide B (GFP) (Sigma) peptide was prepared at 6.4 pmol/μL and 160 fmol/μL in 0.1% formic acid. A dilution series was made using the concentrated trastuzumab digest (19.6 pmol/μL) and GFP solution, then adjusted to final concentrations of 3% acetonitrile and 0.1% TFA, prior to analysis.

GFP peptide was spiked at 0.0% (digest control at 10 pmol/μL), 0.1% GFP (10 fmol/μL), 0.25%, 0.5%, 2.5%, 5.0%, 10% (1 pmol/μL).

RESULTS AND DISCUSSION

In this study, a trastuzumab tryptic digest spiked with GFP enabled us to evaluate the performance of automated UPLC/MS^E peptide mapping workflows within the Biopharmaceutical Platform Solution with UNIFI. To generate the data for this assessment, the trastuzumab digest was supplemented with spiked GFP peptide spanning two orders of magnitude (0.1% to 10% GFP mol/mol) dynamic range, with each level of spiked sample analyzed in triplicate.

UNIFI Software includes a workflow that is specific for accurate mass peptide mapping, constructed of a holistic method that combines UPLC/MS^E acquisition settings, data processing parameters, bioinformatic assignment criteria, and linkage to templates for automated report generation. In this workflow, peptides are assigned by accurate mass, confirmed by accurate mass fragmentation, and assigned to peptides or modified peptides generated by *in silico* digests of one or more proteins defined within the method. Applying this methodology, the GFP peptide was identified and quantified using the automated processing and bioinformatics parameters down to the lowest levels studied (0.1% relative to trastuzumab peptide levels).

The Analysis Center in UNIFI Software provides a central hub to review processed data (Review panel), interrogate the underlying raw data (Investigate panel), examine reports (Report panel), and monitor analytical systems (Acquire panel). The Review panel for the spiked digestion experiment (Figure 1) enables scientists to format processed results to focus on questions about individual samples, to compare binary samples, or to summarize results for specific components across larger sets of data.

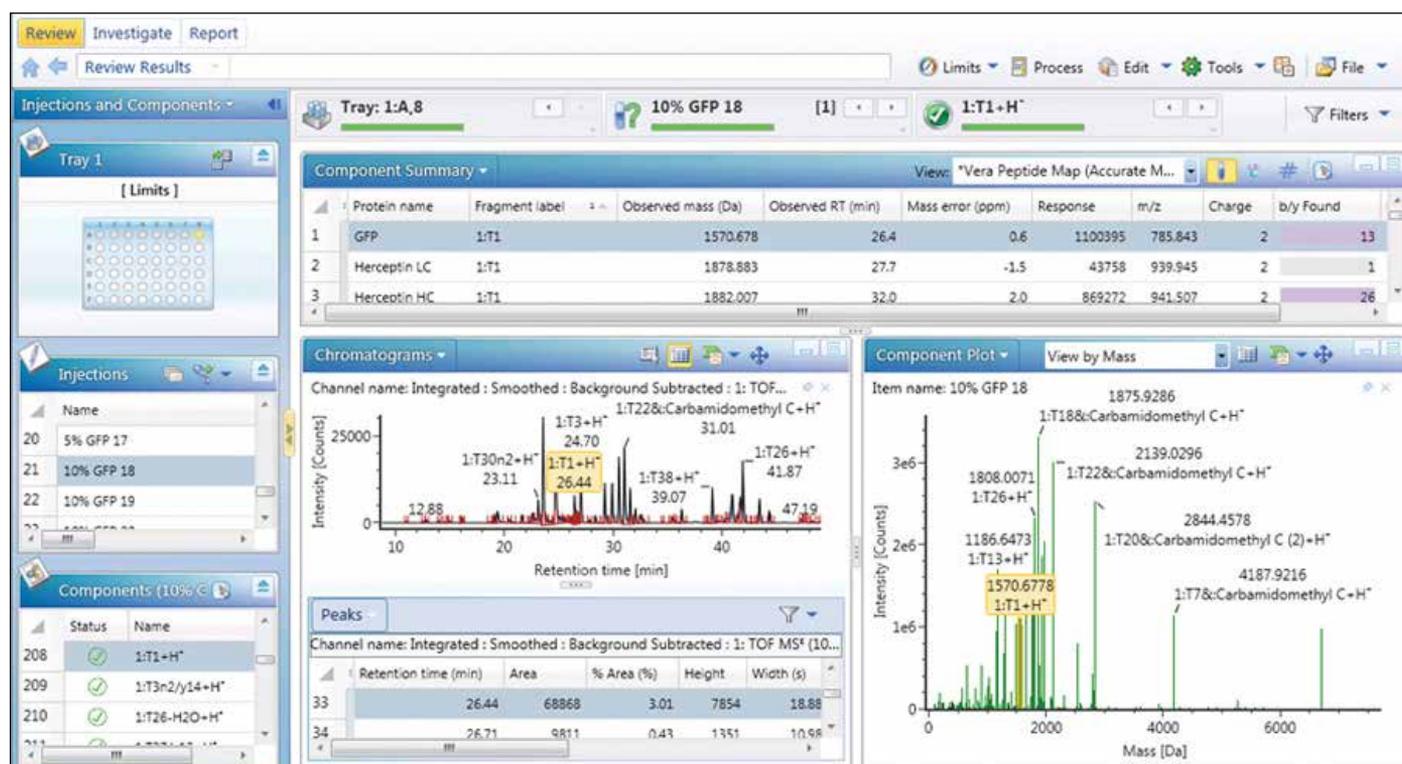


Figure 1. Processed peptide mapping data from the spiked peptide impurity study viewed within the Review tab of the Analysis Center. Highlighted within each of the sub-displays is the spiked GFP peptide.

The spiked GFP peptide is displayed in Figure 3 as the undigested tryptic peptide, T1. Selecting this peptide in the main data table or the associated chromatogram or component plots highlights that component on all the visible panes and enables a researcher to easily scroll through multiple data sets for data relevant to that component. These plots are highly configurable and alternative displays can be used to answer other scientific questions. For example, selecting the Fragmentation Viewer (Figure 2) displays the annotated MS^E spectrum of GFP, enabling the researcher to verify an accurate mass peptide assignment with corresponding characteristic fragment ion information.

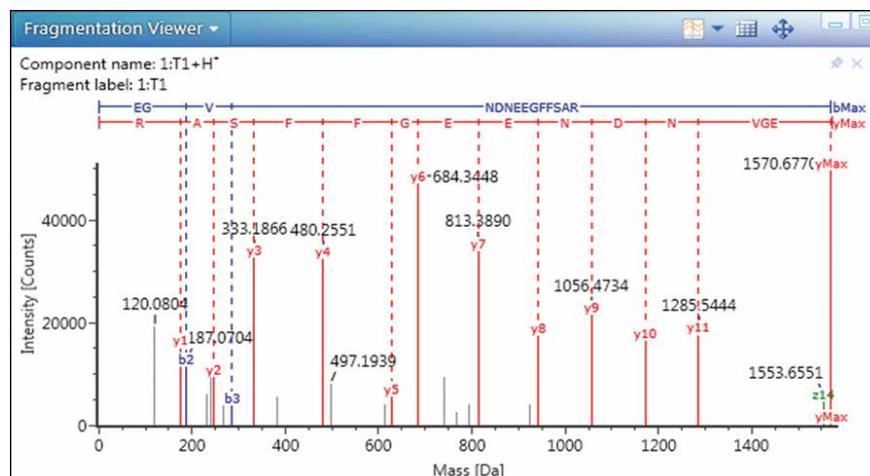


Figure 2. Confirmatory MS^E fragmentation spectrum of the GFP(T1) peptide.

The GFP peptide summary plot (Figure 3) is one of the most informative displays addressing the central questions of the spiked impurity study. The Summary Plot shown in Figure 3 depicts total MS response for the GFP peptide across all experimental samples, on a per-injection basis, and enables us to see that response is proportional to spiked peptide level, and that detection and quantitation was robust across the entire analysis.

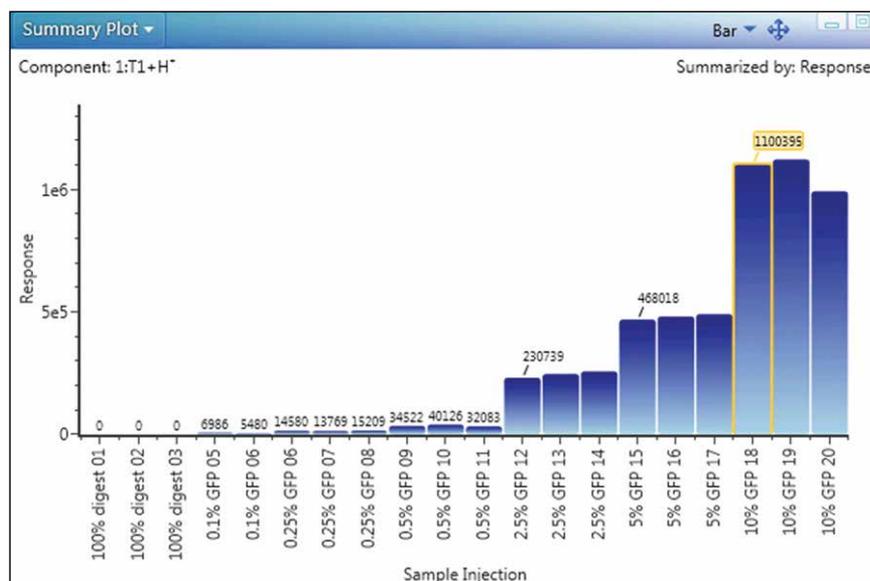


Figure 3. Summary Plot display (MS response) of GFP across all experimental injections.

This ability to focus and summarize results from large sample sets goes beyond the summary plot, as injection replicates, sample replicates, or other data groupings can be utilized to generate tabular result summaries, along with appropriate statistical treatments. These summaries can be generated routinely during automated sample processing (Figure 4, purple box), and displayed within the Review pane.

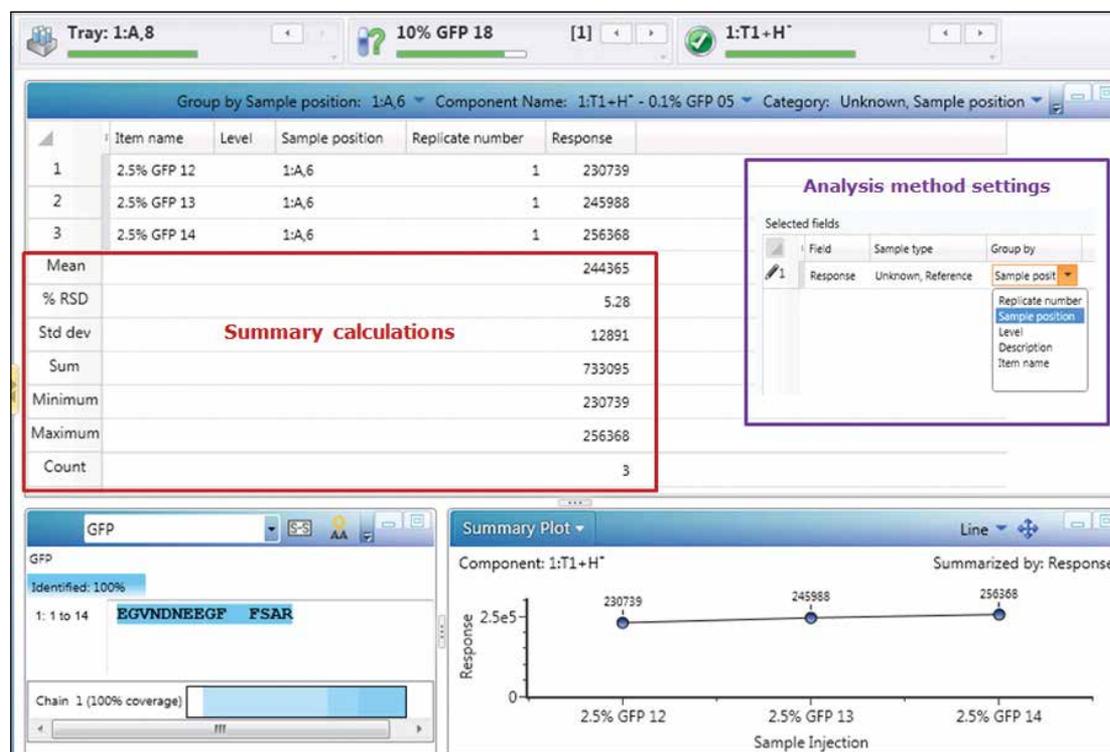


Figure 4. Review tab display featuring the Result Summary for the triplicate analysis of GFP spiked in trastuzumab at the 2.5% level. Highlighted areas represent automated statistical calculations for these injections (red box), and the Analysis Method settings that enabled these calculations to be executed as part of automated processing (purple box).

Adjusting the main data table from a “Component Summary” to “Results Summary” focus enables the summarized display of grouped results for one or more component fields. Figure 4 displays summary results for the GFP peptide for the three injections of the 2.5% spiked sample, enabling automated calculation of average and StdDev and %RSD for this particular triplicate analysis. Executing the tasks of data organization and summarization as part of automated data processing and reporting enables organizations to avoid the transcription and other human errors common with the use of external software packages, and reduces the organizational costs of validating the external tools needed to accomplish this task.

Reporting templates are available for each specific Application Solution (peptide, protein, and glycan analysis) in UNIFI Software. These templates are composed of the individual report objects, which can be rearranged, filtered, and modified to automatically generate the tables, chromatograms, plots and spectra currently used in a researcher’s organization. The object properties as well as the whole report template are configurable by the user, and multiple report templates can be executed for an analysis to answer multiple scientific questions in an efficient format.

A collection of report elements appropriate for the spiked peptide map experiment are displayed within Figure 5. These objects enable a researcher to communicate experimental settings, chromatographic and mass spectral results for individual samples, and the component-focused result summaries available as part of the Review panel functionality described above.

Going from “big data” to communicable information often represents the largest bottleneck in the operations of many biopharmaceutical analytical laboratories. The ability of the Biopharmaceutical Platform Solution with UNIFI to standardize and automate the generation of such information, in both regulated and non-regulated laboratories, enables these organizations to address routine analysis with greater efficiency and effectiveness.

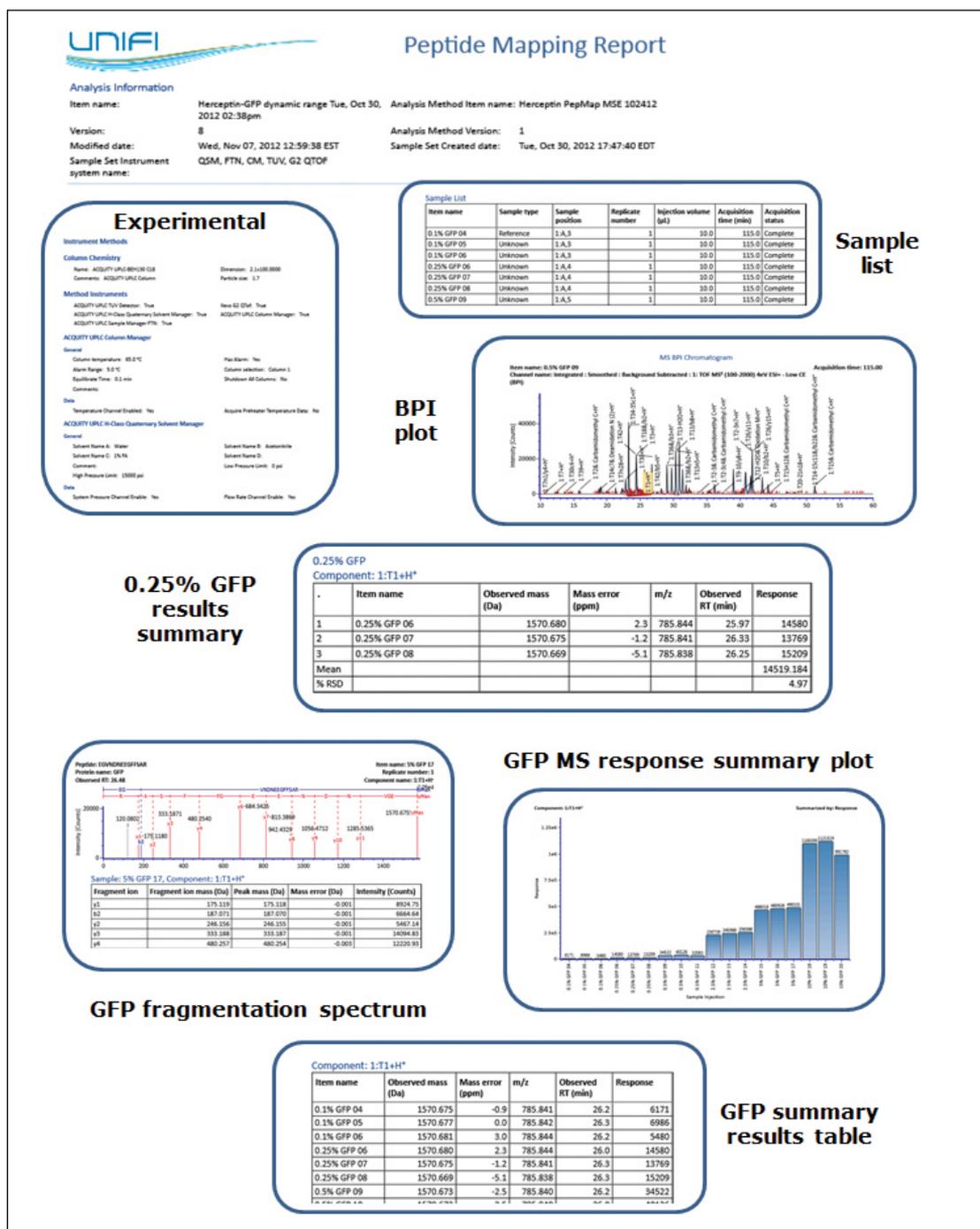


Figure 5. Example peptide mapping report highlighting various report elements commonly applied to display experimental, chromatographic, and spectral mapping results from individual samples and across multiple analyses within the experimental sample set.

CONCLUSIONS

The automated UNIFI peptide mapping workflow was able to identify a spiked peptide impurity down to the lowest concentration tested (0.1% with 100 fmol of digest loaded on-column).

Automated trending and summary statistical calculations executed within UNIFI Software facilitated rapid data review and efficient reporting of the key results from this study.

These results confirmed the utility and reproducibility of UPLC/UV/Tof-MS peptide mapping studies, and their capability to yield quantitative results with high precision and linearity of the MS response.

The ability to integrate and automate such peptide mapping workflows should greatly improve the efficiency of laboratories within regulated and non-regulated biopharmaceutical organizations that are challenged with interpreting complex peptide mapping studies.

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February 2013 720004620EN AG-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

A Streamlined Data Dependent Acquisition (DDA) Peptide Mapping Workflow for Characterizing Therapeutic Proteins Using the Biopharmaceutical Platform Solution with UNIFI

Liuxi Chen and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Biopharmaceutical Platform Solution with UNIFI® provides a DDA workflow for peptide mapping analysis, including FastDDA acquisition, data processing, and automatic report generating. This workflow enables the capability of targeted fragmentation, detailed PTM characterization, and confident sequence confirmation, even for peptides present at low concentrations.

WATERS SOLUTIONS

[Biopharmaceutical Platform Solution with UNIFI](#)

[ACQUITY UPLC® H-Class Bio System](#)

[Xevo® G2-XS QToF Mass Spectrometer](#)

KEY WORDS

Peptide mapping, DDA, mAb, biotherapeutic characterization

INTRODUCTION

LC-MS/MS using data dependent acquisition (DDA) has been widely used to qualitatively characterize therapeutic protein digests. It is used to confirm the primary sequence of proteins and characterize their post-translational modifications (PTMs), such as oxidation, deamidation, and glycation. However, it's also well recognized that this approach has a number of limitations, including under-sampling, a lack of reproducibility, and a lack of in-sample dynamic range. To address this, Waters offers a DDA algorithm called FastDDA. FastDDA is proven to deliver more consistent results between injections and provides greater sequence coverage of proteins.¹

The Biopharmaceutical Platform Solution with UNIFI provides a dedicated DDA peptide mapping workflow, which streamlines data acquisition, processing, and report generation. This streamlined workflow enables an efficient way to analyze a large batch of samples and compile complex results into a comprehensive report.

Here, we illustrate this workflow using a trastuzumab tryptic digest mixture, which has been spiked with a standard peptide leucine enkephalin at 0.1% level. The platform used for this study is comprised of an ACQUITY UPLC H-Class Bio System and an ACQUITY UPLC Tunable UV (TUV) Detector in-line with a Xevo G2-XS QToF Mass Spectrometer. The Xevo G2-XS QToF combines StepWave™ ion optics with an XS collision cell, and significantly increases MS and MS/MS sensitivity without reduction in selectivity.^{2,3}

In this study, we evaluate the MS and MS/MS data quality, especially for low abundant peptides, and the reproducibility of the FastDDA method. In addition, we demonstrate the informatics tools within the UNIFI Scientific Information System that enable efficient reviewing and reporting of the DDA peptide mapping data.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detector:	ACQUITY UPLC TUV
Column:	ACQUITY UPLC BEH C ₁₈ , 300Å, 1.7 μm, 2.1 x 100 mm (p/n 186003686)
Column temp.:	65 °C
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Optical detection:	UV 214 nm
Injection volume:	5 μL
LC gradient table:	

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	0.20	95	5	0	0	6
2	0.20	95	5	0	0	6
40	0.20	57	43	0	0	6
42	0.20	20	80	0	0	6
45	0.20	20	80	0	0	6
46	0.20	95	5	0	0	6
60	0.20	95	5	0	0	6

MS conditions

MS system:	Xevo G2-XS QTof
Capillary voltage:	3.0 kV
Sampling cone:	30 V
Source temp.:	120 °C
Desolvation temp.:	250 °C
Cone gas flow:	0 L/h
Desolvation gas flow:	600 L/h
Acquisition mode:	FastDDA
Mass range (MS and MS/MS):	100-2000 <i>m/z</i>
MS scan time:	0.2 sec
MS/MS scan time:	0.1 sec
Peak detection:	+1, +2, +3, +4, +5, +6
Max. # MS/MS scans/survey:	5

Dynamic peak exclusion:	Acquire and then exclude for 8 sec (± 1.1 Da)
Collision energy:	<i>m/z</i> dependent ramp applied for low and high mass
Stop MS/MS criteria:	TIC 5e ⁸ or 0.4 sec
Lockmass used:	100 fmol/μL of glu-fibrinopeptide B in ([M+2H] ²⁺ , 785.8426)

Data management

UNIFI Scientific Information System v1.8	
Analysis type:	Peptide map (DDA)
Search settings:	1 allowed missed cleavage; carbamidomethyl cysteine (C) is selected as a fix modification; asparagine (N) deamidation and methionine (M) oxidation are set as variable modifications.

Sample preparation

Trastuzumab (1 mg/mL) was denatured in 6.5 M guanidine chloride, 0.25 M tris, pH 7.5. The denatured antibody solution was mixed with 500 mM DTT to a final concentration of 3 mM and incubated at room temperature for 45 minutes, and then alkylated by adding 500 mM iodoacetamide stock solution to a final concentration of 7 mM incubated at room temperature in the dark for 40 minutes. Buffer exchange (0.1 M tris, pH 7.5) was performed with a NAP-5 column (GE Healthcare). Sequencing grade modified trypsin was added to each sample (enzyme to protein ratio 1:25, w/w) and incubated at 37 °C for 5 hours. The digested peptide mixture was diluted to 0.45 pmol/μL. Leucine enkephalin (LeuEnk) was added to the mixture at final concentration of 5 fmol/μL. The injection volume for each LC/MS run was 5.0 μL (2.25 pmol on column per injection).

RESULTS AND DISCUSSION

Trastuzumab tryptic digest mixture spiked with LeuEnk was analyzed in triplicate runs by the automated UPLC®/DDA peptide mapping workflow within the Biopharmaceutical Platform Solution with UNIFI. To evaluate the system's ability to obtain high quality MS/MS data from the low abundant peptides in the peptide mixtures using the FastDDA methods, LeuEnk was added at 0.1% level. The maximum number of MS/MS scans was set as five, which proved to be sufficient for confirming the sequence of the target protein. The exclusion window was set to allow an average of two scans across one chromatographic peak. The charge state reorganization algorithm was used to select 1+ to 6+ ions for fragmentation. In a single run, we observed 95% sequence coverage for light chain and 94% for heavy chain.

Reproducible results were observed in triplicate runs as shown in Figure 1A. The protein sequence coverage map can be displayed for the protein of choice. Criteria for the peptide confirmation can be manually defined by the user based on the purpose of the analysis. The criteria for assigning the identified peptides for current analysis are (1) %matched primary ions is larger than 30%, and (2) mass error on peptide mass is less than 10 ppm. In Figure 1B, confirmed sequences are shown in blue shades. In addition, N-terminal and C-terminal fragments (b/y ions) observed can be displayed on the coverage map as blue (on the top) and red (at the bottom) lines on the sequence map respectively.

LeuEnk peptide, spiked in the sample at 0.1% level, was identified in all three replicate runs with high quality MS/MS data. The annotated DDA spectrum of LeuEnk and extracted ion chromatogram (XIC) of each run is shown in Figure 2A. In addition, peptide modifications present in low abundance were identified with high confidence in this experiment.



Figure 1. A) Sequence coverage comparison in three consecutive runs. B) Sequence coverage map by FastDDA for trastuzumab, provided in UNIFI Scientific Information System, b/y ions observed are also labeled on the map. Blue underline indicates b ions and red underline indicates y ions. The criteria for assigning the identified peptides are (1) %matched primary ions is larger than 30%, and (2) mass error is less than 10 ppm. %matched primary ions = matched primary ions/total number of possible primary ions.

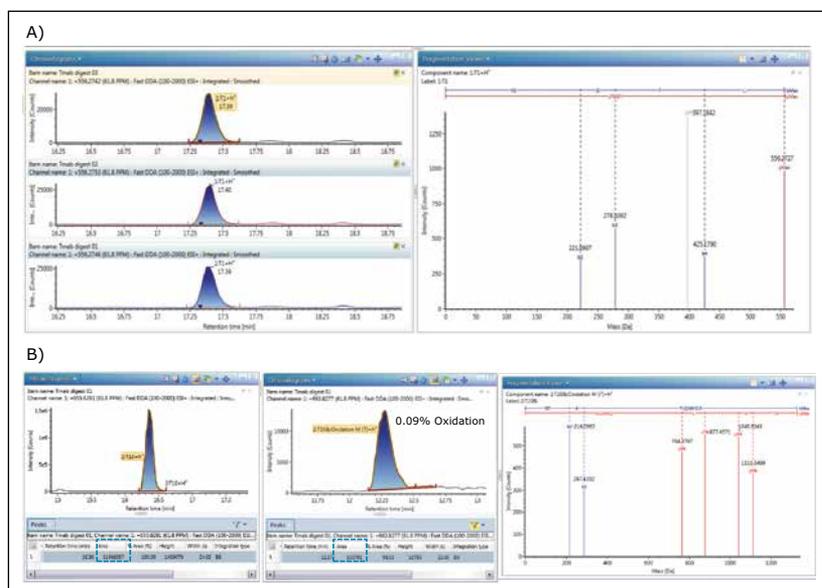


Figure 2. Peptides present at low abundance were identified with high confidence using the FastDDA method. A) LeuEnk was identified in all three injections at 0.1% level (25 fmol load on column). XIC of each run is shown on the left and the annotated MS/MS spectrum is shown on the right. B) XICs of non-modified 2:T10 and oxidized 2:T10, with peak areas shown in the table below. The annotated MS/MS spectrum of oxidized 2:T10 is displayed in the Fragmentation Viewer.

One example of a low abundant 2:T10 peptide (heavy chain, the 10th tryptic peptides from N-terminus) with methionine oxidation was identified at 0.09% level (Figure 2B). Percentage modification can be calculated from peak areas of XICs of non-modified 2:T10 and oxidized 2:T10. Improved instrument speed and sensitivity allows high quality MS/MS spectra, as well as sufficient data points across chromatographic elution peaks. XIC of each component can be displayed in the Review panel (Figure 2B). Fragmentation spectra with the matched b/y ions are shown on the right in the Fragmentation Viewer.

UNIFI Scientific Information System provides a sensible DDA peptide mapping workflow for automatic DDA, data processing, and report generating. In this workflow, peptides are assigned

by accurate mass from the precursors, confirmed with MS/MS fragmentation. The assignment was given to the best matched peptides, or modified peptides generated by in silico digests of proteins defined within the method.

Figure 3 shows the analysis center for the processed results of the DDA peptide mapping data. The Component Summary table at the top of the screen capture shows key attributes from chromatography and mass spectrometry on the identified peptides. On the bottom left, chromatograms of MS and five MS/MS channels are shown. The entire identified components label can be added on all the chromatograms. On the bottom right, the centroid spectra of the MS precursor and MS/MS products are shown to provide a convenient way for on-the-fly spectra review of every identified component.



Figure 3. Review panel of the processed peptide mapping DDA data.

It is important to note that the software is able to automatically assign the specific location of the modifier on the peptide sequence, based on fragmentation pattern. For example, in Figure 4, 1:T3 peptide (the 3rd tryptic peptide from N-terminus) from light chain and its deaminated forms (isoaspartic acid and aspartic acid) were identified with high quality MS/MS spectra; 1:T3&Deamidation N [6] at the top of the fragment display indicates the modification occurs at the sixth amino acid position.⁴

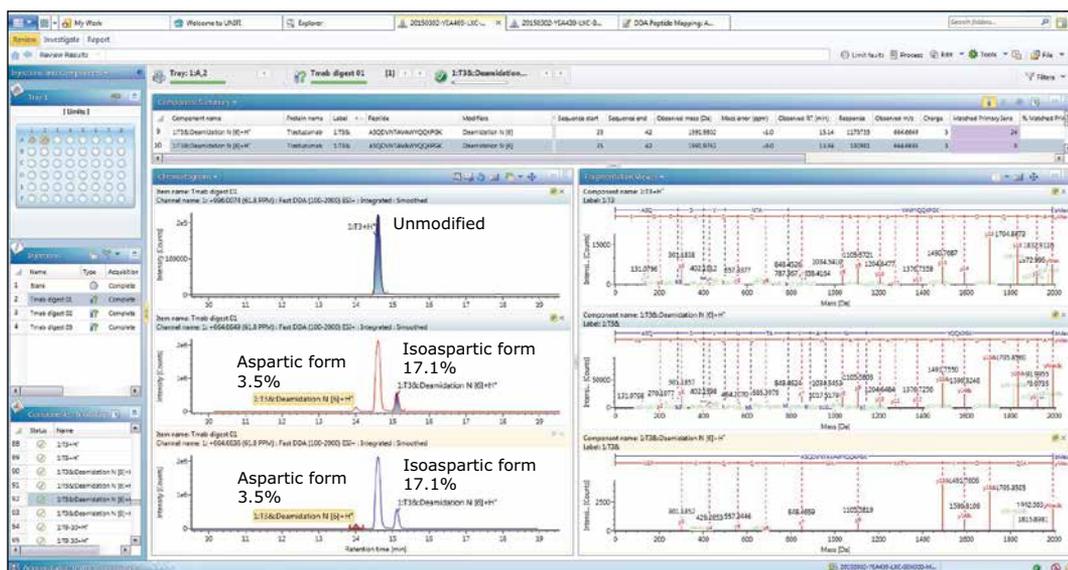


Figure 4. XIC of 1:T3 and deamidation (N) peaks of 1:T3 both the isoaspartic acid and aspartic acid forms are shown on the left, while the corresponding MS/MS spectra of the identified components are displayed on the right.

Reporting templates are available for the UPLC/DDA peptide mapping workflow. Figure 5 provides a snapshot of a typical DDA peptide mapping report; information such as acquisition and sample information, sequence coverage map, identified component plots, and fragmentation viewer, can be organized within a single report format. The object properties, as well as the whole report template, are configurable by the user. Multiple report templates can be executed for an analysis to answer multiple scientific questions in an efficient format.

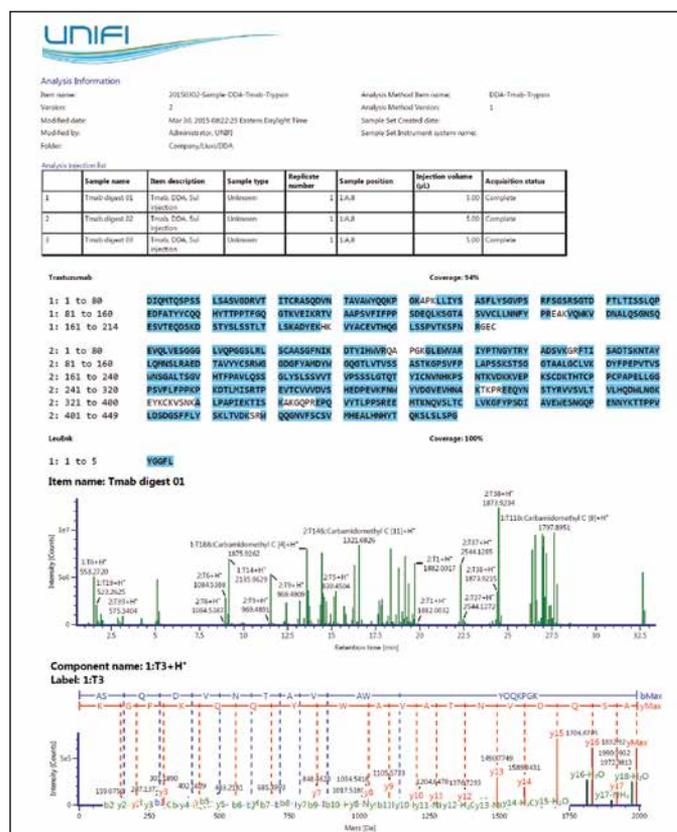


Figure 5. Example of a DDA peptide mapping report.

CONCLUSIONS

The results presented here demonstrate the dedicated DDA peptide mapping workflow within the Biopharmaceutical Platform Solution with UNIFI. The workflow allows seamless integration of data acquisition, processing, and reporting. Combining the FastDDA acquisition method and the high MS and MS/MS sensitivity provided by the Xevo G2-XS QToF, the UPLC/DDA workflow successfully yields high sequence coverage of the mAbs, and confirms the low level spiked-in peptide at 0.1% level and identifies low level modifications like deamidation and oxidation.

The ability to integrate and automate the DDA peptide mapping workflow greatly reduces the time between data collection and drug development decision-making, subsequently improving the efficiency of laboratories that are challenged with interpreting complex peptide mapping results.

References

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Automated Quantitative Analysis of Antibody Drug Conjugates Using an Accurate Mass Screening Workflow in the UNIFI Scientific Information System

Liuxi Chen, Steven Cubbedge, Ying Qing Yu, and Rose Lawler
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Waters® UNIFI® Scientific Information System provides the Accurate Mass Screening workflow to streamline and facilitate the challenging quantitative analysis of antibody drug conjugate (ADC) peptides. This workflow utilizes the UNIFI scientific library as the means to share the peptide and peptide-drug conjugate target component information characterized by the Peptide Mapping workflow within the Biopharmaceutical Platform Solution. This component information is then used to create the target lists and Custom Field to automatically quantify these key attributes of ADCs.

WATERS SOLUTIONS

Accurate Mass Screening workflow with UNIFI

Peptide Mapping (MS^E) Workflow within the Biopharmaceutical Platform Solution

[ACQUITY UPLC® H-Class Bio System](#)

Xevo® G2-S QToF Mass Spectrometer

[Xevo G2-XS QToF Mass Spectrometer](#)

KEY WORDS

Monoclonal antibodies (mAbs), biotherapeutic characterization, antibody drug conjugates, ADC, quantitative analysis, peptide mapping, MS^E

INTRODUCTION

Antibody drug conjugates (ADCs) are a sub-class of biotherapeutics that consist of monoclonal antibodies (mAbs) and cytotoxic drugs linked to mAbs by chemical linkers. ADCs are a complex mixture of conjugates that differ in the number of drugs attached as well as the location of the drug linkage. Previous studies demonstrated that the location, distribution, and site occupancy of the drug molecules play a critical role in the Complementarity Determining Region (CDR) binding efficiency, efficacy, toxicity, stability, and pharmacokinetic profile of ADCs. It is crucial to establish efficient and robust analytical capabilities to characterize the heterogeneity of drug-linker content and modification sites as well as to demonstrate lot-to-lot consistency, stability, and process comparability. Structural characterization, along with qualitative and quantitative analysis of ADCs, is challenging due to the heterogeneous nature of the conjugates and the low abundancies of individually modified species. The quantitation of conjugated peptides and determination of site occupancy ratio has traditionally been done by LC-UV methods, which is limited by the spectroscopic nature of the drug, low sensitivity, and insufficient selectivity. Compared to UV-based methods, MS-based methods can provide improved sensitivity and selectivity. However, MS-based quantitation has its own challenges that often involve monitoring a large number of MS peaks per sample for complex ADC peptides. Currently, efficient quantitation of ADC peptides and direct generation of the key attributes used for ADC comparability studies are impeded by a lack of sophisticated software tools. Waters Accurate Mass Screening (MS^E) workflow within the Screening Platform Solution with UNIFI addresses these challenges and provides targeted component quantitation across samples, Custom Field for key ADC attributes (such as relative conjugation site occupancy ratio), and generation of reports in a streamlined and automated fashion.

EXPERIMENTAL

Liquid chromatography

System:	ACQUITY UPLC H-Class Bio System
Detector:	ACQUITY UPLC Tunable UV (TUV) Detector
Column:	ACQUITY UPLC BEH300 C ₁₈ Column, 300 Å, 1.7 µm, 2.1 mm x 100 mm (P/N 186003686)

Mass spectrometer

Mass spectrometer:	Xevo G2-XS QTof
Acquisition mode:	MS ^E

Informatics

UNIFI 1.8 Scientific Information System

Analysis type:	Accurate Mass Screening (MS ^E) workflow within the Screening Platform Solution with UNIFI, Peptide Mapping (MS ^E) workflow within the Biopharmaceutical Platform Solution.
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Sample information

Antibody drug-conjugated samples were denatured, alkylated, and digested by Asp-N endoproteinase. Leucine enkephalin (LeuEnk) was added to each sample at a final concentration of 50 fmol/ul as an internal standard.

In this application note, lysine-conjugated ADCs were used to demonstrate the utilization of the Accurate Mass Screening workflow to monitor the relative abundance of ADC conjugated peptides across samples and to complete relative conjugation site occupancy ratio calculations automatically. The reported method is universal and can be applied to ADC therapeutics generated by different conjugation chemistries. Multiple features in UNIFI facilitate the quantitation analysis. First, the library of target components, including accurate mass values and optional retention times, is created. This was achieved by processing representative samples using the Peptide Mapping (MS^E) analysis that identifies and labels the various peptide and peptide-drug conjugates. These are then selected and the relevant information entered into the UNIFI scientific library. The target components are therefore stored and easily retrievable to construct analysis methods. Custom Field enables the automated calculation of important product attributes of the ADCs. This is completed by using a Custom Field when setting up the processing method. In addition, UNIFI features a review panel that provides the ability to easily organize and save data review workflow steps, including user-configured plots and tables for consistent data display and straightforward communication. All of the relevant information including the use of result filters is available to the user and can be copied into reporting templates to visualize the results and generate analysis-specific reports.

RESULTS AND DISCUSSION

Step 1: Data acquisition and processing to create the library of target components

LC-MS^E peptide mapping data were acquired using the ACQUITY UPLC H-Class Bio System (with both UV and MS detection) and Xevo G2-XS QToF MS. MS^E data acquisition enables sufficient points across the chromatographic elution peaks and provides MS (Low CE) and MS-MS (High CE) spectra in alternate scans. The data was processed using the Peptide Mapping MS^E workflow. This software identifies the potential peptide and peptide-drug conjugate components through de-novo digestion of a target protein and searches the accurate mass MS and MS-MS data considering adducts and modifications, including the linker and drug. The identified components can then be saved into a user-defined UNIFI scientific library. The Peptide Mapping workflow presents identified components and quality attributes that indicate the relative confidence of the identification. User-defined filters can be created and saved such that these quality attributes can be applied to the components that will be stored within the library and used as the basis for the creation of the target list in the Accurate Mass Screening method.

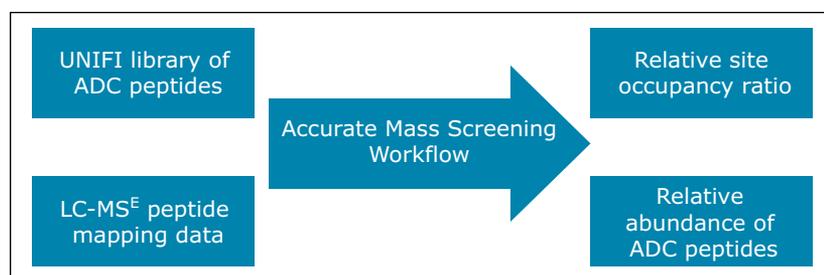


Figure 1. Quantitative analysis workflow of antibody drug conjugates with UNIFI Accurate Mass Screening.

Step 2: Contents and use of the UNIFI scientific library

The UNIFI scientific library is a central library function within the software that contains commercial and user-created libraries. These libraries can be used to store component-centric chemical and physical information as well as detection results. This information can be used to facilitate the creation of analysis methods. Figure 2 shows an example of an ADC peptide library and detection results, including information such as the expected mass-to-charge ratio (m/z), expected retention time, and expected fragment ion m/z . Information can be imported through an excel spreadsheet, input into the library manually, or sent from the results of certain UNIFI analysis types.

The screenshot displays the UNIFI software interface. At the top, there is a 'Search criteria' section with a table containing one entry: 'Name' with the operator 'equals' and a search value. Below this is a search bar with the text 'ADC-AspN-PeptideOnly, ADC-AspN-...' and a 'Show 100 Results' button. The main area shows 'Search results (63 items found)' with a table listing items 6 through 10. Each item includes a name (e.g., '1:09+H⁺'), a library name ('ADC-AspN-PeptideOnly'), and a 'Has detection results' checkbox. Below the search results is a 'Detection Results' section with a table showing two entries. The first entry has a priority of 1, intensity of 1, formula of '4335.0239 +H', neutral mass of 4335.0239, charge of +1, fragmentation type of 'None', expected m/z of 4336.1112, observed m/z of 43.969, expected RT of 43.969, observed RT of 43.969, ionization technique of 'ESI+', and detail type of 'MS/MS'. The second entry has a priority of 2, intensity of 2, formula of 'CID', neutral mass of 1064.7832, charge of 0, fragmentation type of 'CID', expected m/z of 1064.7832, observed m/z of 43.969, expected RT of 43.969, observed RT of 43.969, ionization technique of 'ESI+', and detail type of 'MS/MS'.

Priority	Intensity	Formula	Neutral Mass (Da)	Adduct	Charge	Fragmentation type	Expected m/z	Observed m/z	Expected RT (min)	Observed RT (min)	Ionization technique	Detail type
1	1		4335.0239	+H	+1	None	4336.1112	43.969	43.969	43.969	ESI+	MS/MS
2	2					CID	1064.7832	43.969	43.969	43.969	ESI+	MS/MS

Figure 2. An example of an ADC peptide library entry.

Step 3: Accurate Mass Screening workflow method settings

a. Manage Components table

First, the Manage Components table is created. Screening components can be imported directly from the scientific library. Figure 3 shows an example Manage Components table. Please note that in order to recognize the conjugated peptide with its unconjugated peptide counterpart, the peptide sequence must be included as an “item tag” for each component. This item tag is stored in the library along with the peptide sequence from the result fields in the Peptide Mapping MS^E analysis. The internal standard can be added to the component table as shown in Figure 3. The expected retention time (RT) field is not required; however it can increase screening accuracy if the value is known from a previous analysis. The screening time window can be defined by the “target by retention time” setting in the analysis method. The expected neutral mass (Da) value for each component is required for the screening workflow. Mass-to-charge (m/z) values of different charge states or peptide adduct ions (m/z) are automatically calculated from the neutral mass during data processing.

Manage Components								
Create Import Paste Results Delete Edit Fragments... Edit Adducts... Add To Common Fragments Add To Neutral Losses								
Component name	Label	Item tags	Expected RT (min)	Expected neutral mass (Da)	Expected wavelength (nm)	Internal standard?	Use internal standard	
1	IS		18.82	555.2683	214.0	<input checked="" type="checkbox"/>		
2	1:D6&Carbamidomethyl C [13]+H ⁺	DEQLKSGTASVCLLNFPYREAKVQWKV	31.30	3378.7394	214.0	<input type="checkbox"/>	IS	
3	1:D6&Carbamidomethyl C [13], Drug+Linker [24]+H ⁺	DEQLKSGTASVCLLNFPYREAKVQWKV	43.05	4335.1039	214.0	<input type="checkbox"/>	IS	
4	1:D6&Carbamidomethyl C [13], Drug+Linker [5]+H ⁺	DEQLKSGTASVCLLNFPYREAKVQWKV	43.70	4335.1039	214.0	<input type="checkbox"/>	IS	
5	2:D16+H ⁺	DIAVEWESNGQPENNYKTPPVL	25.55	2600.2395	214.0	<input type="checkbox"/>	IS	
6	2:D16&Drug+Linker [17]+H ⁺	DIAVEWESNGQPENNYKTPPVL	44.70	3556.6039	214.0	<input type="checkbox"/>	IS	

Figure 3. Manage Components table in the Accurate Mass Screening workflow.

b. Processing parameters

Figure 4 shows the “processing sections” of the accurate mass screening workflow. The quantitative analysis of ADC peptides is facilitated through multiple features of UNIFI, some of which are highlighted and described in detail in Figure 4. The users have the flexibility to define the channel (UV or MS) or ions used for quantitation (most intense monoisotopic or sum of all adducts and isotopes). MS components can be quantified using the 2D peak (2D extracted ion chromatograms (XIC) area or height) or 3D peak volume. In addition, all the quantitation settings can be set on a per-component basis to allow flexibility in the application of different settings for separate target groups.

The screenshot displays the 'Peak Processing Settings' window with several sections highlighted by orange boxes and callouts:

- Targeted Screen Settings:** Includes 'Target by Retention Time' and 'Target by Mass'. A callout states: "Discovery settings allow users to validate the ADC peptides by confirming the common fragment ions or common neutral loss."
- Discovery Settings:** Includes 'Mass Defect Settings', 'Halogen Match Settings', 'Common Fragment Settings', and 'Common Neutral Loss Settings'. 'Common Fragment Settings' is highlighted.
- Quantitation Settings:** Includes 'Calibrate' and 'Quantify'. A callout states: "Quantitation settings allow user to define the method for calibration and choose 2D or 3D peaks to quantify."
- Analysis Specific Settings:** Includes 'Adducts', 'Lock Mass', 'Processing Rules', and 'Chromatographic Performance Settings'. 'Adducts' is highlighted. A callout states: "Adducts allow users to define max number of protonation or metal ions adducts."
- Custom Fields and Limits:** Includes 'Custom Fields', 'Summary Calculations', and 'Limit Checks'. 'Custom Fields' is highlighted. A callout states: "Custom formulas can be defined to summarize the peaks at different retention times and facilitate the site occupancy ratio calculation."

Figure 4. Processing parameters overview of the Accurate Mass Screening workflow.

Step 4: Setting up Custom Fields

Custom Field is designed to perform the specific calculations for target analysis. The Custom Field can be stored in the analysis method with the results directly available for viewing, plotting, and reporting. Automated calculations within the analysis method eliminate the tedious extra steps for manual data manipulation and re-processing. Custom Field and analytical methods are protected by a secure framework via configurable user access controls based upon roles that facilitate their use in routine analysis, particularly in regulated laboratories.

Custom Field and the formulae to support the calculation of ADC attributes in a quantitative analysis are shown in Table 1. The Custom Field is formulated generically so that it can be applied to other types of ADC peptide analyses. The text marker “drug” is used to identify the conjugated peptide components in the screening results. As long as the component name of the conjugated peptides contains “drug” in the Manage Components table, the Custom Field will find these components and apply the determined values. “Calculate amount” is a standard field generated by UNIFI processing that is obtained by normalizing the intensity of each identified quantitative response against the spiked-in internal standard response. The peptide sequence values are included in the “item tag” field, so that the peptide components with the same sequence can be summed using the “Peptide Tot AMT” Custom Field. Using these four Custom Fields, users can effectively monitor the amount of conjugated peptides and relative site occupancy ratios across sample sets.

Custom field name	Description	Data type	Formulas
DRUG	Identify the conjugated peptides components	Component	$IF(FIND(\text{Component name}, \text{“Drug”}), 1, 0)$
Peptide-Drug AMT	Sum of conjugated peptide amount	Component	$SUM(\text{Calculated amount}, \text{Item tags}=\text{VALUE}(\text{Item tags}, \text{CURRENT}) \& \text{DRUG}=1)$
Peptide Tot AMT	Sum of amount of conjugated peptide and unconjugated peptide	Component	$SUM(\text{Calculated amount}, \text{Item tags}=\text{VALUE}(\text{Item tags}, \text{CURRENT}))$
Site occupancy ratio	% amount of the conjugated peptide in the total amount for the peptide based on the same sequence	Component	$\text{Peptide-Drug AMT} / \text{Peptide Tot AMT}$

Table 1. Custom Field settings for the conjugated peptide amount and site occupancy ratio calculations. Blue indicates “functions.” Red indicates “fields.” Green indicates a vector of a function. Site occupancy ratio = $\text{area}(\text{conjugated pep. peak}) / [\text{area}(\text{unconjugated pep. peak}) + \text{area}(\text{unconjugated pep. peak})]$.

Step 5: Results review

The detection and calculation results are displayed in the UNIFI review tab as shown in Figure 5. It consists of three areas:

- The Component Summary table which includes the user-specified result fields in this example for all of the identified components which were selected through the use of a filter.
- The user-configured Chromatogram panel that displays the XICs of the selected component(s). In this example it contains the three XICs for the two diastereomers of the conjugated peptide 2:D13 and the unconjugated peptide 2:D13 (Heavy chain, the thirteenth Asp-N peptides from N-terminus).
- The summary plot can be used to plot the value of any component field across the samples in a batch. This includes Custom Field, which is illustrated by the plotting of the relative site occupancy ratio plotted as a bar chart from duplicate injection of three ADC samples.

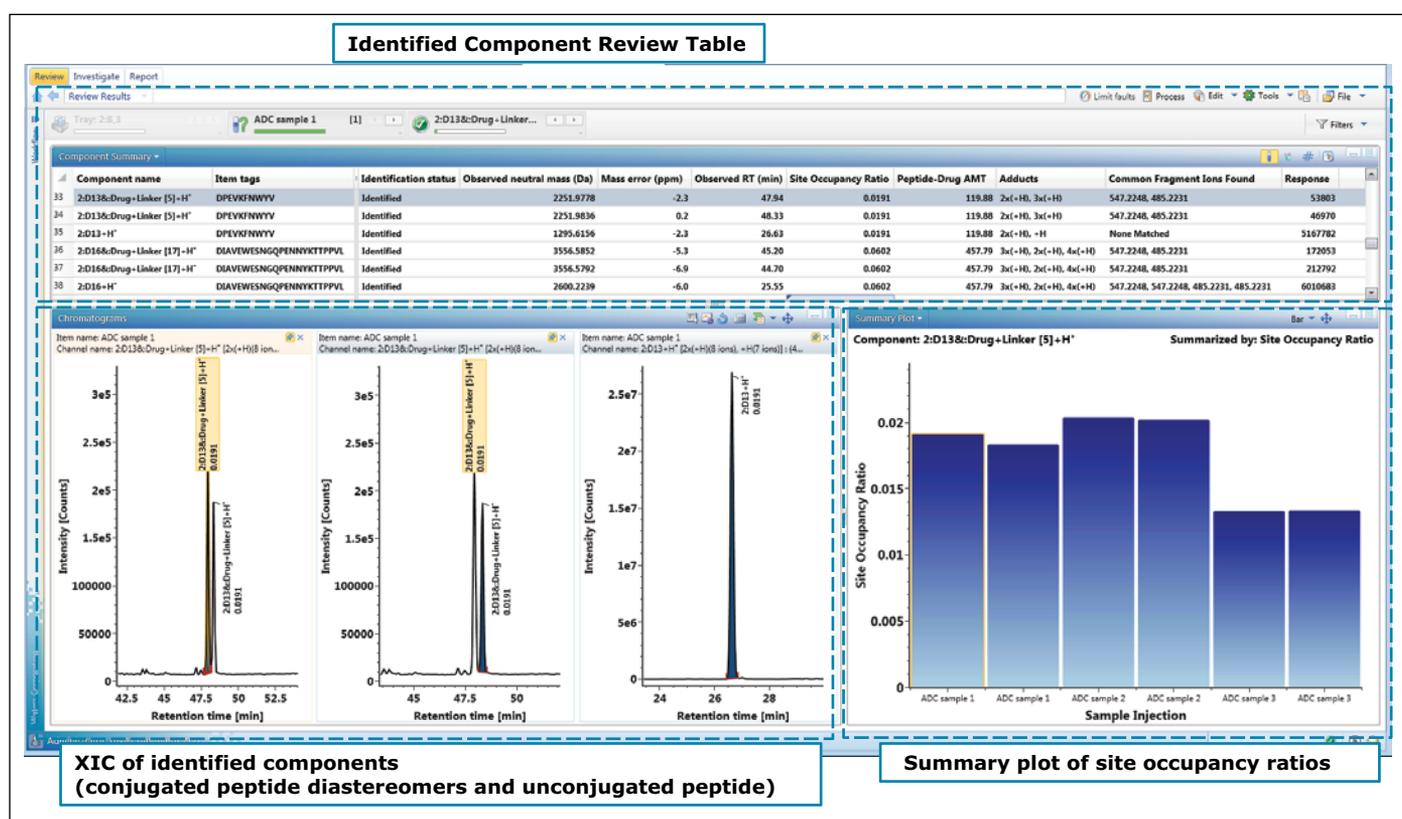


Figure 5. Identified Component Review table of the three ADC samples.

CONCLUSIONS

This application note describes how to perform ADC peptide quantitation using the Accurate Mass Screening workflow in UNIFI and further demonstrates the capability of comparing the key product attributes of ADCs across samples through this automated workflow. This eliminates tedious and labor-intensive manual data processing, which reduces associated human errors. Furthermore, the defined workflow in UNIFI can be saved and transferred to promote high quality data generation, consistent processing, and reporting for complex ADC samples. Combined with the UNIFI Peptide Mapping MS^E workflow, this will enable scientists to overcome the analytical challenges for both structural characterization as well as qualitative and quantitative analysis of complex ADCs. Collectively the Accurate Mass Screening workflow (quantitative tool) combined with UNIFI scientific library and Custom Field is a great addition to the traditional Peptide Mapping MS^E workflow (qualitative tool) within the same UNIFI architecture to facilitate the challenges of ADC biotherapeutics research and development.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Automated Disulfide Bond Mapping, and Comparing Innovator and Biosimilar mAbs Using UNIFI

Henry Shion, Min Du, and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

This application note describes a UPLC-MS^E analysis of monoclonal antibody (mAb) disulfide bond mapping using the Waters® Biopharmaceutical Platform Solution with UNIFI.® This study demonstrates that UNIFI – a compliance-ready software – is designed to support biopharmaceutical laboratories in performing fast peptide mapping and monitoring of mAb disulfide linkages.

WATERS SOLUTIONS

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[Xevo® G2-S and Xevo G2-XS QToF MS Systems](#)

[Intact mAb Mass Check Standard](#)

[ACQUITY UPLC BEH Column](#)

KEYWORDS

Biotherapeutics, infliximab, innovator, biosimilar, Waters Intact mAb Mass Check Standard, peptide mapping, non-reduced digestion, disulfide bonds, post-translational modifications (PTMs), ACQUITY H-Class Bio, Xevo, Waters Biopharmaceutical Platform Solution with UNIFI, compliance-ready

INTRODUCTION

Disulfide bond formation is critical for the structure, stability, and biological functions of therapeutic proteins.¹ Disulfide bond linkage assignment, localization, and monitoring are therefore of great importance to ensure process consistency and product integrity during biotherapeutic drug development. Regulatory agencies, including the EMA and FDA, have specific requirements for mapping the disulfide bonds in biotherapeutics, such as monoclonal antibodies (mAbs).^{2,3}

In this application note, we describe a streamlined disulfide bond mapping analysis workflow using the Waters Biopharmaceutical Platform Solution with UNIFI. It combines UPLC®, UV, and QToF MS characterization technologies for automated data acquisition, processing, and reporting under the compliance-ready architecture of the UNIFI Scientific Information System. As shown in Figure 1, this workflow was used for the disulfide bond mapping analysis of the Waters Intact mAb Mass Check Standard, and for three batches each of infliximab innovator and biosimilar samples.^{4,5} This approach provides biopharmaceutical laboratories with a compliance-ready tool for fast mapping and monitoring of the disulfide linkages of mAbs.



Figure 1. Disulfide bond mapping using the Waters Biopharmaceutical Platform Solution with UNIFI. The workflow encompasses automated UPLC-MS^E data acquisition, processing, and reporting.

EXPERIMENTAL

LC conditions

Column:	ACQUITY UPLC BEH C ₁₈ , 1.7 μm, 2.1 mm x 100 mm (P/N 186002352)
Column temp.:	65 °C
Mobile phase A:	water*
Mobile phase B:	acetonitrile*
Mobile phase C:	1% formic acid in water*
Mobile phase D:	not used
Detection:	ACQUITY UPLC Tunable UV (TUV), 280 nm

LC gradient table:

Time	Flow rate	Composition				Curve
		A	B	C	D	
0.0	0.20	89.0	1.0	10.0	0.0	Initial
60.0	0.20	48.0	42.0	10.0	0.0	6
61.0	0.20	10.0	80.0	10.0	0.0	6
64.0	0.20	10.0	80.0	10.0	0.0	6
65.0	0.20	89.0	1.0	10.0	0.0	6
75.0	0.20	89.0	1.0	10.0	0.0	6

Total run time: 75 minutes

*LC-MS grade water, acetonitrile, and formic acid are highly recommended for mobile phase preparations.

MS conditions

Data acquisition mode:	Positive sensitivity
Capillary:	2.5 kV
Sampling cone:	50 V
Source offset:	40 V
Source temp.:	100 °C
Desolvation temp.:	250 °C
Cone gas flow:	0 L/Hr
Desolvation gas flow:	800 L/Hr
Data acquisition mass range:	$m/z = 100$ to 2000 amu
Lockmass:	Glu Fibrinopeptide B at 100 fmol/μL in 50:50 water-acetonitrile, 0.1% formic acid

Data management

Biopharmaceutical Platform Solution with UNIFI

Sample preparation

Three batches of the infliximab innovator product (REMICADE®) were purchased from Janssen (Horsham, PA). The product was produced in a SP2/0 mouse cell line. Three batches of infliximab biosimilar samples produced by an alternative mammalian cell line (Chinese hamster ovary, CHO) were obtained from an outside collaborator. All of the samples were stored at -80 °C before analysis. The concentration of all the batched samples was at 21 mg/mL.

For the Waters Intact mAb Mass Check Standard, a 3 mg/mL stock solution was prepared by dissolving the mAb in 1 M Tris (Sigma Aldrich P/N T2444, pH 7.5). The solution was then sonicated for five minutes before following the non-reduced protein sample digestion protocol as described next.

Non-reduced sample digestion protocol

For the infliximab and biosimilar candidate batches, 25 µL of the stock solution (at 21 mg/mL) was mixed with 150 µL of 1 M Tris and 325 µL of 8 M GdnHCl (Sigma Aldrich P/N 50937) in order to prepare a 1 mg/mL mAb solution. For the Intact mAb Mass Check Standard, 175 µL of the stock solution was mixed with 325 µL of 8 M GdnHCl in order to prepare a 1 mg/mL mAb solution. The samples were denatured for one hour at 37 °C. After cooling down to room temperature, 7 µL of 0.5 M iodoacetamide (Sigma Aldrich P/N 11149) was added and the samples were incubated in the dark for 30 minutes.

Next, the alkylated protein samples were buffer exchanged into a pH 7.5 digestion buffer (0.1 M Tris) using an illustra NAP-5 Column (GE Healthcare). The NAP-5 Column was equilibrated per the manufacturer's instructions (equilibration volume = 10 mL of digestion buffer; the NAP-5 Column holds 3 mL of solution at a time). 0.5 mL of the alkylated protein solution was transferred into the NAP-5 Column after the equilibration step, allowing the mAb solution to flow completely through the column. The flow was directed to a waste container. Only the desalted mAb collected was eluted with 0.50 mL of the digestion buffer. Solubilized trypsin (Promega P/N V5111, 20 µg) was added to the buffer-exchanged mAb solution (about 500 µg mAb, trypsin-to-protein ratio ~1:25). The samples were digested at 37 °C for four hours. The trypsin reaction was stopped by adding 0.5 mL of a quench solution (474 µL water, 25 µL acetonitrile, 0.5 µL trifluoroacetic acid). The final concentration of the mAb was estimated at 0.5 mg/mL. Triplicate LC-MS analyses were performed for each mAb digest.

RESULTS AND DISCUSSION

The Waters Biopharmaceutical Platform Solution with UNIFI supports a variety of analytical workflows designed for comprehensive biotherapeutic protein analysis. These workflows include intact protein, subunit, and peptide mapping analyses.⁵⁻⁹ Disulfide bond analysis is completed using the peptide mapping workflow with disulfide bond searching parameters selected (Figure 2). There are two search criteria for the disulfide peptide mapping: constrained and relaxed. The "constrained" option in UNIFI "limits the generation of disulfide-bonded peptides to digestion products, missed cleavages, and semi-digestion products." The "relaxed" option "allows the consideration of all possible peptide products."⁴ Users can link disulfide bonds in the protein sequence if there is prior knowledge. Without linked cysteines in the protein sequence, both search criteria will look for all scrambling situations. The possible disulfide peptide targets increase dramatically when no data search restrictions are set – therefore increasing process time. The fastest processing with the most confident confirmatory assignments is achieved through use of "constrained" criteria with minimum PTMs and linking known C-C bonds in the sequence.

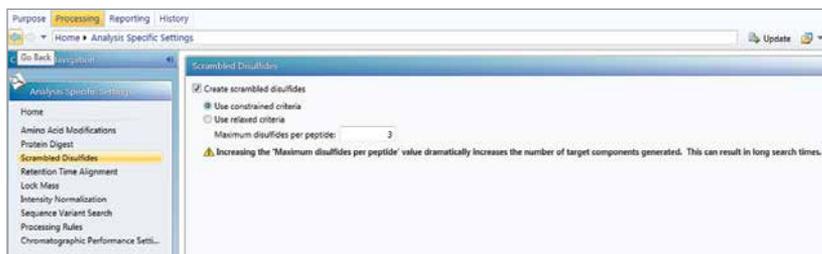


Figure 2. Disulfide bond searching parameters defined in a peptide mapping analysis method in UNIFI.

Search results are displayed in a review panel after data processing. Figure 3 shows the processed results from the Intact mAb Mass Check Standard non-reduced tryptic digestion mapping experiment. Only disulfide-containing peptides are displayed after application of a data filter. All seven unique disulfide bonds were found. There are many ways to display the result – e.g. in a sequence coverage map, component table, or component plot. The selected disulfide bond-containing peptide (1:T21-2:T17-3:T17-4:T2) is highlighted in all modes of display.

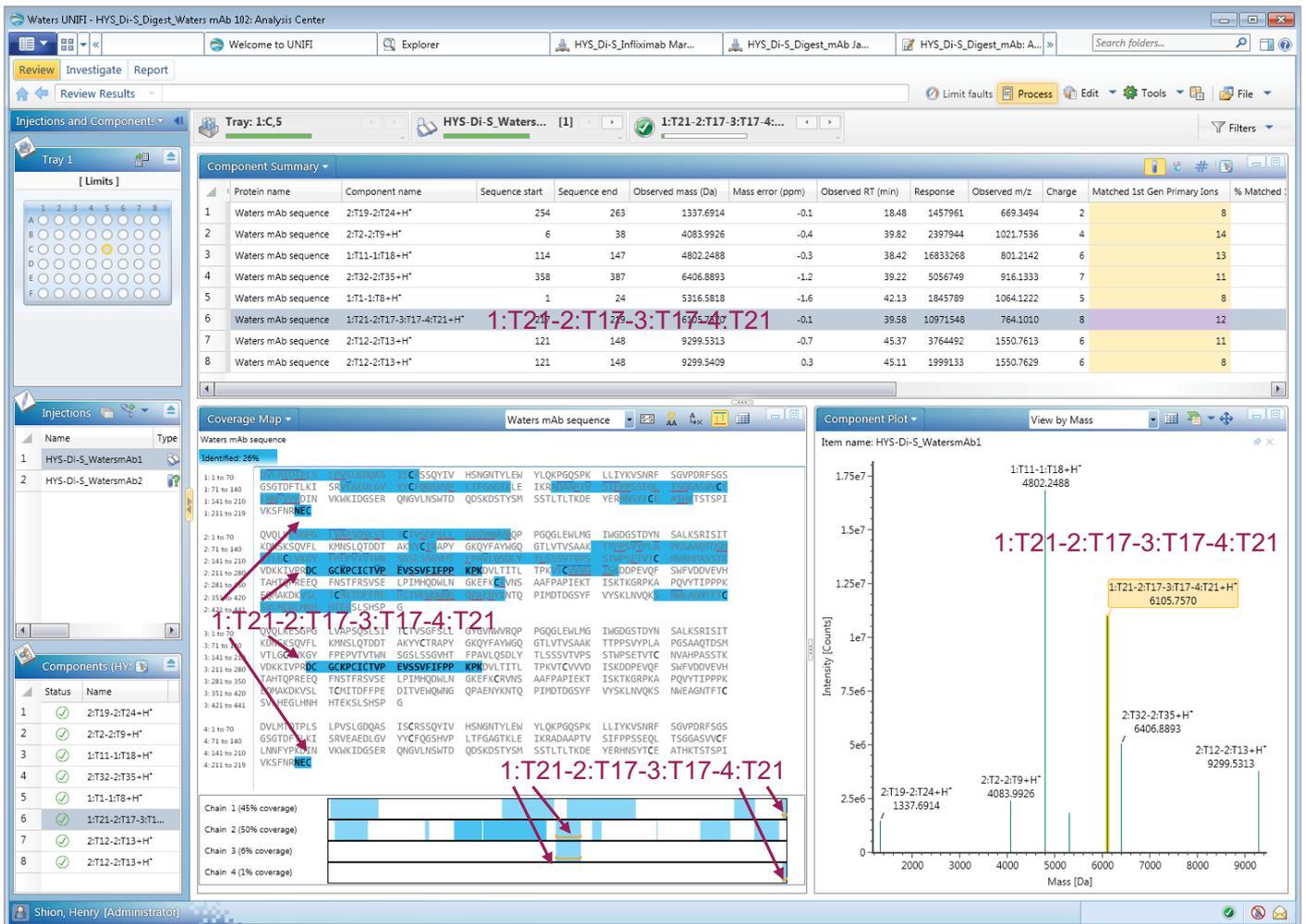


Figure 3. Only disulfide bond-containing peptides are displayed using a simple filter in the UNIFI review panel.

The disulfide bond-containing peptides are confirmed from the high collision energy spectra. Figure 4 shows the MS-MS spectrum of the highlighted peptide (1:T21-2:T17-3:T17-4:T21) from Figure 3.

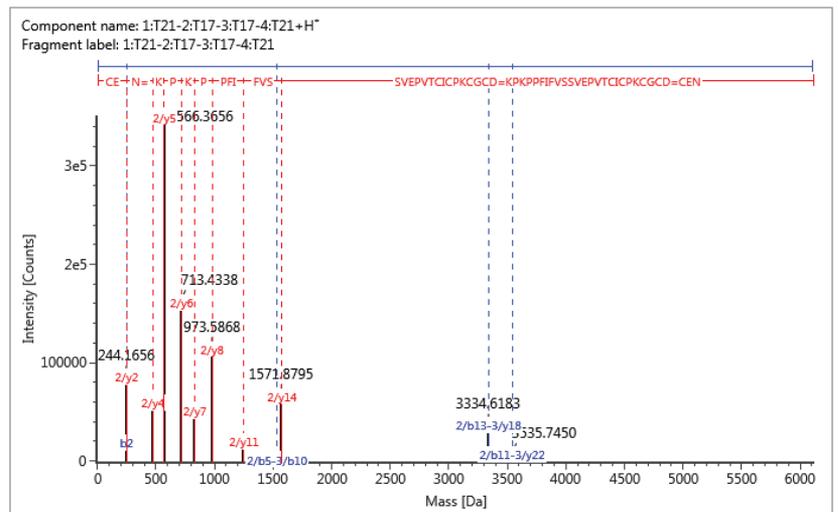


Figure 4. MS-MS spectrum of the disulfide bond-containing peptide of 1:T21-2:T17-3:T17-4:T21 from an MS^E experiment. Because the mAb was not reduced, this particular peptide contains both inter- and intra-peptide disulfide bonds connecting duplicated tryptic peptides from heavy and light chains.

Because the peptide mapping workflow in UNIFI has a binary comparison ability for chromatograms or spectrum displays, we can now look at the innovator and biosimilar peptide maps in comparison mode. Figure 5 is a mirror image plot of the MS total ion chromatogram (TIC) comparison between an infliximab innovator batch and a biosimilar candidate batch. Figure 6 shows a mirror image plot of the confirmed disulfide-linked peptides comparing an infliximab innovator and biosimilar batch. After reviewing all of the peptide mapping results and carefully examining the data using the binary comparison tool, no differences were observed between any of the batches of infliximab and biosimilar candidates in terms of disulfide linkages.

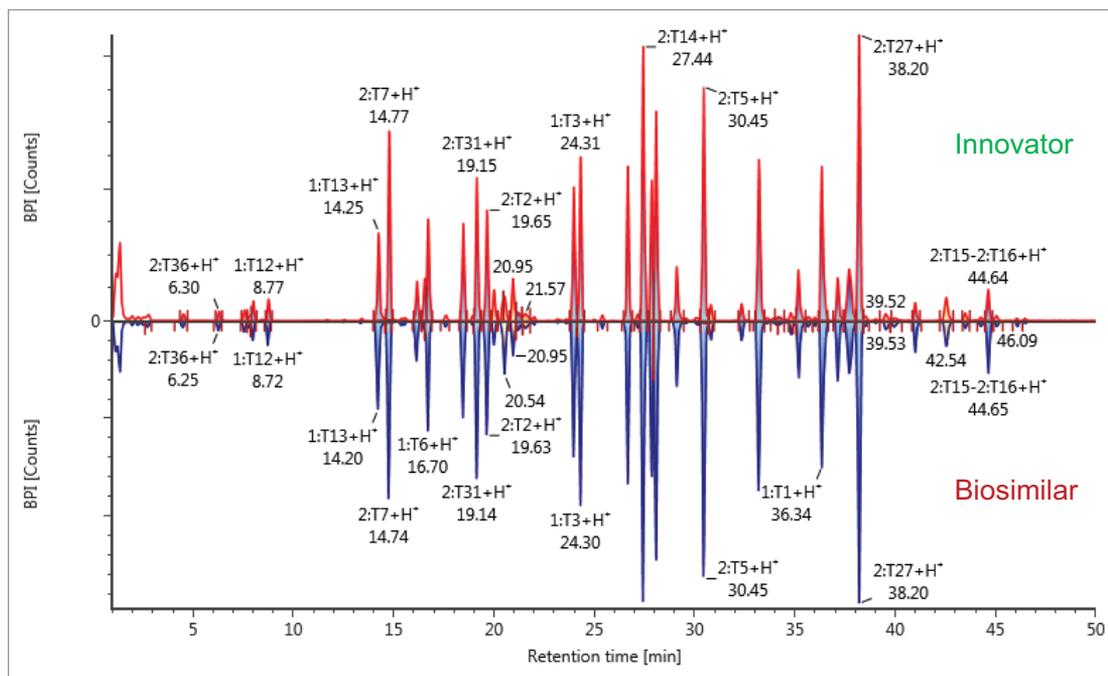


Figure 5. Mirror image plot of MS BPI chromatograms comparing an infliximab innovator and biosimilar sample. This plot demonstrates that no major differences in peptide level between the samples were observed.

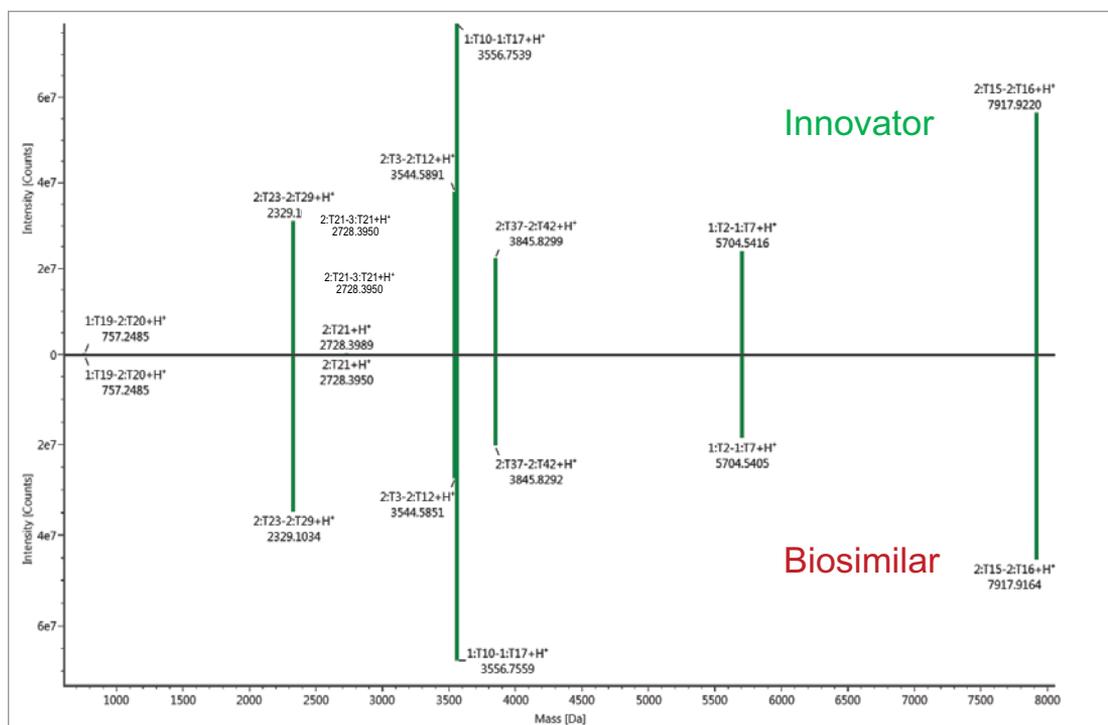


Figure 6. Mirror image plot of the confirmed disulfide-linked peptides comparing an infliximab innovator and biosimilar sample. No differences were found.

The summary trending plot is another important and useful feature for data reviewing in UNIFI. Figure 8 shows the response trending plot of the disulfide bond-containing peptide 2:T15-2:T16. It provides an overview response comparison of the selected disulfide bond-containing peptides across all of the samples with triplicate injections. Elevated responses of peptides were observed in the innovator batches compared to the biosimilar batches. The plot also shows high reproducibility from injection to injection.

Reporting is also a key feature of UNIFI. Customizable templates for automated report generation allow scientists to organize information in a fit-for-purpose fashion and communicate experimental results quickly and effectively. Key results from a typical disulfide bond mapping report are shown in Figure 9. In this instance, the report contains experimental information from the analysis, identified disulfide bonds, locations and linkages on the sequence map, examples of MS chromatograms, MS-MS spectra, and the trending plot of one of the identified disulfide bond-containing peptides.

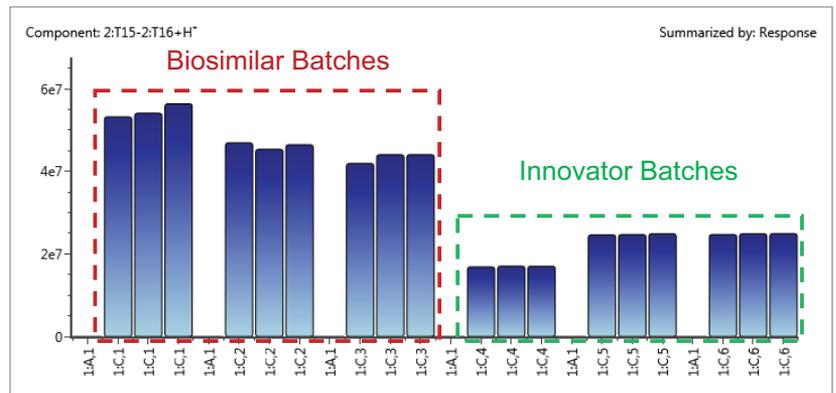


Figure 8. Trending plot of the disulfide bond-containing peptide 2:T15-2:T16. The total MS ion count response for this particular disulfide-linked peptide is plotted across all three sets of innovator and biosimilar samples, with triplicate injections.

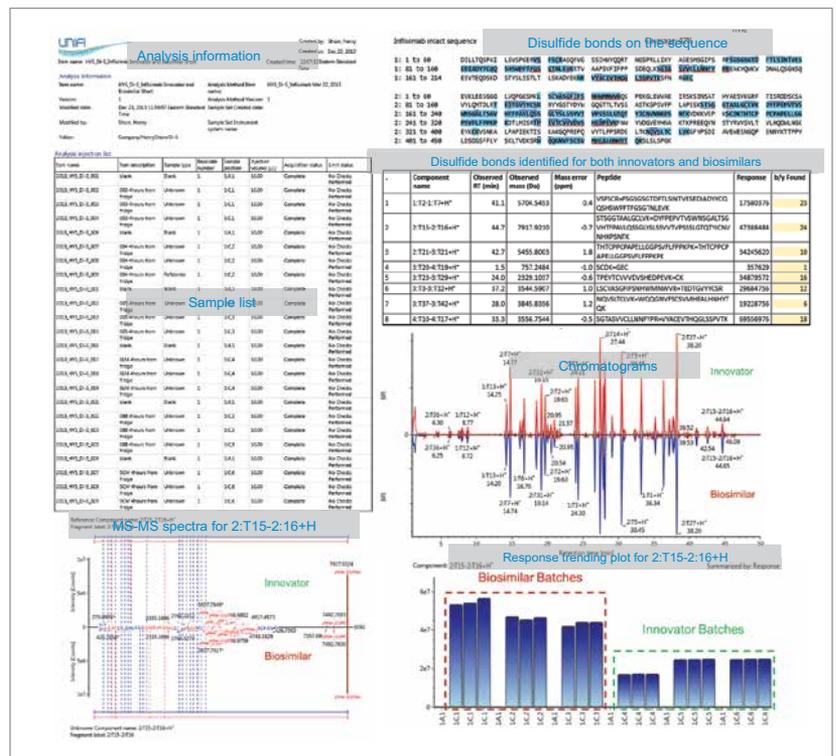


Figure 9. Example of a typical disulfide bond mapping report, which contains experimental information, identified disulfide bonds, locations and linkages on the sequence map, examples of MS chromatograms, MS-MS spectra, and the trending plot of one of the identified disulfide bond-containing peptides.

CONCLUSIONS

Biopharmaceutical organizations demand streamlined analytical platforms. These platforms need to increase the throughput of the characterization of biotherapeutics and to demonstrate batch-to-batch or innovator-to-biosimilar comparability in order to meet regulatory guidelines. Disulfide bond linkages are a key product quality attribute; however, mapping disulfide bonds using LC-MS techniques requires highly skilled scientists and is a time-consuming process. The peptide mapping workflow in UNIFI is designed to lower the technical barrier for scientists. UNIFI addresses industry challenges and provides fit-for-purpose toolsets that effectively reduce analysis time. The described workflow streamlines the UPLC-MS/MS data acquisition, processing, reviewing, and reporting process for efficient disulfide bonds assignment, localization, and monitoring.

This application note demonstrated successful application of the described workflow to identify all expected disulfide bonds from the Waters mass check standards. Additionally, it demonstrated the comparability between innovator and biosimilar infliximab samples. Combined with the UNIFI compliance-ready architecture, this disulfide mapping workflow can help biopharmaceutical organizations overcome analytical challenges, and complete fast mapping and monitoring of disulfide bonds during the development and manufacturing of their biotherapeutic products.

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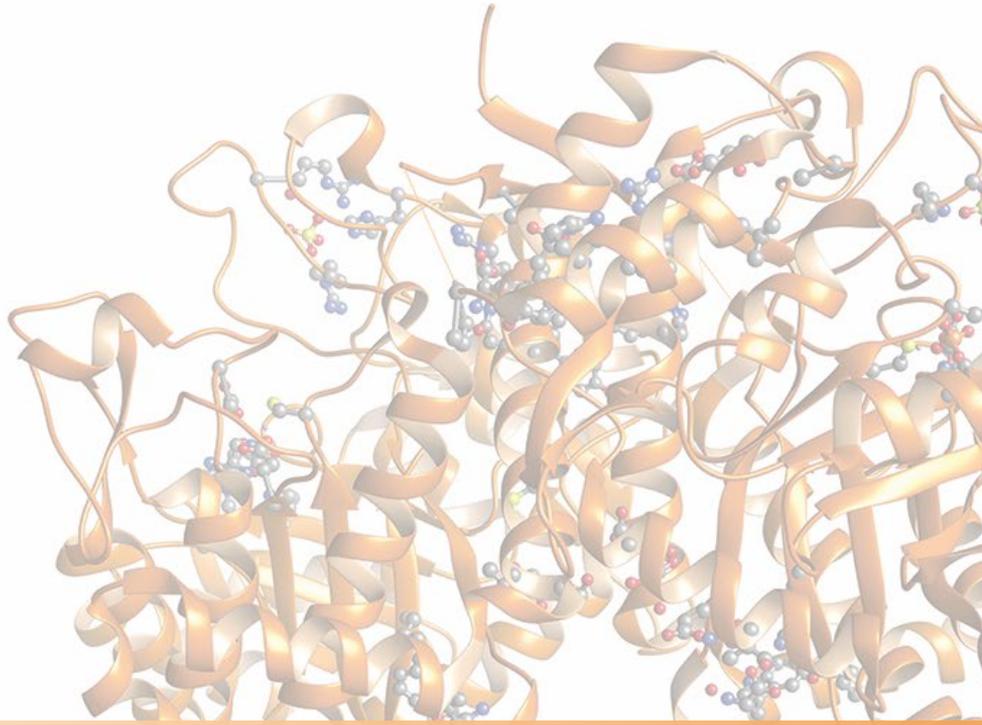
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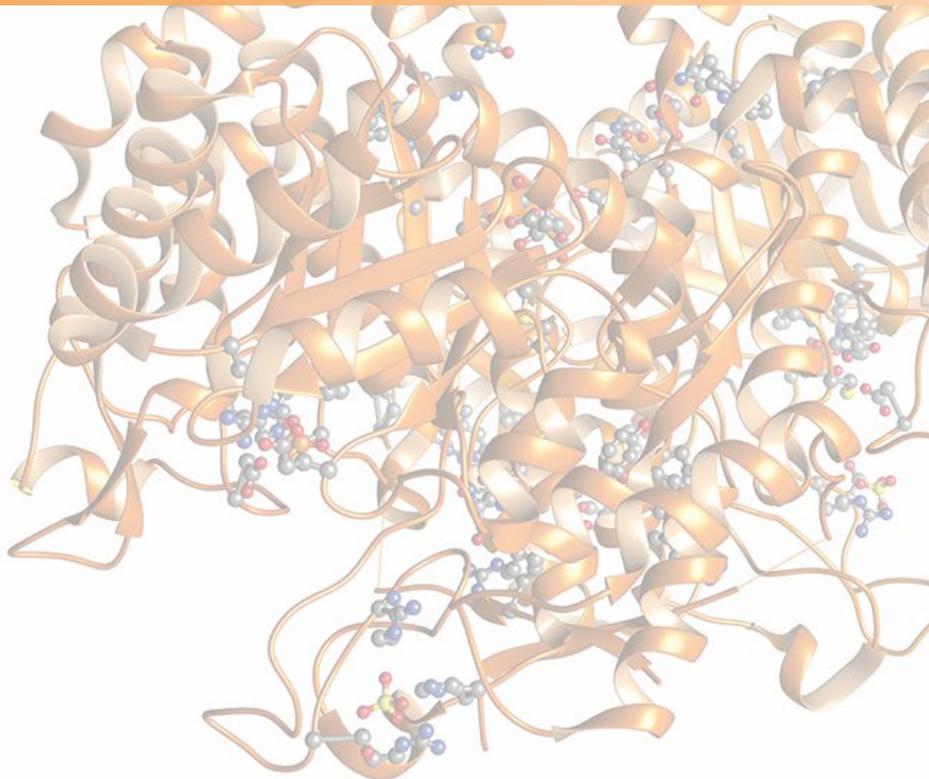
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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
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RELEASED GLYCAN ANALYSIS

Chapter 3



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

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[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 *RapiFluor*-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

The screenshot shows the FLR settings interface with the following configurations:

- General Settings:**
 - Mode: 2D Channels
 - Sampling rate: 10 points/sec
 - Data channels: 1
 - Filter time constant: Normal (0.2 Sec)
 - On inject start: Auto zero
 - Gain: Enable (value: 1)
 - Data units: Emission
 - Lamp state: Lamp on
- Mode Parameters:**

ID	Name	Excitation (nm)	Emission (nm)	Data Mode	Comment
1	Channel A	330	420	Channel A	No Comment

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.nibr.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.

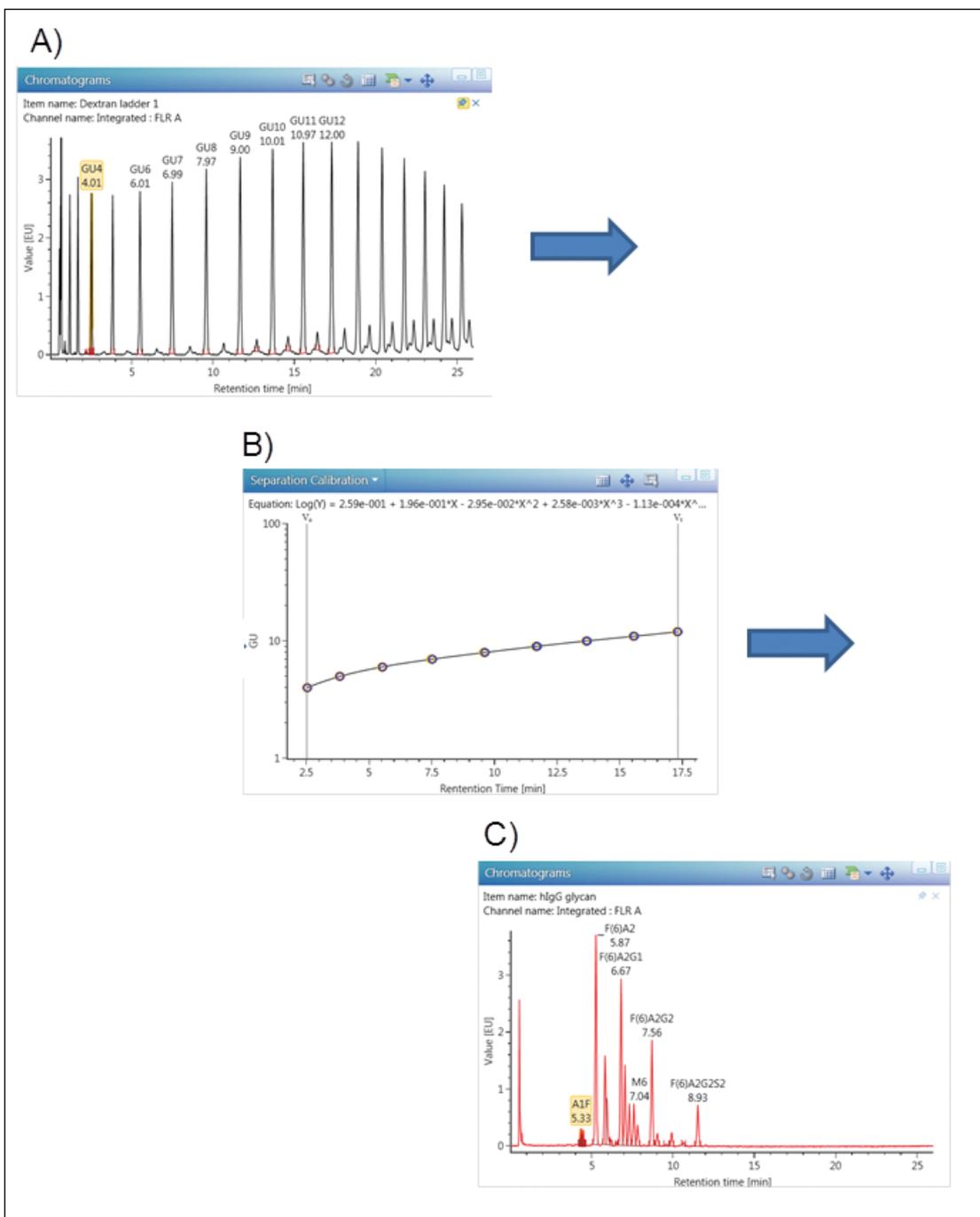


Figure 1. Glycan analysis workflow. A) 2-AB Dextran Calibration Ladder was used as a separation standard. B) The Glucose Unit (4-12) calibration from the separation standard was fitted by a fifth order polynomial curve. C) Glycan peaks from an unknown sample were assigned GU values from this curve. The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

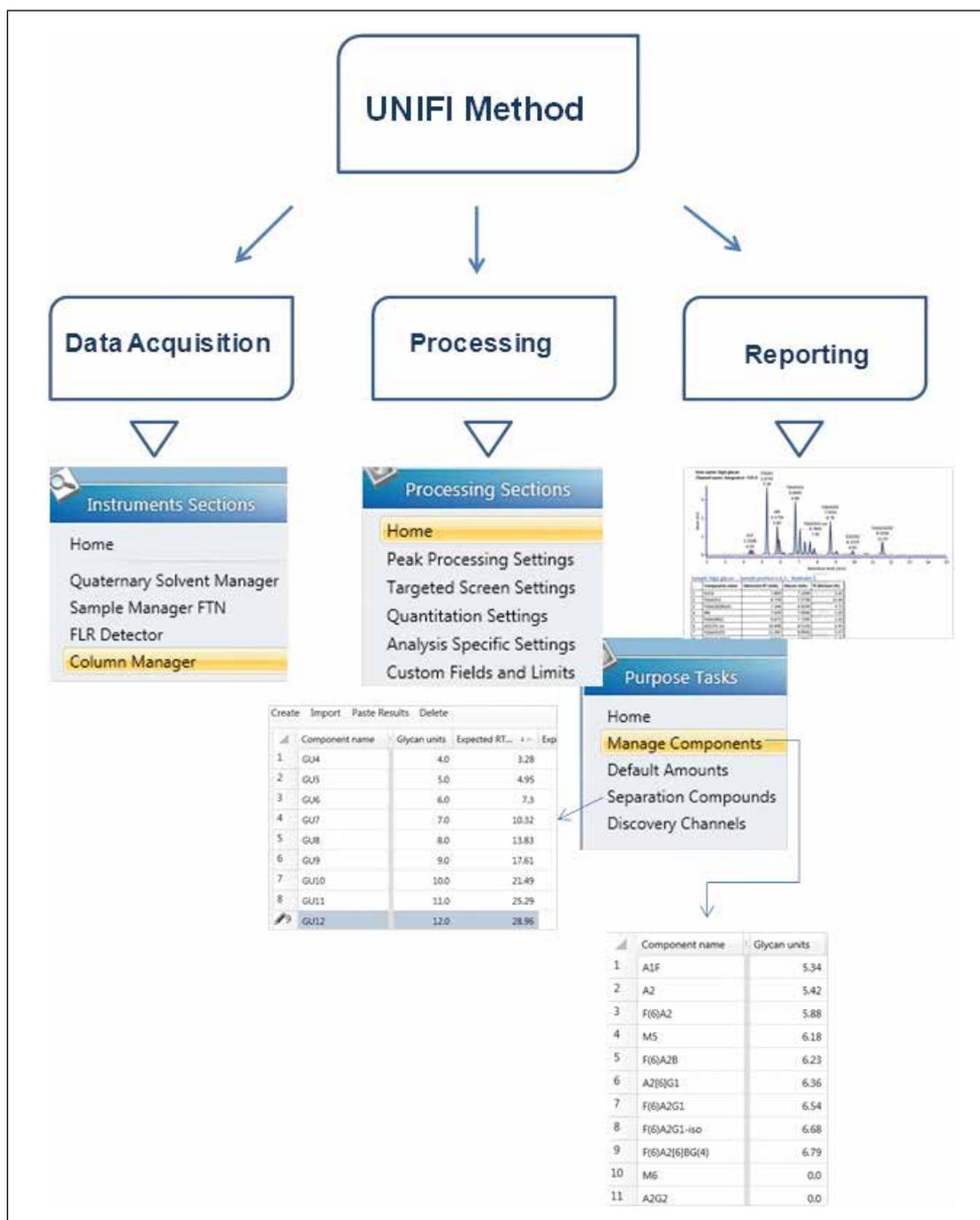


Figure 2. The UNIFI released glycan analysis method.

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.

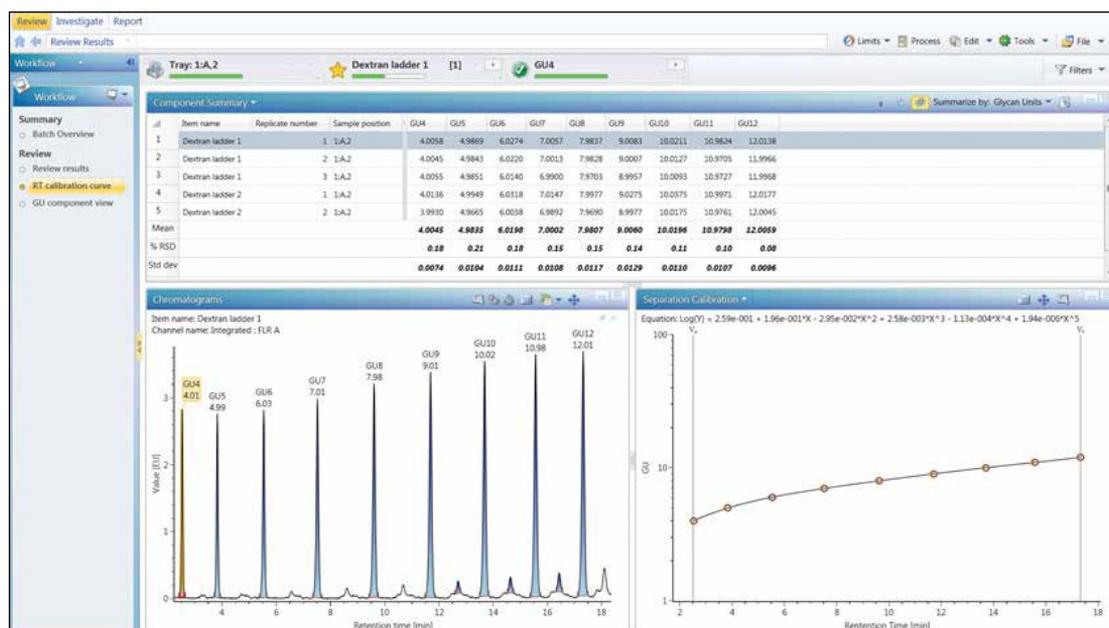


Figure 3. In UNIFI Software, the review panel summarizes GU values (GU 4–12) obtained across 5 injections of the 2-AB Dextran Calibration Ladder standard. The chromatogram of the first injection and calibration curve results for all injections are displayed below the summary table.

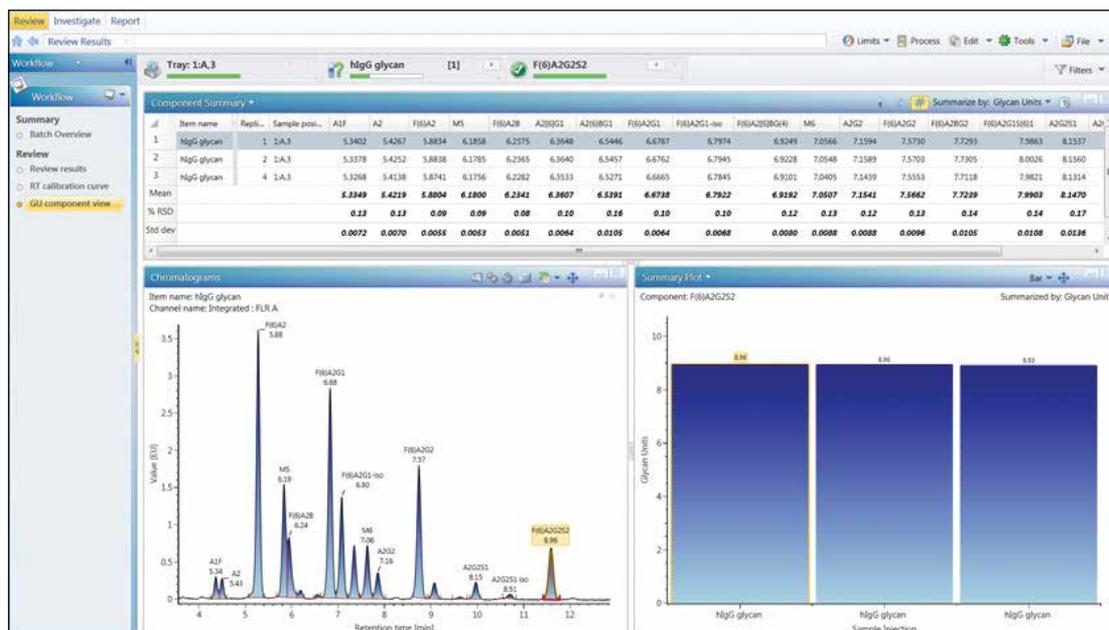


Figure 4. A component summary table in the review page that compares the GU value for all the identified glycans across a triplicate injection of a glycan mixture sample. Summary calculations for individual glycans across triplicate analysis of the glycan test standard are presented in the component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

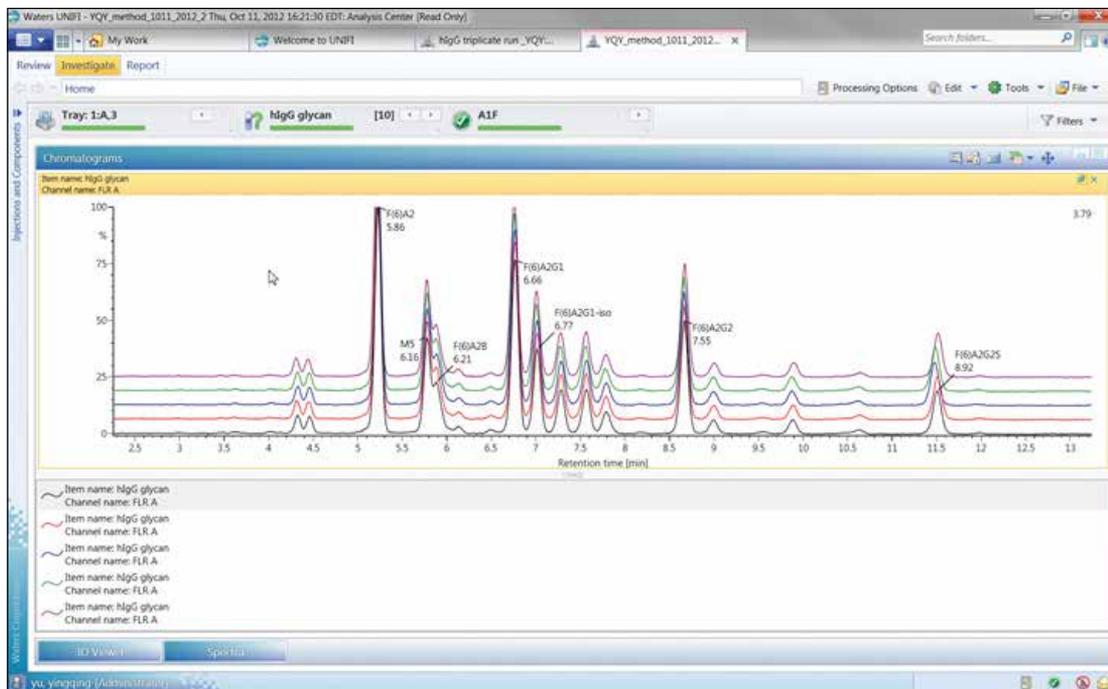


Figure 5. The Investigate panel of the experiment demonstrates glycan profile reproducibility for five HILIC UPLC FLR analyses of the glycan mixture.

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.

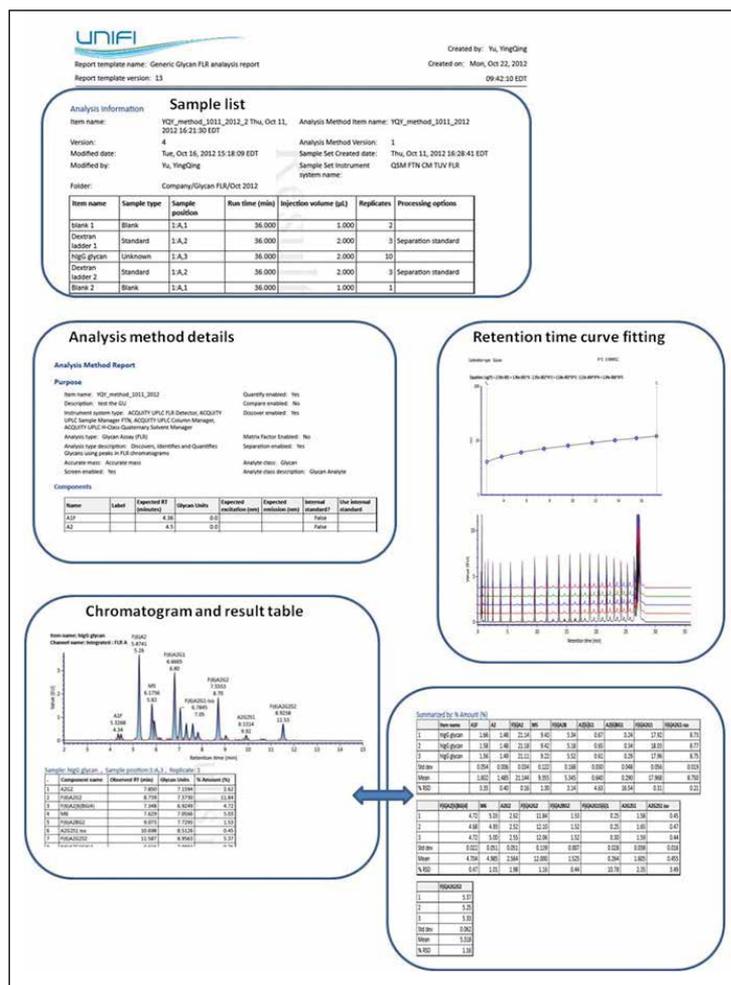


Figure 6. A typical UNIFI released glycan report.

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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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

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

General Settings						
Mode:	2D Channels	Sampling rate:	10	points/sec		
Data channels:	1	Filter time constant:	Normal	0.2	Sec	
On inject start:	<input checked="" type="checkbox"/> Auto zero					
Gain:	<input checked="" type="checkbox"/> Enable					
	1					
Data units:	Emission					
Lamp state:	<input checked="" type="checkbox"/> Lamp on					
Mode Parameters						
Name	Excitation (nm)	Emission (nm)	Data Mode	Comment		
1 Channel A	330	420	Channel A	No Comment		

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 commercialized 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 spline curve fit are applicable for retention time calibration.

The screenshot displays two panels from the UNIFI software interface:

- Separation Compounds:** A table with columns for Component name, Expected GU, and Expected RT (min). It lists components GU4 through GU12.
- Separation Calibration:** A settings panel with the following options:
 - Analysis will create new Separation Calibration Curve, if Standard Samples are used
 - Settings used in the calibration computations:
 - Ignore 'Calibration RT Correction' (if any) during computation of curve
 - V0 value: [input field]
 - Vt value: [input field]
 - Calibration curve fit type: **Fifth order** (selected in dropdown)
 - Average by expected y-value
 - Settings used to display the curve:
 - X axis on separation calibration curve: Time (selected in dropdown)
 - X axis units: minutes (selected in dropdown)

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.

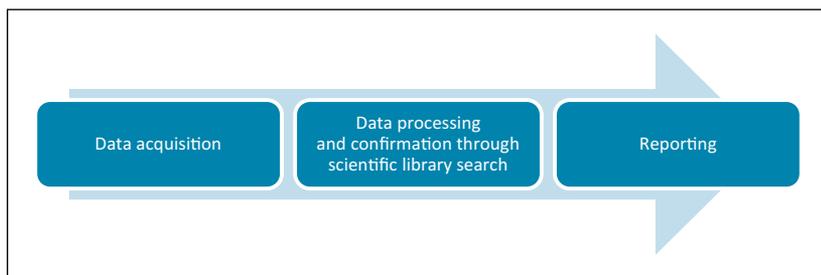


Figure 1. The glycan assay workflow, using FLR with MS confirmation.

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.

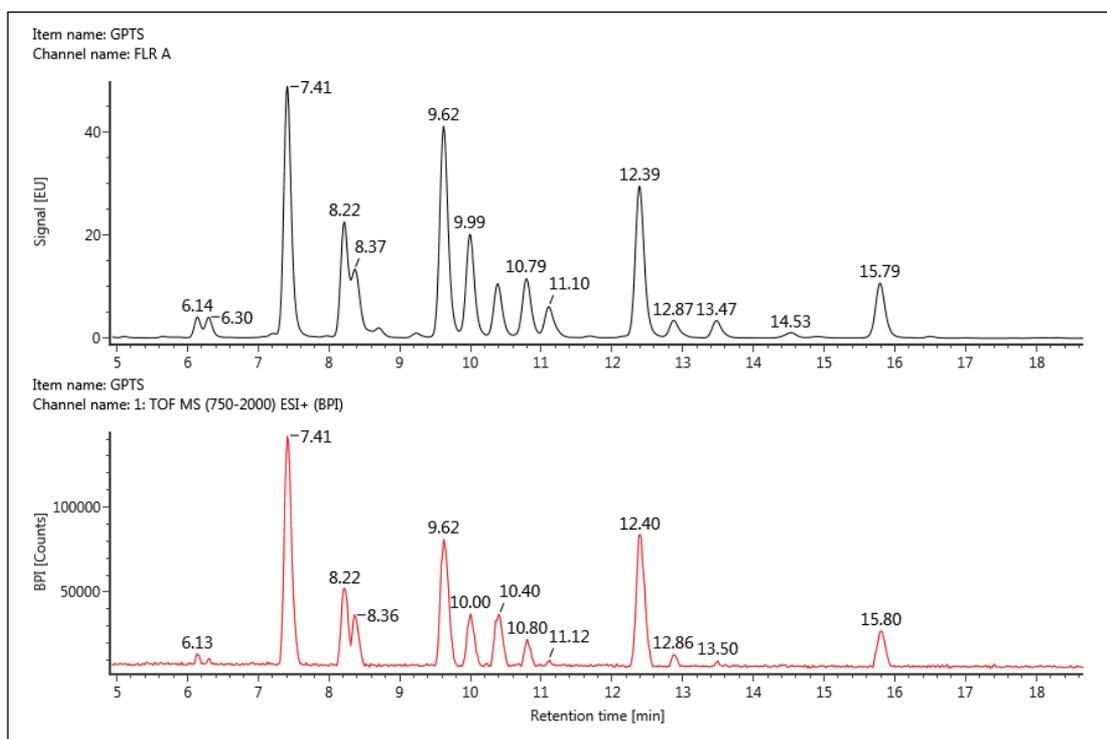


Figure 2. UPLC-FLR/MS chromatogram of 2-AB Glycan Performance Test Standard. The FLR chromatogram is shown at the top, and the BPI MS chromatogram is shown at the bottom. The BPI MS trace was time aligned with the FLR during data acquisition.

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.

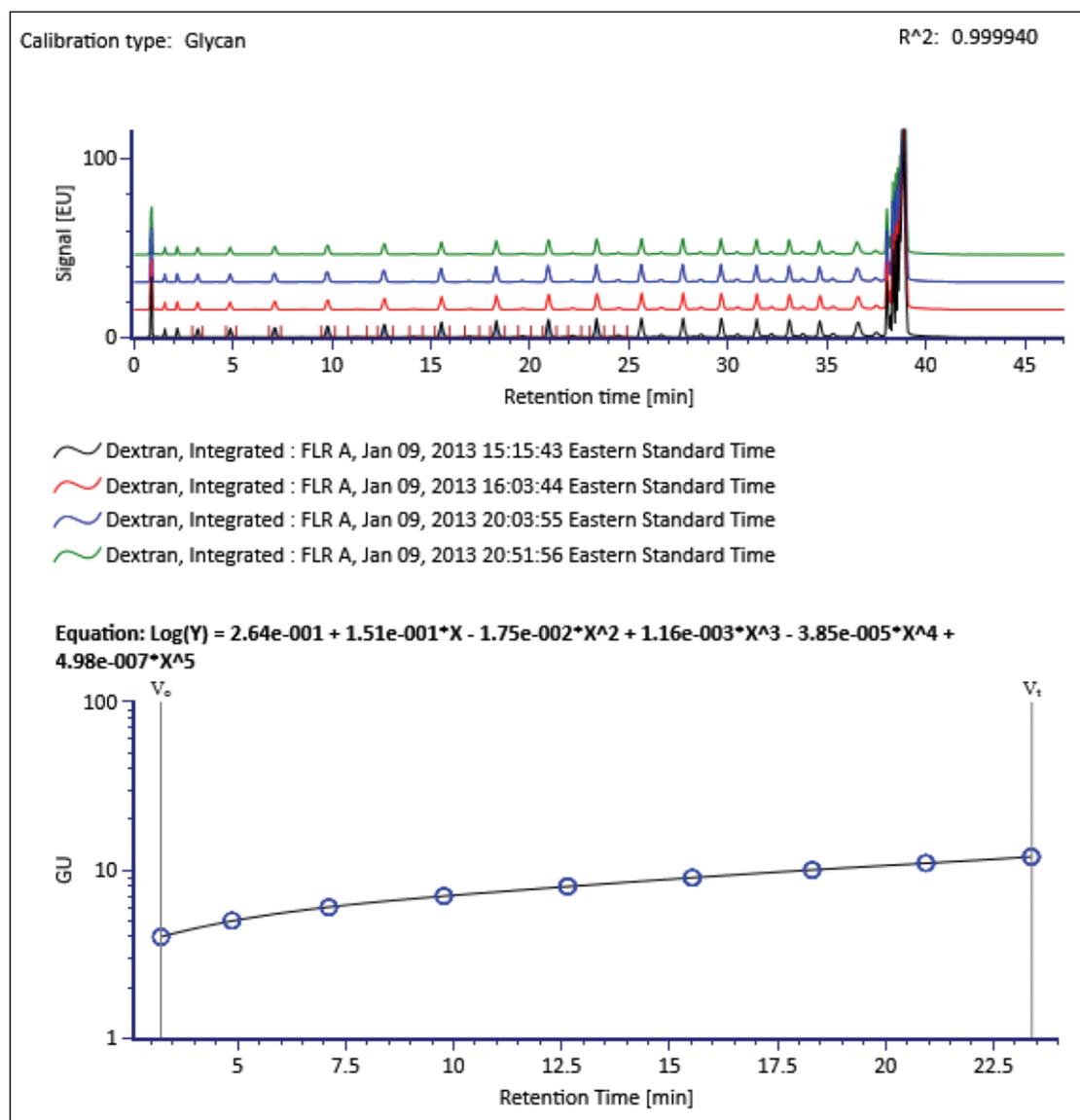


Figure 3. An example of dextran ladder calibration is shown. The top chromatogram shows the overlay of four injections of dextran ladder collected before and after two experimental sample runs. The bottom plot is the fifth order curve generated from these injections. The R² (0.999963) and overlaid data 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.

The screenshot shows the 'Library Search (Glycans)' interface. At the top, there is a description: '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 compound.' Below this, there is a checkbox for 'Enable library search' which is checked. A 'Reagent:' dropdown menu is set to '2AB' with a note '(Used to adjust mass used for MS confirmation)'. There are buttons for 'Duplicate', 'Delete', and 'Clear'. A table of search criteria is shown with two entries: 'Glucose unit equals 7 ± 0.15' and 'Glycan attributes equals Has not Antenna 3'. Below the criteria table, there is a 'Search in:' dropdown set to 'Waters Glycan GU Library', a 'Show:' dropdown set to '20 Results', and a 'Search' button. The results table below shows six entries with columns for Name, Formula, GU value, GU value standard deviation, and Monoisotopic mass.

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

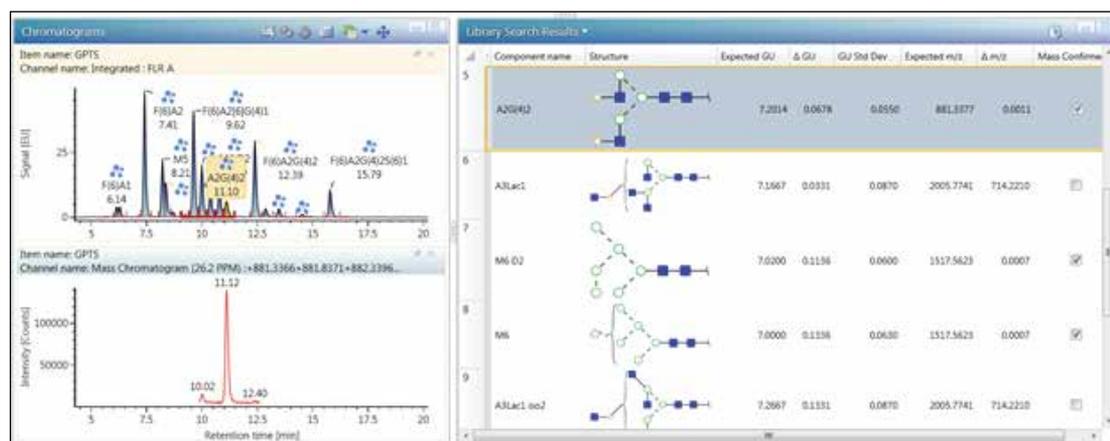


Figure 5. Waters Glycan GU Library search result from the review window. On the left is the processed FLR chromatogram and XIC MS of a highlighted glycan; on the right is the library search result of the selected glycan peak. Information such as the structure (with linkage assignment), expected GU, expected mass, Δ GU and Δ mass 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.

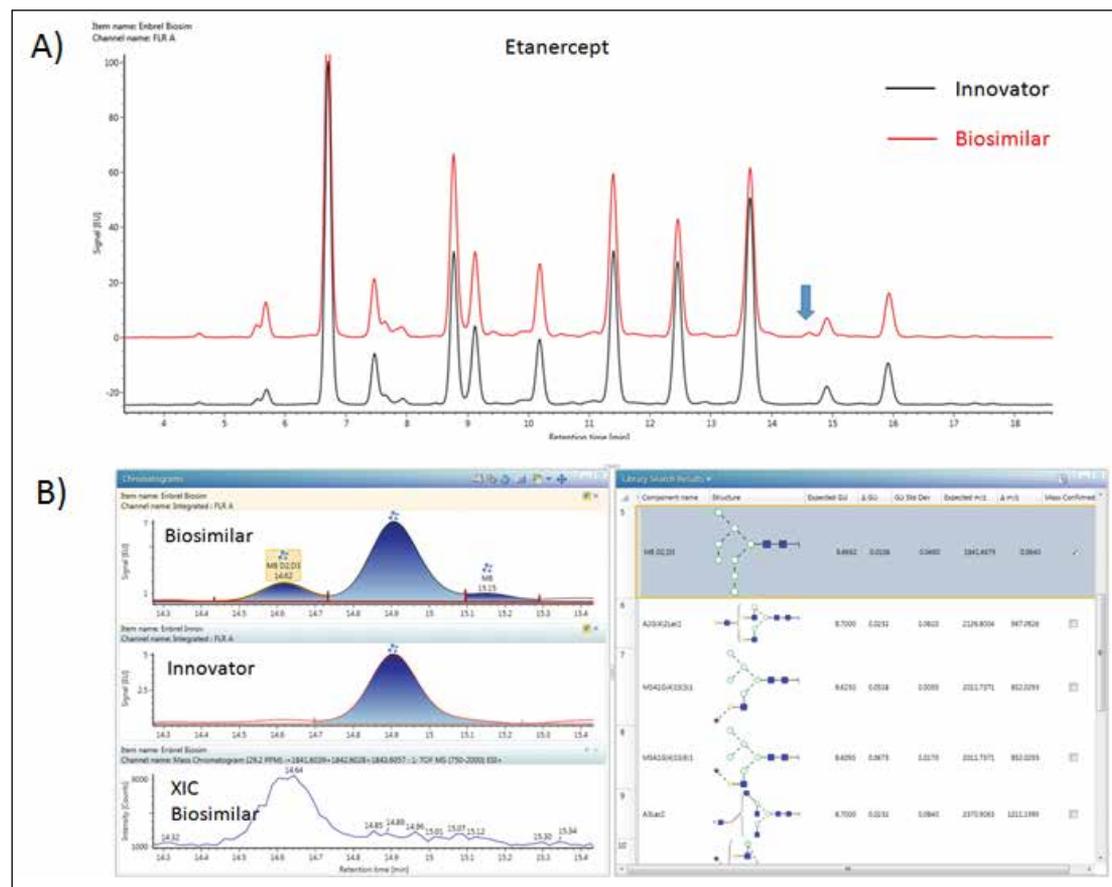


Figure 6. A) Overlay chromatogram of the N-glycans from the innovator and a biosimilar candidate etanercept. The blue arrow highlights the Man8 glycan that is only observed in the biosimilar candidate. B) The Waters Glycan GU Library search result shows that the marked peak from (A) is assigned to Man8. 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.

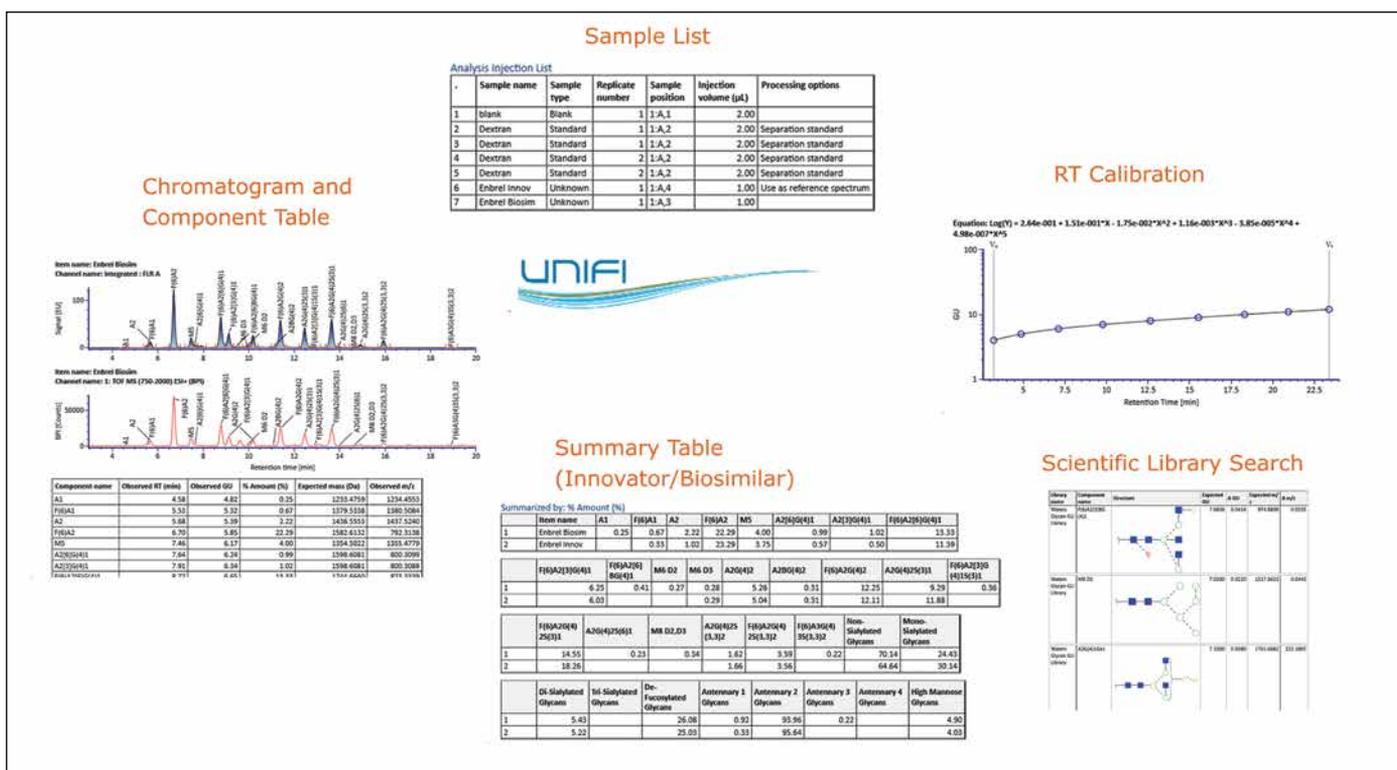


Figure 7. An example of a UNIFI report for the N-glycan profiling and comparison between innovator/biosimilar etanercept glycans. Key information such as sample list, retention time calibration curve, the scientific library search result for each identified glycan, summary table on relative % amount for innovator/biosimilar N-glycans, 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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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Optimization of GlycoWorks HILIC SPE for the Quantitative and Robust Recovery of N-Linked Glycans from mAb-Type Samples

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

APPLICATION BENEFITS

- GlycoWorks HILIC μ Elution Plate provides an efficient mechanism for glycan cleanup
- Quantitative and consistent recoveries for a diverse range of N-glycans with optimized SPE conditions
- Optimized SPE conditions also confer excellent method robustness
- Availability of a 2-AB labeled glycan standard for ensuring the performance of sample preparation and analysis methods

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC GST Amide
 (BEH Glycan) Column

GlycoWorks™ 96-well HILIC
 μ Elution Plate

Glycan Performance Test Standard

KEY WORDS

N-glycans, GlycoWorks, BEH glycan,
 Glycan Performance Test Standard, HILIC,
 HILIC SPE

INTRODUCTION

More than half of all proteins are estimated to be glycosylated.^{1,2} This post-translational modification, involving the attachment of oligosaccharides, plays a very significant role in many biological processes.³ Therapeutic antibodies are a salient example of a set of proteins affected by glycosylation, given that their efficacy and immunogenicity can be considerably attenuated by changes in their glycan profile. Glycan profiles of therapeutic antibodies are often, therefore, a critical quality attribute (CQA) that must be assessed during cell line selection and monitored during development and batch releases.

A highly effective analysis platform for evaluating N-glycans from glycoproteins involves the release of glycans by PNGase F, their labeling with fluorescently active 2-aminobenzamide (2-AB), subsequent separation by hydrophilic interaction chromatography (HILIC), and detection by fluorescence (FLR), as shown in Figure 1:³⁻¹⁰

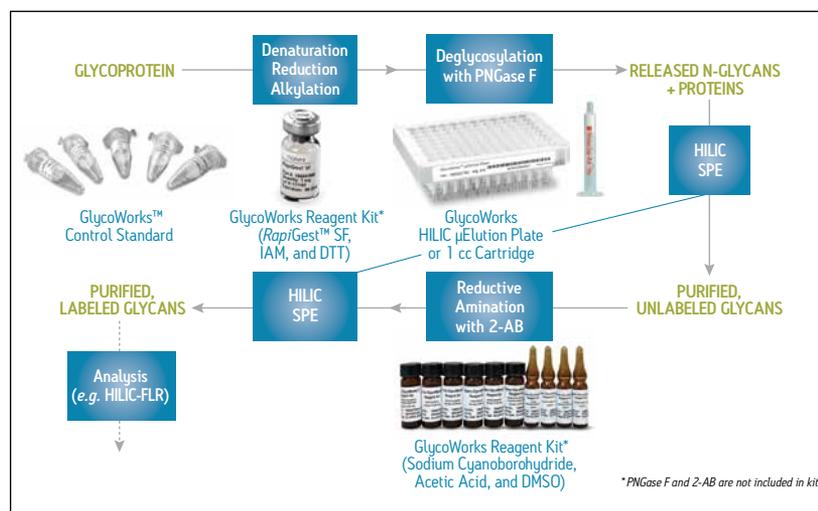


Figure 1. Schematic for preparing 2-AB labeled glycans from a glycoprotein using GlycoWorks. Consumables that are included as part of the GlycoWorks solution are highlighted in blue. Note that PNGase F and 2-AB are not included as part of the GlycoWorks Reagent Kit.

EXPERIMENTAL

Sample description

For the labeled oligosaccharide recovery studies, the Glycan Performance Test Standard ([p/n 186006349](#)) was mixed with 2-AB labeled trisialylated A3 (ProZyme) in water to make a solution of 3 pmol/ μL . Aliquots (10 μL) of this mixture were diluted with 15 μL of acetonitrile (ACN) to make control samples. Aliquots (10 μL) were also dried under vacuum to prepare lyophilization control samples. In addition, 10- μL aliquots were processed by HILIC SPE according to the protocol found in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual ([p/n 715004079](#)). Various eluents were studied and are noted in Figures 3 through 6. Dried glycans were reconstituted in 10 μL of water and 15 μL of ACN prior to injection.

For the unlabeled oligosaccharide recovery studies, unlabeled Man5 and trisialylated A3, obtained from ProZyme, were reconstituted in water, and mixed to equal molarity (6.7 μM). Aliquots (6 μL) of this mixture were diluted with 6 μL of ACN to make control samples. Aliquots (6 μL) were also dried under vacuum to prepare lyophilization controls. In addition, 6 μL aliquots were processed by HILIC SPE, according to the protocol found in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual. Elution was performed with an eluent comprised of 100 mM ammonium acetate (NH_4OAc) in 5% ACN (pH 7). Dried glycans were reconstituted in 6 μL of water and 6 μL of ACN prior to injection.

A schematic for the HILIC SPE steps used in this study is shown below:

WASH 200 μL H_2O
CONDITION 200 μL 85% ACN
LOAD 10 μL sample + 100 μL ACN (2-AB labeled) 6 μL sample + 44 μL 25 mM NH_4HCO_3 (pH 8) + 350 μL ACN (unlabeled)
WASH 3 x [200 μL 85% ACN]
ELUTE 3 x [50 μL 100 mM NH_4OAc , 5% ACN, pH 7] (unless otherwise noted)
LYOPHILIZE AND RECONSTITUTE

Note: Eluates were transferred to micro-centrifuge tubes then lyophilized. Thereafter, samples were reconstituted and transferred to sample vials for injection.

The procedure for preparing samples for this analysis can be complicated. GlycoWorks products help make the workflow more straightforward by bringing together many of the needed consumables. Moreover, GlycoWorks products provide a solution to the cleanup steps that are needed throughout the process of preparing labeled glycans for analysis. In particular, HILIC solid-phase extraction (SPE)^{11,12} has been developed to purify released glycans from proteins and buffer/formulation constituents, which can disrupt derivatization. HILIC SPE can also purify labeled glycans after derivatization from excess reagents, which can potentially interfere with downstream chromatography, reduce the lifetime of a column, and thereby impair method robustness.

This application note evaluates HILIC SPE sample preparation to ensure quantitative recovery of both unlabeled and labeled N-glycans. A test mixture, including a complex array of 2-AB labeled human IgG glycans spiked with both high mannose and trisialylated glycans, was used to interrogate and optimize SPE recoveries as well as study the robustness of optimized elution conditions. In addition, an LC-MS assay was employed to demonstrate the quantitative recovery of unlabeled glycans during HILIC SPE with the optimized conditions.

Method conditions (unless otherwise noted)**LC conditions**

System:	ACQUITY UPLC H-Class Bio with a 20-cm Column Heater
Detection:	Waters® ACQUITY UPLC FLR Detector
Excitation:	330 nm
Emission:	420 nm
Scan rate:	10 Hz
Time constant:	0.2 s
Gain:	1.00
Column:	ACQUITY UPLC GST Amide (BEH Glycan), 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	2.5 µL (HILIC-FLR), 10 µL (HILIC-MS)
Flow rate:	0.5 mL/min (0.25 mL/min for the highly aqueous regeneration step in the gradient)
Mobile phase A:	100 mM Ammonium formate, pH 4.4
Mobile phase B:	Acetonitrile (ACN)
Sample collection plate:	1 mL Round Well Collection Plate (p/n 186002481)
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)

Gradients:

HILIC-FLR	Time (min)	% A	% B	Flow rate (mL/min)
	0	22.0	78.0	0.5
	38.5	44.1	55.9	0.5
	39.5	80.0	20.0	0.25
	44.5	80.0	20.0	0.25
	46.5	22.0	78.0	0.5
	50.0	22.0	78.0	0.5

HILIC-MS	Time (min)	% A	% B	Flow rate (mL/min)
	0	27.9	72.1	0.5
	19.25	50.0	50.0	0.5
	20.25	80.0	20.0	0.25
	25.25	80.0	20.0	0.25
	27.25	22.0	78.0	0.5
	31.00	22.0	78.0	0.5

MS conditions

Mass spectrometer: Xevo® G2 Q-Tof

Ionization mode:	ESI+
Analyzer mode:	Sensitivity
Capillary voltage:	3.20 kV
Cone voltage:	37 V
Source temp.:	100 °C
Desolvation temp.:	350 °C
Cone gas flow:	0.0 L/h
Desolvation gas flow:	800 L/h
Calibration:	NaI, 1 µg/µL from 50 to 2000 <i>m/z</i>
Acquisition:	700 to 3000 <i>m/z</i> , 1 Hz scan rate
Lock mass:	0.5 µM [Glu ¹]-fibrinopeptide in 50:50 ACN/water, 0.1% formic acid

Data management

UNIFI® and MassLynx® software

RESULTS AND DISCUSSION

Optimizing the recovery of glycans from GlycoWorks HILIC SPE

A test mixture, capable of rigorously interrogating the recovery of N-glycans from GlycoWorks HILIC SPE, was prepared by combining the Glycan Performance Test Standard with 2-AB labeled trisialylated A3 glycans. The Glycan Performance Test Standard is comprised of 2-AB labeled N-glycans derived from pooled human serum IgG spiked with high mannose glycans (Man5 and Man6). The addition of the trisialylated A3 glycans further extends the complexity of this mixture, as the A3 glycans are larger, more acidic, and bind more strongly in a HILIC-based separation than glycans commonly found on human or human-like IgG. Figure 2 shows a HILIC-FLR analysis of this modified test mixture using an ACQUITY UPLC GST Amide (BEH Glycan) Column along with UNIFI Software for instrument control and data interpretation.

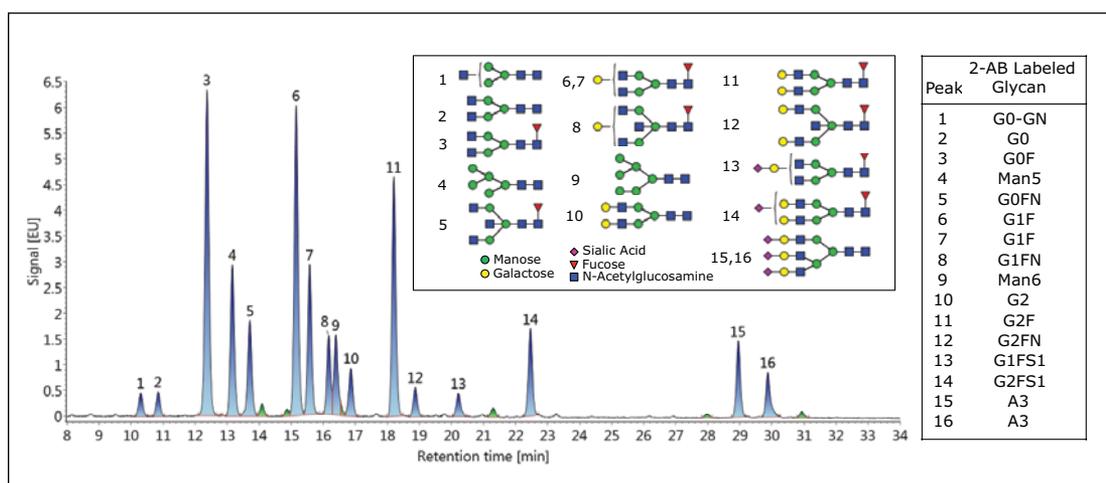


Figure 2. HILIC-FLR analysis of 2-AB labeled glycan performance test standard and trisialylated A3 glycans. 3 pmol of sample injected in 2.5 μ L onto an ACQUITY UPLC GST Amide (BEH Glycan), 1.7 μ m, 2.1 x 150 mm Column. Peaks detected by UNIFI processing are shaded in blue (expected component) or green (discovered component).

Based on this analytical approach, the HILIC SPE of the GlycoWorks solution was evaluated. A silica-based aminopropyl sorbent is contained in the GlycoWorks Kit (p/n 176003090). This sorbent was selected from several tested because it is highly polar and, consequently, useful for HILIC separations. Since this sorbent possesses a weakly basic surface and potential for anion exchange, it was, however, assumed that the relative and total recovery of glycans from a GlycoWorks HILIC SPE device could be particularly sensitive to elution conditions. To evaluate this step, elution from the GlycoWorks HILIC sorbent was studied in detail. 2-AB labeled glycans were loaded onto a 96-well HILIC μ Elution Plate according to the protocol provided in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual.¹³ Various eluents were then employed for elution of the labeled glycans, and recoveries for each major species in the test mixture was 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. A series of eluents comprised of 20% ACN and increasing concentrations of ammonium bicarbonate (NH_4HCO_3 , pH 8–9) were first investigated. A volatile salt was chosen, due to requisite lyophilization steps. Interpretation of the recoveries led to the observation that the recovery of the glycans was biased, based on eluent choice, with smaller, neutral species recovered better than larger, acidic species. With an eluent comprised of simply 20% ACN/80% water (H_2O) and no other components, acidic glycans in the test mixture were either poorly recovered or not recovered at all; meanwhile, neutral glycans were obtained with reasonable recovery ($\geq 70\%$). The addition of NH_4HCO_3 , to concentrations of 25 mM or higher minimized this apparent and non-desired ionic retention mechanism. Nevertheless, even with 100 mM NH_4HCO_3 , there was a noticeable correlation between recovery and the hydrophilicity, or glucose unit (GU) values, of the glycans (Figure 3A).

Biased recovery, or speciation, can be problematic for a sample preparation procedure. In addition to not providing an accurate representation of the species present in the sample, it can be indicative of a method that is not robust and that the relative abundance profiles obtained may not be reproducibly determined, particularly with respect to the most poorly recovered species. As a result, a study was performed to improve these observed 2-AB labeled glycan recoveries. Given that retention of polar analytes to a polar sorbent is dominated by hydrogen bonding and ionic interactions, eluents with more aqueous content (decreased ACN concentrations) were evaluated (Figure 3B). As predicted, NH_4HCO_3 eluents comprised of lower concentrations of organic solvent yielded both higher and less biased recoveries of the glycan profile. Within the range of this study, an eluent composition of 25 mM NH_4HCO_3 /5% ACN was found to produce optimal recoveries.

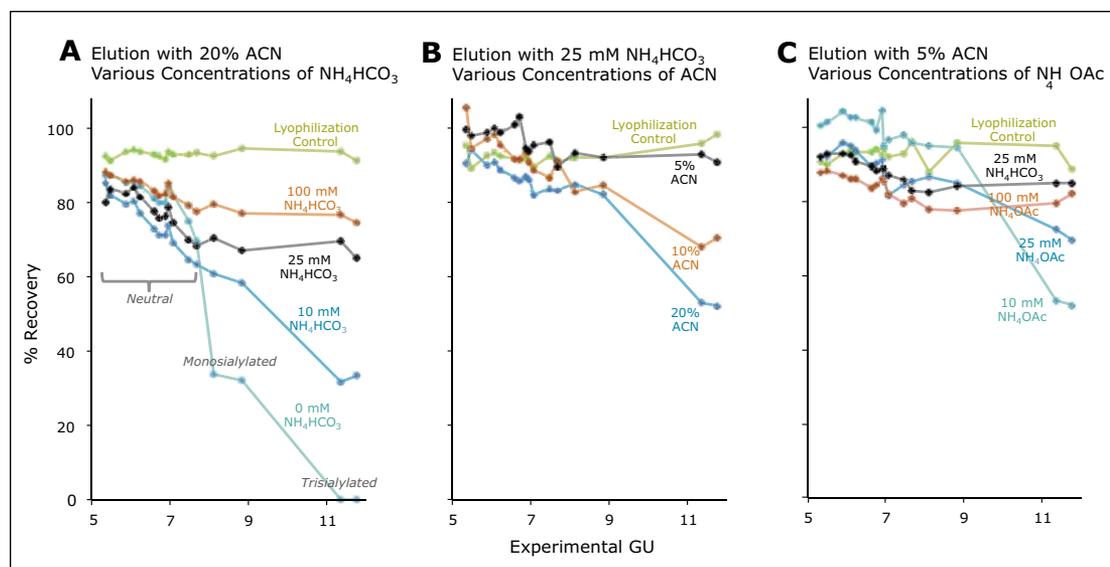


Figure 3. Recovery of 2-AB labeled N-glycans (Figure 2) from GlycoWorks HILIC SPE 96-well μ Elution Plates (30 pmol of glycans processed). Percent recoveries as a function of experimentally determined glucose units (GU) are shown for various elution conditions. Values are based on the average of three replicate analyses.

Unfortunately, eluents containing NH_4HCO_3 posed a challenge in this application as their basicity (typically pH 8 but increased toward pH 9 upon exposure to air) may result in noticeable dissolution of the silica SPE particles and problematic levels of precipitate in the reconstituted samples. To eliminate this potential issue and establish a more robust procedure, we investigated alternative eluents based on neutral solutions of ammonium acetate (pH 7). The effect of ammonium acetate (NH_4OAc) eluents on the recoveries of the 2-AB labeled glycans is shown in Figure 3. A 100-mM NH_4OAc , 5% ACN eluent was selected as the optimal elution condition, since it provided high as well as relatively unbiased analyte recoveries, similar to those obtained using the 25-mM NH_4HCO_3 , 5% ACN eluent.

The set of chromatograms shown in Figures 4A and 4B demonstrates that the test mixture, before and after HILIC SPE treatment, exhibits highly consistent glycan profiles. Relative abundance determinations for control samples as well as a processed sample are shown in Figure 4C. Compared to the control, differences in relative abundances were $\leq 7\%$ across the entire profile. For example, the relative abundance of GOF (peak 3) was determined to be 17.9% and 18.6%, before and after SPE, respectively. The relative abundance of trisialylated A3 (peak 16) before and after SPE was 2.8% and 2.7%, respectively (Figure 4C). These optimized elution conditions provide quantitative recoveries for both glycans typical of human IgGs and heavily sialylated glycans, as demonstrated with the recovery of the A3 glycan. With these conditions, the GlycoWorks HILIC μ Elution Plate is well suited for the preparation of N-glycans from a range of glycoproteins, including those with primarily low GU value neutral glycans as well as those decorated with high GU value, heavily sialylated glycans.

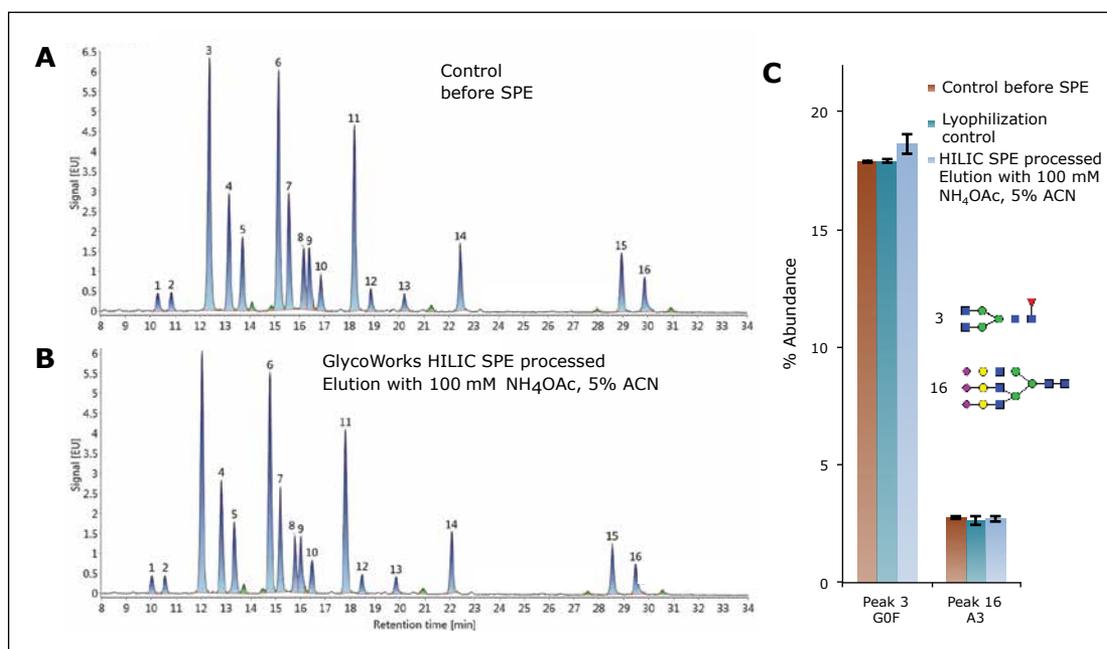


Figure 4. Glycan profile (A) before, and (B) after GlycoWorks HILIC SPE with the optimized elution conditions. Representative chromatograms and relative abundances (C) measured for two 2-AB labeled glycans (low and high GU values) from the test mixture before and after GlycoWorks HILIC SPE are displayed ($n=3$).

Robustness testing of optimized SPE elution conditions

The GlycoWorks HILIC μ Elution Plate was optimized to yield desired recoveries and, more importantly, to be robust. Elution conditions were purposely optimized so that even relatively large changes in critical elution parameters, namely organic concentration and ionic strength, would have minimal effect on the obtained glycan profile. To demonstrate this, the HILIC SPE method was subjected to robustness testing. Glycan profiles obtained using SPE eluents with the optimized concentrations of ACN and NH_4OAc concentrations were compared to those obtained with eluents comprised of ACN and NH_4OAc concentrations varied by 10%. The impact of changes in ionic strength and ACN concentration were purposely compounded in these studies. A strong eluent with 110-mM NH_4OAc , 4.5% ACN as well as a comparatively weak eluent with 90-mM NH_4OAc , 5.5% ACN were employed. Figure 5 shows the relative abundances for each of the major constituents in the test mixture obtained using these varied conditions. The glycan profiles obtained were comparable to the conditions tested. The largest percent change observed between relative abundances from the optimal to extreme conditions was only 7%, corresponding to the recovery of trisialylated A3 (peak 16). This result demonstrates that clean-up of 2-AB labeled glycans using the GlycoWorks HILIC μ Elution Plate with the optimized elution conditions exhibits noteworthy ruggedness, and is, therefore a robust solution for N-glycan preparations even in quality control applications.

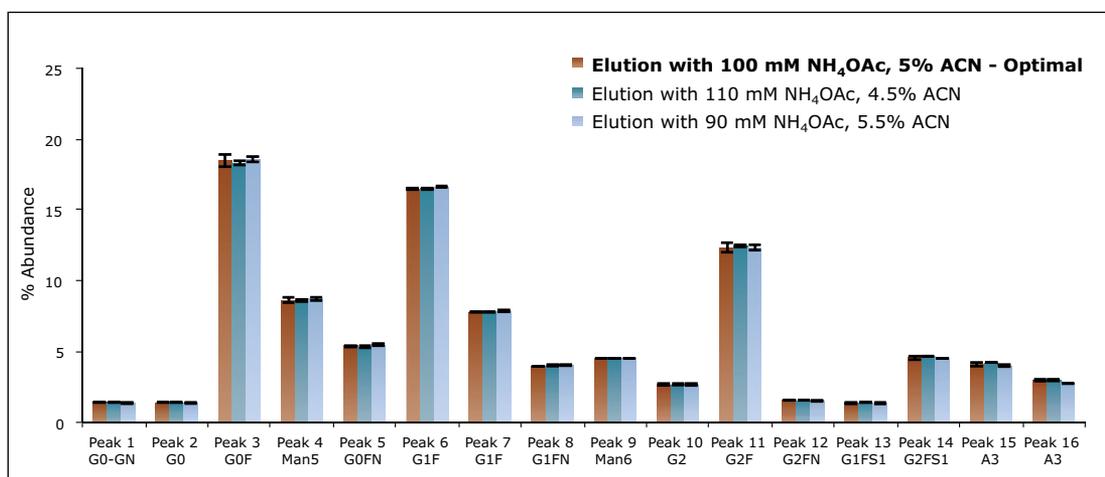


Figure 5. Robustness testing of the SPE elution conditions. Glycan profile for the test mixture obtained after GlycoWorks HILIC SPE with the optimized elution conditions and elution conditions wherein critical parameters were varied by 10% ($n=3$).

Assaying unlabeled glycans before and after GlycoWorks HILIC SPE by LC-MS

The GlycoWorks HILIC μ Elution Plate is also suggested for the initial purification of unlabeled glycans cleaved from the target glycoprotein via enzymatic digestion. 2-AB labeled glycans, as previously studied, are slightly less hydrophilic than unlabeled glycans due to the hydrophobicity of the benzamide fluorescent tag. To confirm that recoveries of unlabeled glycans were similar in comparison to the recoveries of 2-AB labeled glycans, an additional study was performed. A HILIC-MS assay was established to determine the relative abundances of two unlabeled glycans representing the extremes of most IgG N-glycan profiles. The mixture tested was comprised of equal amounts of a neutral, low GU value glycan (Man5) and an acidic, high GU value glycan (trisialylated A3). An extracted ion chromatogram (XIC) obtained for this mixture with a Xevo G2 QToF is shown in Figure 6A. Interestingly, two major peaks were observed for both unlabeled Man5 and A3, indicating the presence of different isoforms. Mass spectral windows wide enough to capture both protonated and salt adduct species of the unlabeled glycans were used to construct the chromatogram. XICs obtained in this manner were integrated, and the obtained peak areas were used to calculate relative abundances of the unlabeled glycans before and after HILIC SPE (Figure 6B). As with 2-AB labeled glycans, the profile of the unlabeled glycan mixture before and after SPE was highly comparable, indicating that the optimized GlycoWorks HILIC SPE process also yields minimally biased recoveries of unlabeled glycans.

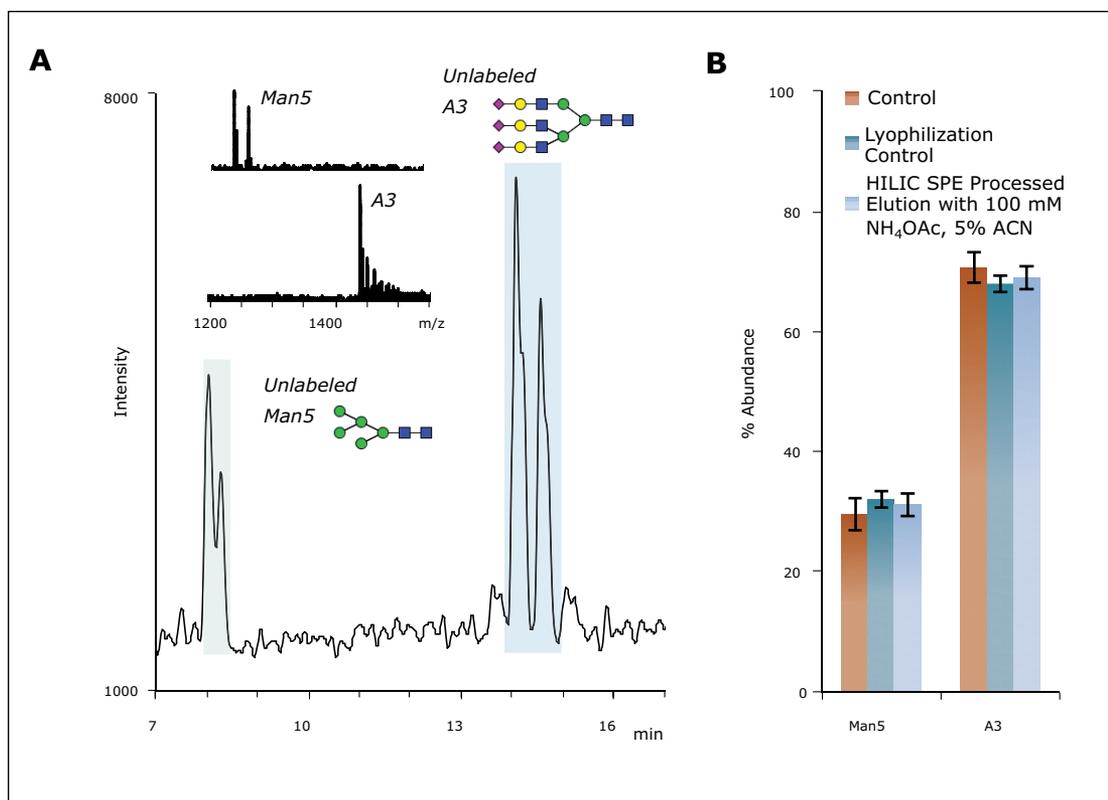


Figure 6. Assessing the effect of GlycoWorks HILIC SPE on the distribution of unlabeled glycans. An extracted ion chromatogram (XIC, 1235-1270+1435-1500 m/z) from a HILIC-ESI-MS analysis of a mixture containing a low GU, neutral glycan (Man5) and a high GU, acidic glycan (trisialylated A3) is shown on the left (A). Relative abundances determined via such an analysis for the mixture before and after GlycoWorks HILIC SPE are shown on the right (B). In this study, 40 pmol of each unlabeled glycan were processed by GlycoWorks HILIC SPE and 10 μ L of a 12- μ L reconstitution for a total maximum load of 33 pmol of each glycan was loaded onto an ACQUITY UPLC GST Amide (BEH Glycan), 1.7 μ m, 2.1 \times 150 mm Column for analysis.

CONCLUSIONS

HILIC SPE was rigorously studied and optimized to provide quantitative recoveries of 2-AB labeled and unlabeled N-glycans. A test mixture containing a diverse array of 2-AB labeled N-glycans was employed to interrogate GlycoWorks HILIC μ Elution Plate performance, and develop optimized elution conditions for a robust and reproducible method. In ruggedness testing of the optimized SPE, only minimal changes in a glycan profile were observed despite significant changes in the critical parameters of the SPE eluent. Moreover, an LC-MS assay showed that unlabeled glycans, like 2-AB labeled glycans, are recovered with minimal bias using the newly optimized elution conditions. These studies highlight the development of the GlycoWorks solution and its value in facilitating the release, labeling, and purification of N-glycans.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Single-Use and High-Throughput HILIC SPE Device Formats and an IgG Control Standard for Facilitating N-Glycan Analyses

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

GOAL

To demonstrate aspects of the GlycoWorks™ workflow including its flexibility to accommodate different SPE device formats and the availability of a human IgG control standard to use during method confirmation and troubleshooting.

BACKGROUND

Glycosylation of proteins is a highly significant post-translational modification that can modulate both protein structure and function. The glycosylation of biotherapeutics is understandably a structural feature that must be thoroughly characterized and monitored, particularly since changes in a glycan profile can correspond to changes in efficacy and/or immunogenicity.

A commonly employed method for evaluating N-glycans from glycoproteins involves the release of glycans by PNGase F, their labeling with fluorescently active 2-aminobenzamide (2-AB), subsequent separation via hydrophilic interaction chromatography (HILIC), and detection by fluorescence (FLR) (Figure 1). Sample preparation can be complicated during this workflow. To make this process more straightforward, Waters® introduced GlycoWorks, bringing together many of the consumables that are needed to prepare N-glycans for analysis. Most notably, GlycoWorks provides HILIC SPE devices for the pre- and post-labeling cleanup steps that

GlycoWorks facilitates the preparation of N-linked glycans for analysis by providing the analyst with options in SPE device format (low/high-throughput) as well as an IgG control standard for method confirmation and troubleshooting.

are important for ensuring method robustness. The following work demonstrates two salient aspects of the GlycoWorks workflow: that it can accommodate the use of different SPE device formats (single-use cartridges and high-throughput plates), and that the included human IgG control standard can be used for method confirmation and troubleshooting.

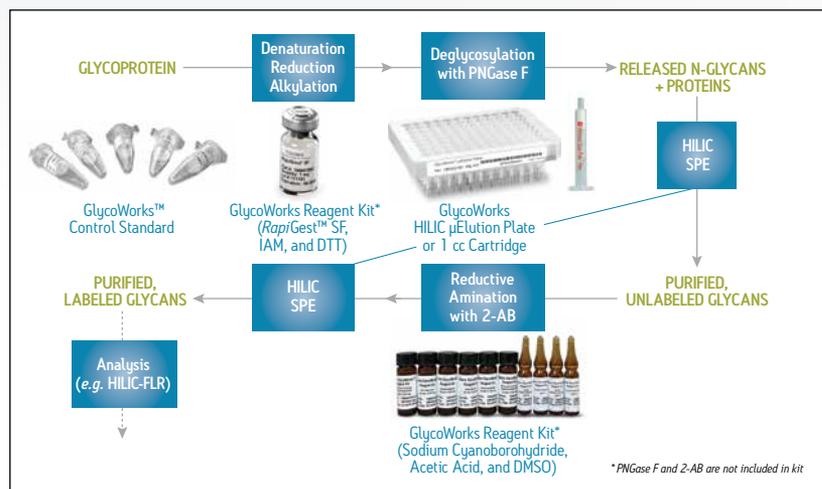


Figure 1. Schematic for preparing 2-AB labeled glycans from a glycoprotein using GlycoWorks. Consumables included as part of the GlycoWorks solution are highlighted in blue. Note that PNGase F and 2-AB are not included as part of the GlycoWorks Reagent Kit.

THE SOLUTION

2-AB labeled N-glycans were prepared from the GlycoWorks Control Standard (p/n 186007033), a human IgG, using two different SPE device formats with protocols provided in their respective [care and use manuals](#). Figure 2A shows a HILIC-FLR chromatogram obtained for the glycans of the Control Standard using the GlycoWorks HILIC μ Elution 96-well Plate (p/n 186002780), an ACQUITY UPLC GST Amide (BEH Glycan) Column, and UNIFI® for instrument control and data interpretation. Assignments for nine of the most abundant species in this glycan profile were made by means of glucose unit (GU) values, and by comparisons to the Glycan Performance Test Standard (p/n 186006349) which, like the Control Standard, is based on human IgG. A chromatogram obtained when using GlycoWorks HILIC 1-cc

Cartridges (single-use) (p/n 186007080) is shown in Figure 2B. Visual inspection of the two chromatograms indicated that highly similar results are obtained regardless of the chosen SPE device format. To confirm this, assigned peaks were integrated and peak areas were used to determine the relative abundances of the nine aforementioned glycan species (Figure 2C). This analysis revealed no significant differences in the relative levels of these glycans between samples prepared using the two different formats.

During this study, the GlycoWorks Control Standard was also employed to evaluate the effect of sample quantity on the glycan profile. HILIC-FLR chromatograms obtained when using the HILIC μ Elution Plate to prepare glycans from 25 μ g and 2.5 μ g of IgG are shown in Figure 3. Comparable results were obtained. However, the total recovery for the 2.5- μ g sample was lower on average, likely due to low-level non-specific surface losses. The relative abundances were determined and found to be highly similar, differing by $\leq 11\%$ despite the fact that the quantity of sample processed had changed ten-fold. The largest deviation corresponded to peak nine (G2FS1), which was observed to be present at a level of 8.5% versus 7.6% when processing 25 μ g and 2.5 μ g of IgG, respectively.

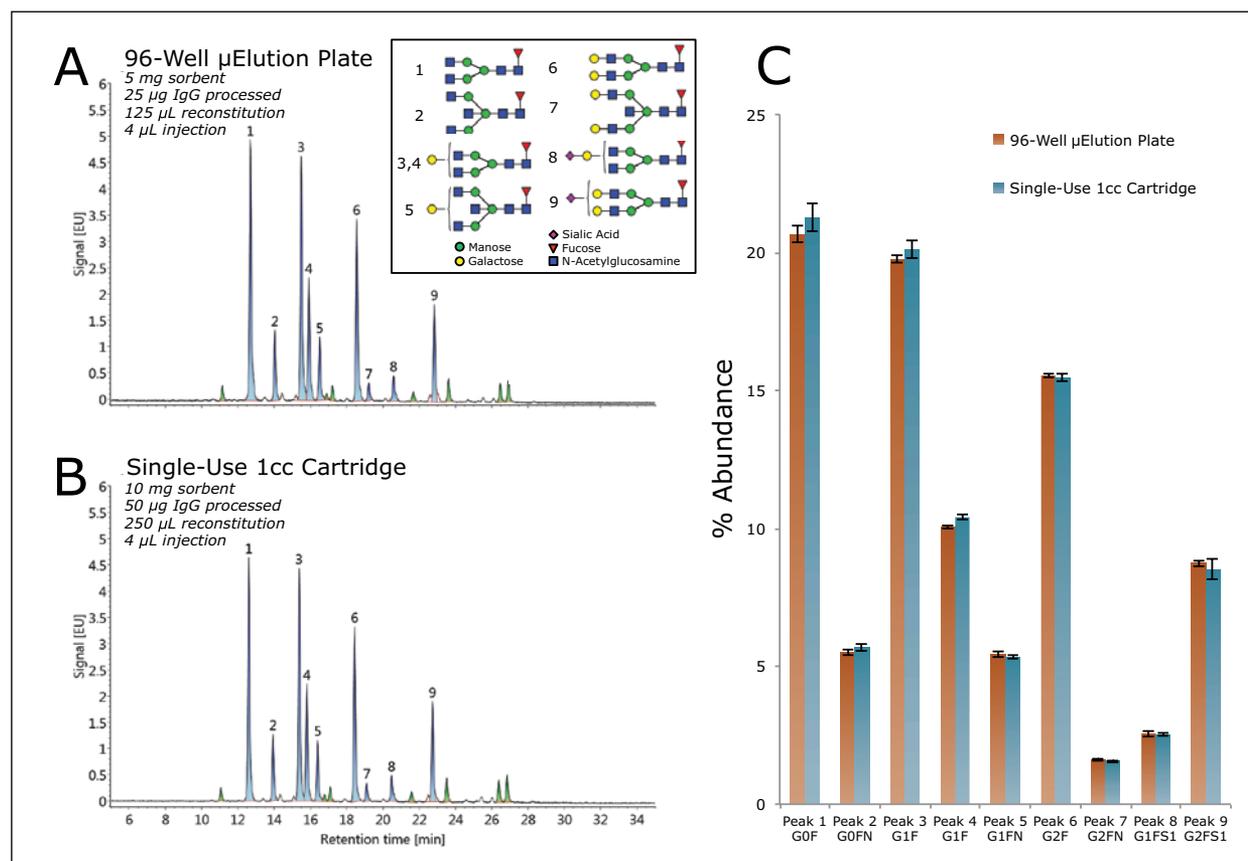


Figure 2. HILIC-FLR Analysis of 2-AB Labeled Glycans released from the GlycoWorks Control Standard using an ACQUITY UPLC GST Amide (BEH Glycan), 1.7 μ m, 2.1 x 150 mm Column. The left panel displays chromatograms obtained for samples processed using (A) GlycoWorks HILIC 96-well μ Elution Plate, and (B) a single-use HILIC 1-cc cartridge. Peaks detected by UNIFI processing are shaded in blue (expected component) or green (discovered component). The integrated peak areas were used in the calculation of the relative abundances displayed on the right (C). (n=3)

SUMMARY

The GlycoWorks workflow accommodates the use of two different SPE device formats: one suited for high-throughput needs (96-well μ Elution plate) and another for low-throughput applications (1-cc cartridge). Most importantly, the results demonstrate that highly similar glycan profiles are measured regardless of the GlycoWorks SPE format chosen. The results were based on applying the GlycoWorks workflow to analyze the N-linked glycans of the GlycoWorks Control Standard. This standard is included in the GlycoWorks Kits and may be used to confirm that the analysis is yielding appropriate results, or potentially aid in troubleshooting the procedure.

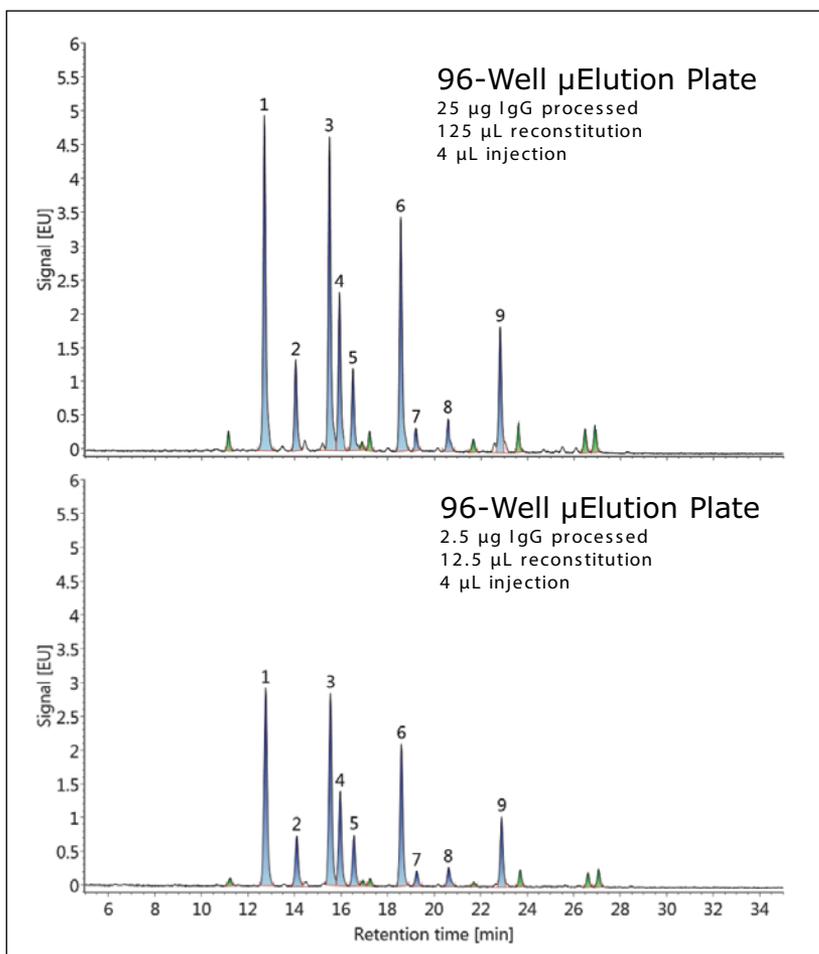


Figure 3. Adaptability of the GlycoWorks workflow and a HILIC SPE device for processing varying quantities of glycoprotein. HILIC-FLR chromatograms are shown of 2-AB labeled glycans prepared from 25 and 2.5 μ g of the GlycoWorks Control Standard.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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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, *RapiFluor-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 *RapiFluor-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.

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, GU⁵) 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 RapiFluor-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.

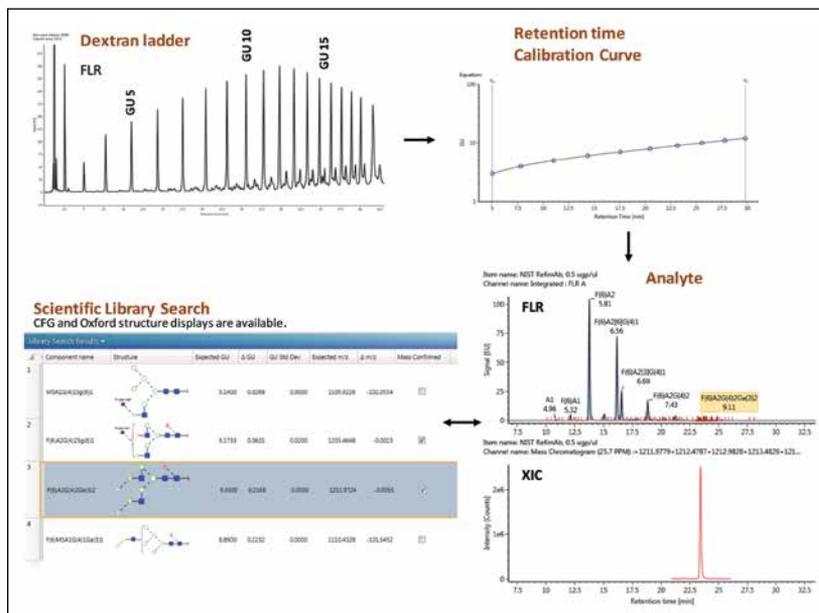


Figure 2. Workflow 1: Glycan FLR with MS confirmation.

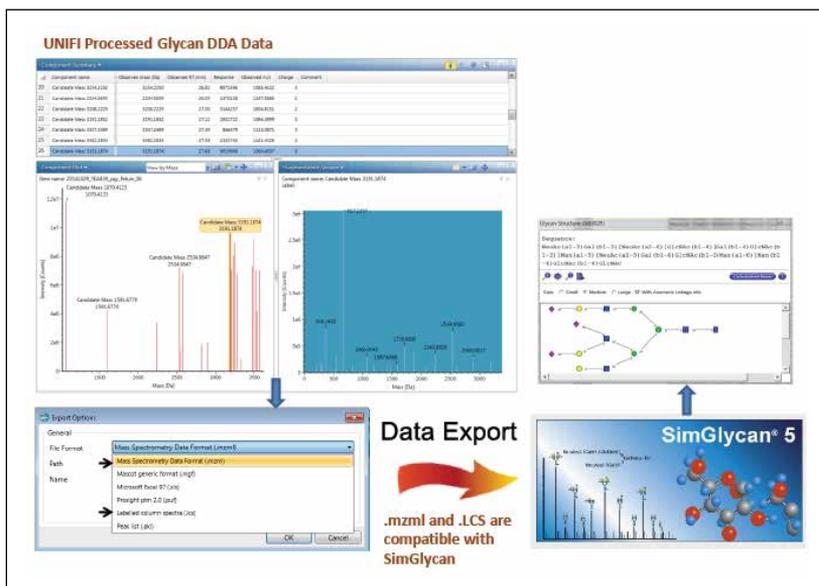


Figure 3. Workflow 2: Glycan DDA workflow in UNIFI Software. Collision-induced dissociation (CID) of glycans in data dependent acquisition (DDA) mode is processed and exported to SimGlycan.

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.

Analytical method for Glycan Application Solution in UNIFI

Sample preparation: N-glycans were prepared using the GlycoWorks RapiFluor-MS N-Glycan Kit (p/n [176003713](#))

System: Biopharmaceutical Platform with UNIFI

LC settings for RapiFluor-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: $\lambda_{ex} = 265 \text{ nm}$, $\lambda_{em} = 425 \text{ nm}$

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 RapiFluor-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.

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 34 Maple Street
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 T: 1 508 478 2000
 F: 1 508 872 1990
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Applying a Novel Glycan Tagging Reagent, *RapiFluor-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, *RapiFluor-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 *RapiFluor-MS* labeled glycans.

WATERS SOLUTIONS

GlycoWorks™ *RapiFluor-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, *RapiFluor-MS* has been developed by Waters. *RapiFluor-MS* contains a rapid tagging reactive group, an efficient fluorophore, and a functional group that imparts high ionization efficiency.[†] 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.[†] 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 *RapiFluor-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 *RapiFluor*-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 *RapiFluor*-MS labeling step and glycan extraction using an SPE device. The entire sample preparation procedure took 30 minutes.

LC conditions

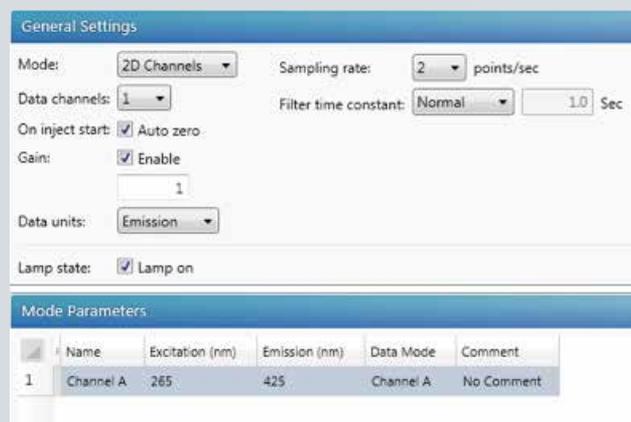
All chromatographic mobile phases are prepared using LC/MS compatible solvents and reagents.

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:



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: H₂O) 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 *RapiFluor*-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 *RapiFluor*-MS labeled N-Glycans released from 0.5 μ g 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 *RapiFluor*-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 *RapiFluor*-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 *RapiFluor*-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.

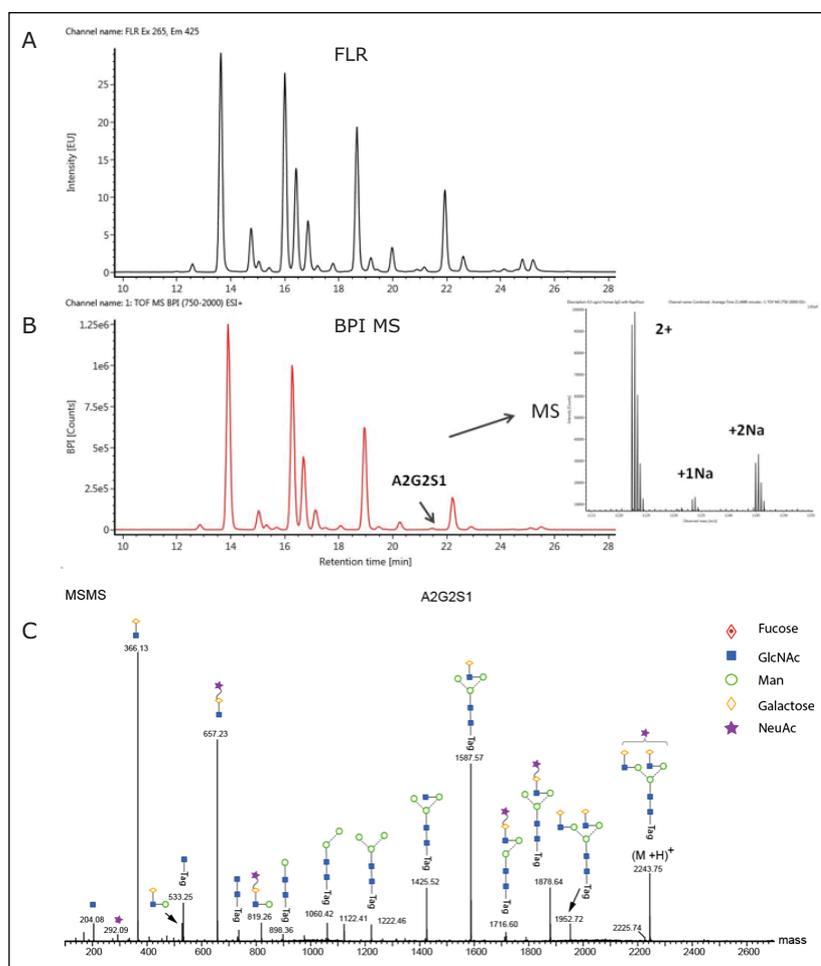


Figure 2. UPLC/FLR/MSMS analysis of *RapiFluor* labeled human IgG N-glycans. A) FLR data channel. B) BPI MS data channel. The MS spectrum of a low intensity ion was inserted (A2G2S1). The dominant ions were doubly charged with minor sodium adduct ions. C) Deconvoluted MS/MS spectrum of A2G2S1 was displayed.

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 *RapiFluor*-MS labeling chemistry, sufficient amount of precursor ions were obtained for subsequent MS/MS fragmentation.

Overall, we demonstrated that *RapiFluor*-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.

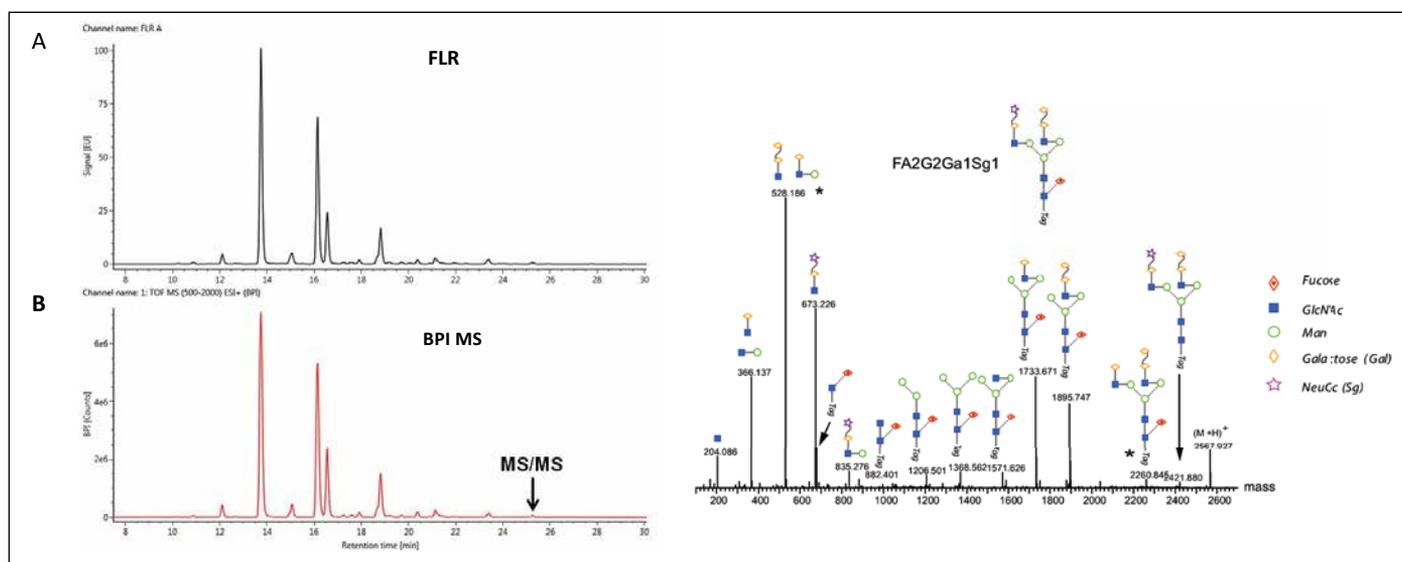


Figure 3. UPLC/FLR/MSMS analysis of *RapiFluor*-MS labeled mouse IgG1 N-glycans. A) FLR data channel. B) BPI MS data channel. One of the last eluting glycans were selected for MS/MS fragmentation. The deconvoluted fragmentation data from FA2Ga1Sg1 was displayed in C); "Ga" stands for galactose and "Sg" 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 *RapiFluor*-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 *RapiFluor*-MS label and the QToF MS with StepWave Technology allow confident identification and characterization of minor but critical glycoforms from mAbs.

References

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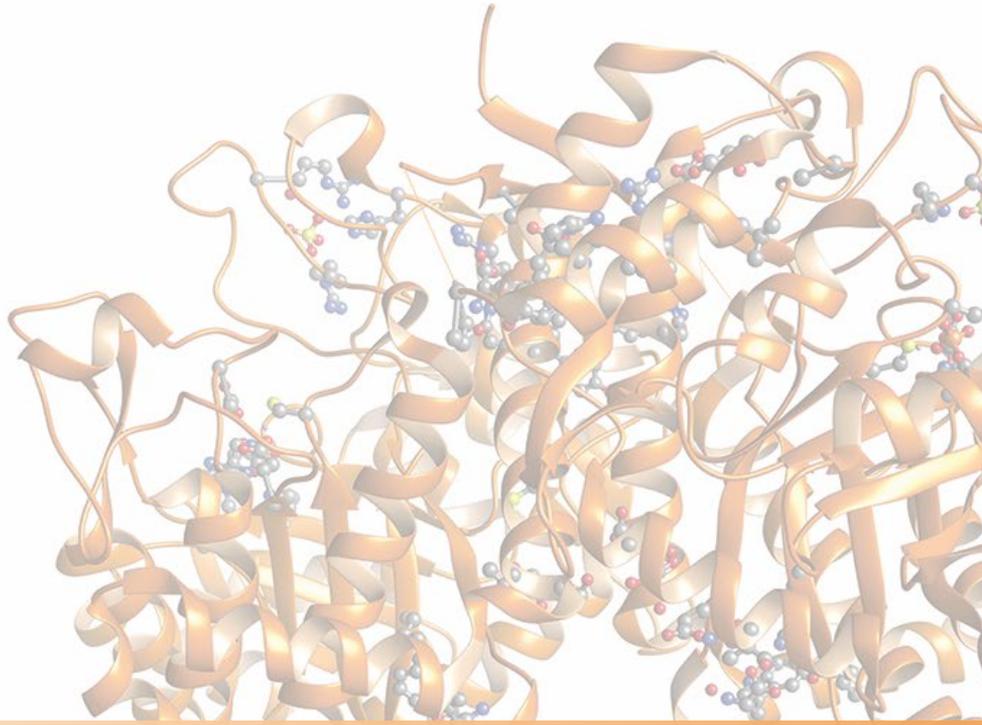
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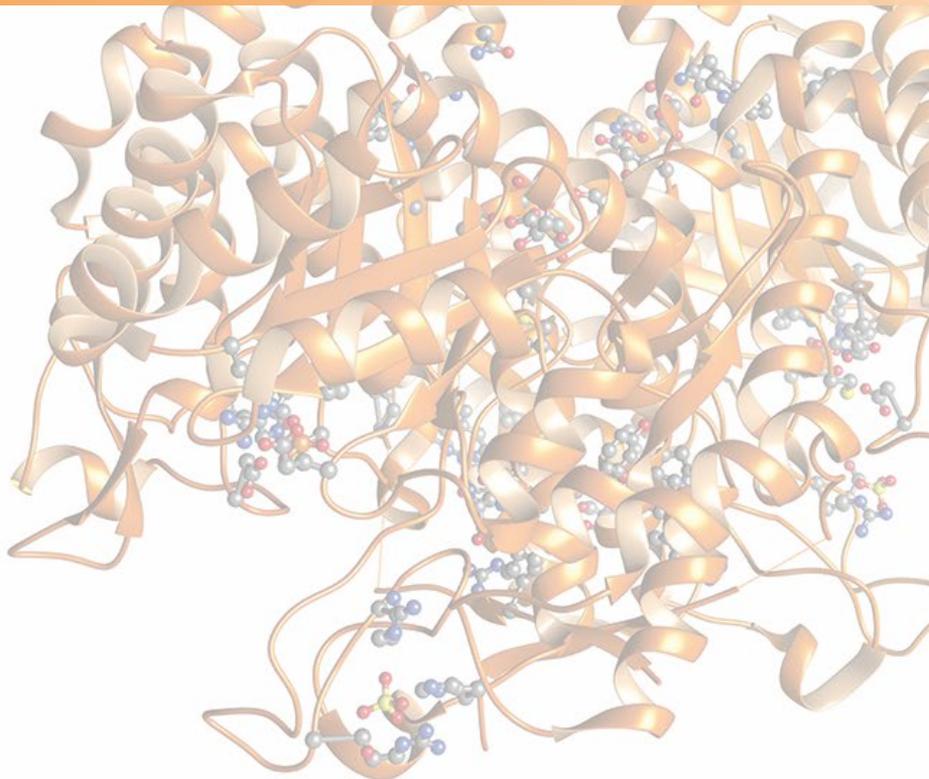
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34 Maple Street
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T: 1 508 478 2000
F: 1 508 872 1990
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BIOSEPARATIONS

Chapter 4



Developing Robust and Efficient IEX Methods for Charge Variant Analysis of Biotherapeutics Using ACQUITY UPLC H-Class System and Auto•Blend Plus

Robert Birdsall, Thomas Wheat, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Increased productivity through the automation of analytical techniques
- Robust method development that delivers consistent and reproducible results for confirmation and quantification of biotherapeutic charge variants
- Reproducible and simplified method development without the need to prepare additional buffers

WATERS SOLUTIONS

Biopharmaceutical Platform Solution
with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet
(TUV) Detector

Protein-Pak™ Hi Res SP SCX Column

UNIFI Scientific Information System

KEY WORDS

Auto•Blend Plus™ Technology,
cation exchange, antibody, IEX, SCX,
chromatography, bioseparation, protein,
method development, robustness

INTRODUCTION

Charge variant analysis is critical for characterizing and monitoring quality attributes of therapeutic proteins. Protein modification such as deamidation, N-terminal pyroglutamation, isomerization, sialylated glycans, and C-terminal lysine clipping all contribute to charge variant formation.¹ In some cases, such changes affect binding, biological activity, patient safety, and shelf lifetime of therapeutic proteins.

The biopharmaceutical industry relies on tools such as ion exchange chromatography (IEX) and isoelectric focusing (IEF) gel electrophoresis to characterize charge variants. Ion exchange chromatography has been particularly useful in the development of biotherapeutics due to its ease of use, wide applicability, and high resolution.

In-depth characterization of charge heterogeneity of therapeutic proteins from the biopharmaceutical development process requires robust and efficient IEX methods. Method development involves a thorough evaluation of all possible experimental parameters such as buffer/ionic strength, buffer pH, salt gradient, flow rate, and column temperature. However, systematic evaluation on the impact of individual experimental parameters on the separation performance often requires a time-consuming and iterative process that involves preparing and testing discreet buffers of varying composition.

Variation in buffer preparation can lead to inconsistent results, consequently increasing method development time. Waters Auto•Blend Plus Technology takes advantage of the ACQUITY UPLC H-Class System's quaternary solvent management capabilities, and uses pure solutions and concentrated stocks to address these challenges. Calculation of the percentage of each stock to blend to achieve the desired pH is performed by the Auto•Blend Plus Technology, reducing error, consumable use, and development time.

With such integrated features, the Biopharmaceutical Platform Solution with UNIFI is well suited for robust method development and can be easily automated for increased productivity. The objective of this application note is to demonstrate the efficiency and robustness of Auto•Blend Plus Technology for optimization of an IEX method for charge variant separations. A chimeric monoclonal antibody, infliximab, was used as a model therapeutic protein to showcase the application.

EXPERIMENTAL

Sample description

A Waters Protein-Pak Hi Res SP, strong cation exchange column (4.6 x 100 mm, 7 μ m, P/N 186004930) was conditioned as outlined by the manufacturer. MES monohydrate (P/N AC327761000), MES sodium salt (P/N AC397351000), sodium chloride (P/N S640-500) were purchased from Fisher Scientific. The chimeric mAb evaluated in this study was used as received for all experiments at a concentration of 20 μ g/ μ L.

LC conditions

LC system:	ACQUITY UPLC H-Class with Auto•Blend Plus
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit
Column:	Protein-Pak Hi Res SP, 4.6 x 100 mm, 7 μ m
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	3 μ L
Flow rate:	0.5 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 M Ω H ₂ O
Buffer conditions:	20 mM MES, pH 6.8
Gradient:	25 mM to 65 mM NaCl in 25 minutes (see Figure 2)

Informatics for data collection & processing

UNIFI Scientific Information System, v 1.6

RESULTS AND DISCUSSION

Auto•Blend Plus Technology

Method development of ion exchange chromatography (IEX) techniques often involves a time-consuming trial and error methodology. The iterative process involves preparing multiple buffers at a specific pH and ionic strength, followed by testing of each buffer system until an adequate separation is achieved.

The Auto•Blend Plus Technology system is integrated software that comes standard with an ACQUITY UPLC H-Class System. It is designed to take the guesswork out of method development and increase productivity in the analysis of charge variants. Auto•Blend Plus helps analysts configure the quaternary solvent management system to blend pure solutions and concentrated stocks to achieve a desired gradient (Figure 1). The end user is presented with an easy-to-use gradient table interface, where the gradient is expressed directly in terms of pH and ionic strength. The software automatically calculates the percentage of acid and base required for the specified pH using the known pK_a value of the chosen buffer system or an empirical calibration table (Figure 2).

Auto•Blend Plus Technology allows for multiple buffer compositions to be tested from a single set of pure components and can be easily automated to improve productivity.

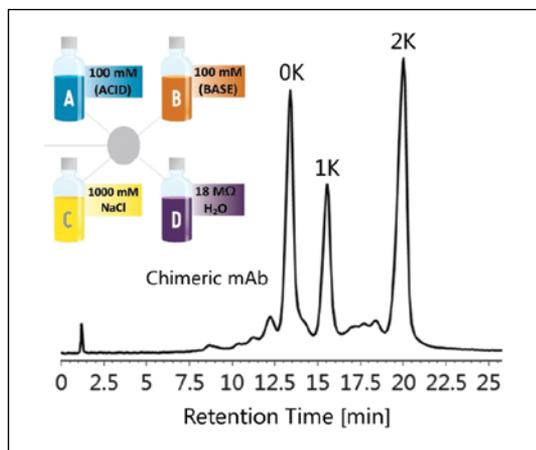


Figure 1. Auto•Blend Plus Technology uses the ACQUITY UPLC H-Class quaternary solvent manager to blend individual pure buffers from the reservoirs to deliver robust separations of charge variants in therapeutic proteins. Here, it is used in the separation of C-terminal lysine truncation variants in a chimeric monoclonal antibody.

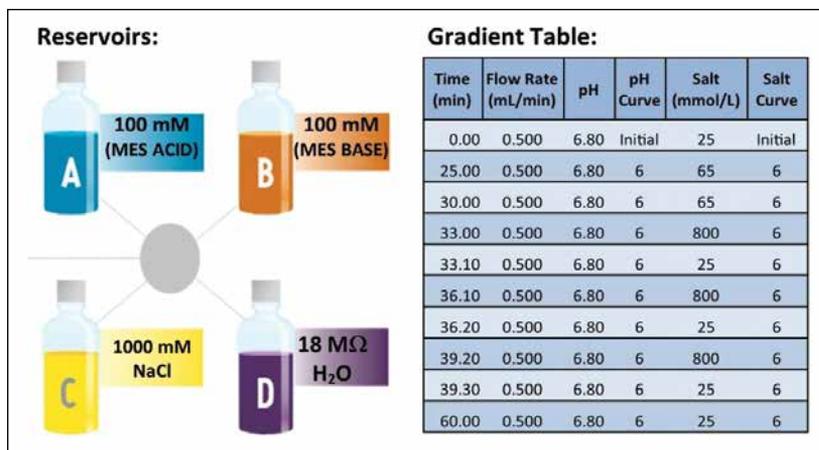


Figure 2. Illustration of a typical Auto•Blend Plus Technology reservoir setup with accompanying gradient table for separation of a chimeric monoclonal antibody.

Robust method development

Robustness is a measure of the ability of a separation method to maintain reproducible results with the introduction of small changes in the system. For ion exchange chromatography, these parameters can include pH, protein mass load, and reproducibility. For pharmaceutical companies a robust method can increase productivity with less time spent on method validation. These parameters were explored to evaluate the robustness of method development using the Auto•Blend Plus Technology.

Protocol for validating and qualifying Auto•Blend Plus

Auto•Blend Plus Technology enables easy system validation and qualification when transferring methods between instruments, analysts, and labs.

Three separate MES buffer systems were prepared and tested using the outlined protocol, below. From Table 1, it can be readily seen that the experimental pH from each buffer system is in good agreement with the desired test pH. The precision among the three separate buffer systems results in reproducible chromatograms as shown in Figure 3. Auto•Blend Plus Technology can readily be adapted to qualification protocols, minimizing time spent on system validation.

Install solutions

- A: 100 mL of 1.0 M MES monohydrate in 900 mL HPLC grade H₂O
- B: 100 mL of 1.0 M MES sodium salt in 900 mL HPLC grade H₂O
- C and D: HPLC grade H₂O

Cross-calibrate pH meter

- Low pH reference: Mix 1.8 mL from A, 0.2 mL from B, 8 mL from C
- Medium pH reference: Mix 1 mL from A, 1 mL from B, 8 mL from C
- High pH reference: Mix 0.2 mL from A, 1.8 mL from B, 8 mL from C
- Record pH

Test solutions

- Low: 0.5 mL/min at low pH reference (pH 5.13); Salt concentration: 0
- Medium: 0.5 mL/min at medium pH reference (pH 6.12); Salt concentration: 0
- High: 0.5 mL/min at high pH reference (pH 7.10); Salt concentration: 0

Collect samples

- Run to waste for 10 minutes
- Collect effluent in scintillation vial for 20 minutes
- Repeat for all three test solutions

Measure pH

- Confirm pH meter calibration
- Measure and record pH for each test solution

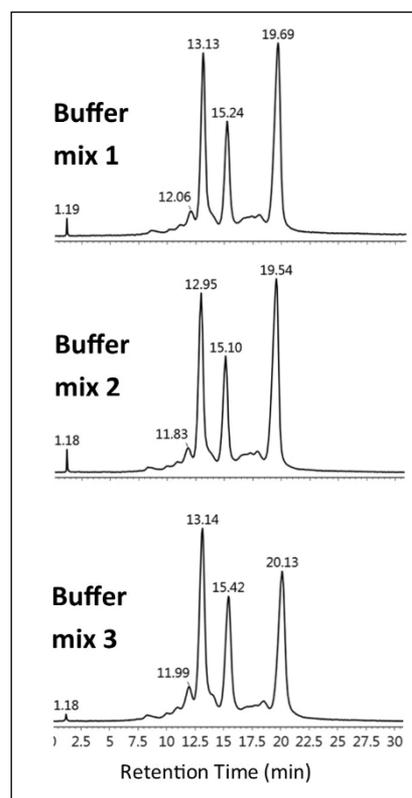


Figure 3. Separation of C-terminal lysine truncation variants with three different preparations of MES buffer over a two-week time period using Auto•Blend Plus Technology.

Test pH	Buffer mix 1	Buffer mix 2	Buffer mix 3	Mean	Std. Dev.	%RSD
5.13	5.10	5.02	5.10	5.07	0.05	0.91
6.12	6.19	6.05	6.19	6.14	0.08	1.32
7.10	7.23	7.06	7.23	7.17	0.10	1.37

Table 1. Experimental pH results for three MES buffer preparations.

Maintaining consistent separation performance with increasing sample concentration

Retention time and column performance can be affected by the amount of protein being injected onto the IEX column.

The effects of protein mass load on column performance were tested by injecting between 1-10 μL of the chimeric mAb stock solution in 1 μL intervals. Total peak area was integrated from 5-30 minutes for each injection. Reproducible retention times were observed over a 9-fold increase in mass load ranging from 20-180 μg of protein as shown in Figure 4. Coupled with the Auto•Blend Plus Technology, the ACQUITY UPLC H-Class System provides a high degree of fidelity for accurate quantification and characterization of charge variants in biotherapeutics.

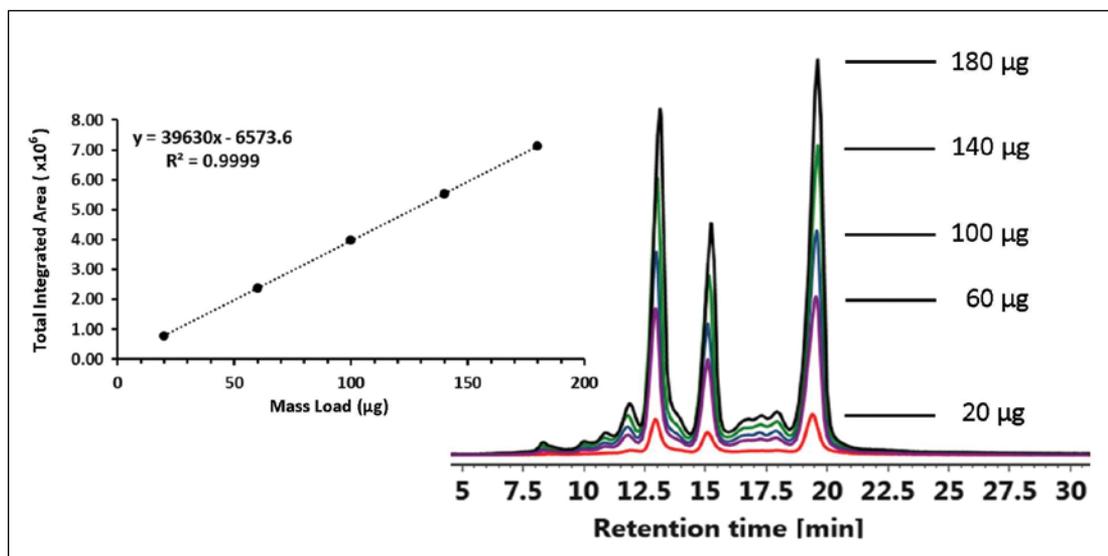


Figure 4. Chromatogram overlays of a chimeric monoclonal antibody separation with increasing protein concentration. The total peak area was integrated as a measure of precision as shown in the plot of integrated area vs. mass load.

Achieving highly reproducible separations across analysis replicates

Automation of analytical techniques can minimize error in method development as well as increase productivity.

Auto•Blend Plus Technology was evaluated over 40 injections to simulate an unattended analysis over three days. Chromatograms are shown at injection 1, 20, and 40. The 60-minute separations as outlined in Figure 2 are comprised of a 30-minute separation gradient and a 30-minute cleaning and reconditioning phase. Integration intervals of five peak areas including the three main C-terminal lysine truncation variants are represented by the vertical drop lines in each chromatogram. Calculated areas of each peak area and total area are listed in Table 2.

It can be seen that Auto•Blend Plus Technology offers reproducible results well within U.S. FDA guidelines² with covariance of the individual peaks below 12% and the total peak area covariance below 9%. The ability to automate Auto•Blend Plus Technology, combined with its reproducibility, offers a reliable approach to robust method development for the characterization of charge variants in biotherapeutics.

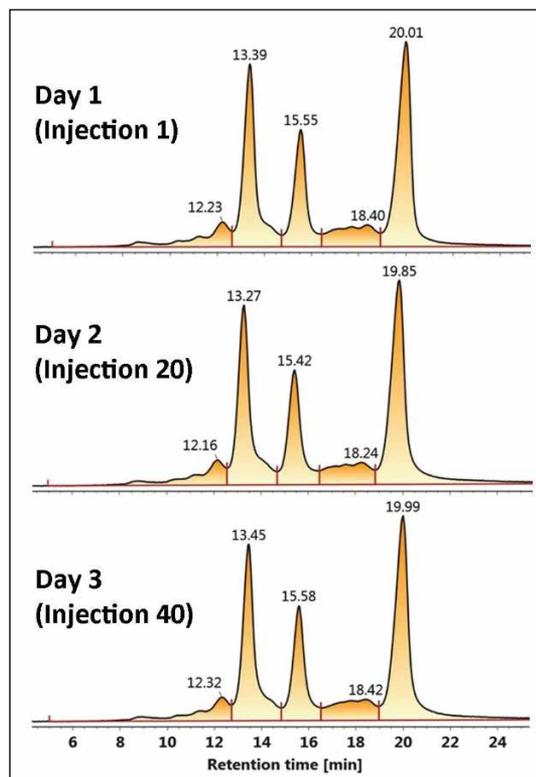


Figure 5. Separation of charge variants of a chimeric monoclonal antibody at three different time intervals over three days. Integration intervals represent the five peak areas calculated in Table 2.

Injection #	Peak 1 Area	Peak 2 Area	Peak 3 Area	Peak 4 Area	Peak 5 Area	Total
1	252260.0	788195.0	504001.0	296130.0	1052614.0	2893200.0
20	203498.0	660040.0	427519.0	237894.0	940898.0	2469849.0
40	214836.0	686459.0	437254.0	255744.0	974813.0	2569106.0
Avg	223531.3	711564.7	456258.0	263256.0	989441.7	2644051.7
SD	25517.4	67665.7	41632.2	29835.9	57276.6	221402.7
% RSD	11.42	9.51	9.12	11.33	5.79	8.37

Table 2. Integrated peak area results.

CONCLUSIONS

Analysis of charge heterogeneity profiles that arise during the development process of biopharmaceuticals require robust methods that can be automated, readily deployed, and quickly adapted to meet the demand of fast paced biopharmaceutical industry.

The combination of Auto•Blend Plus Technology with the ACQUITY UPLC H-Class System, which represent a UV-based option of the Biopharmaceutical Platform Solution with UNIFI, simplifies method development by allowing multiple buffer compositions to be tested from a single set of pure components. These features, combined with the ability to automate the process, makes Auto•Blend Plus a powerful tool for robust method development and increasing productivity.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Charge Variant Analysis of Therapeutic Monoclonal Antibodies Using a pH Gradient Generated by Auto•Blend Plus

Robert Birdsall and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Increased productivity through the automation of analytical method parameter evaluations
- Thorough method development for charge separation using pH gradients for confirmation and quantification of biotherapeutic charge variants
- Efficient pH or ionic strength screening using concentrated stocks

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP SCX Column

KEY WORDS

Auto•Blend Plus,™ cation exchange, antibody, IEX, SCX, bioseparation, therapeutic protein, method development, pH gradient, UPLC

INTRODUCTION

Charge-based separation methods play an important role in characterization studies and quality control strategies for biotherapeutics.¹⁻⁴ For the analysis of charged species of antibodies, ion exchange chromatography (IEX) has a widespread use in the biopharmaceutical industry for its ability to resolve species related to protein conformation, size, sequence variants, glycosylation, and post-translational modifications. The capability to perform protein characterization under non-denaturing conditions combined with the ability to isolate charge variants easily has both contributed to the popularity of IEX in charge variant analysis of biotherapeutics.

Protein separations by IEX methods routinely utilize salt (ionic strength) or pH gradients to elute the protein from the IEX column. Although somewhat different in the separation mechanisms of the two eluting methods, method parameters such as column types, mobile phase composition, and pH (or salt concentration) gradients often need to be evaluated to yield the optimal separation for each individual antibody.⁵ However, the evaluation of the selected method parameters often requires a time consuming, iterative process that involves preparing and testing discrete buffers of varying compositions. This requirement imposes a great challenge to the method development process, and calls for an intelligent setup/process that decreases time spent on method development, and improves the efficiency of the workflow.

Auto•Blend Plus Technology uses the ACQUITY UPLC H-Class System's quaternary solvent manager to blend individual pure solutions and concentrated stocks from the reservoirs to deliver pH gradients for the separations of charge variants in therapeutic proteins. The technology allows the analyst to evaluate multiple buffer compositions from the concentrated stocks; enabling the evaluation process to be easily automated to increase the productivity of charge variant analysis. Auto•Blend Plus Technology allows for the use of mixed buffers with different pka values to increase the buffering capacity and extend the effective working pH range that a single buffering species cannot deliver.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class System with Auto•Blend Plus
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	Protein-Pak Hi Res SP, 7 μ m, 4.6 x 100 mm (p/n 186004930)
Column temp.:	25 $^{\circ}$ C
Sample temp.:	4 $^{\circ}$ C
Injection vol.:	3 μ L
Flow rate:	0.50 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM sodium phosphate dibasic
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 M Ω H ₂ O
Buffer concentration:	20 mM
Gradient:	pH 5.2 to 7.9 in 30 minutes (Figure 2); pH 6.5 to 7.2 in 15 minutes (Figure 6)

Informatics for data collection & processing

UNIFI Scientific Information System, v 1.6

In addition to these benefits, the unique design of Auto•Blend Plus Technology provides the flexibility to allow analysts to switch between pH or salt gradients during the method development process for the determination of optimal separation parameters. The objective of this application note is to demonstrate the performance of Auto•Blend Plus for optimizing IEX methods for charge variant separations using pH gradients. A therapeutic monoclonal antibody, infliximab, was used as a model protein to evaluate the functionality.

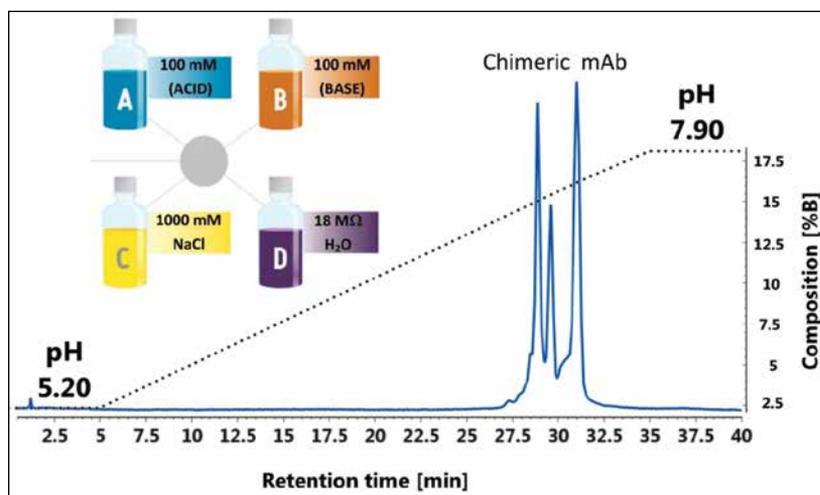


Figure 1. Automated delivery of an extended pH gradient range by the Auto•Blend Plus Technology. The chromatogram shows a gradient spanning from pH 5.20 to 7.90 is formed for the separation of lysine truncation charge variants of a chimeric monoclonal antibody (infliximab).

Sample Preparation

A Waters Protein-Pak Hi Res SP, strong cation exchange column (7 μ m, 4.6 x 100 mm, p/n 186004930) was conditioned as outlined by the manufacturer. MES monohydrate (p/n A69892), sodium phosphate dibasic (p/n S5136), and sodium chloride (p/n S1679) were purchased from Sigma Aldrich. The pH gradients generated by Auto•Blend Plus were monitored on-line using a GE Healthcare Monitor pH/C-900 similar to previous work (p/n 720004149en). Calibration was performed at flow rates of 1 mL/min with the column off-line using the reference pH values from the empirical table data. The mAb samples evaluated in this study were used as received for all experiments at a concentration of 20 μ g/ μ L.

RESULTS AND DISCUSSION

Flexible method development with the Auto•Blend Plus

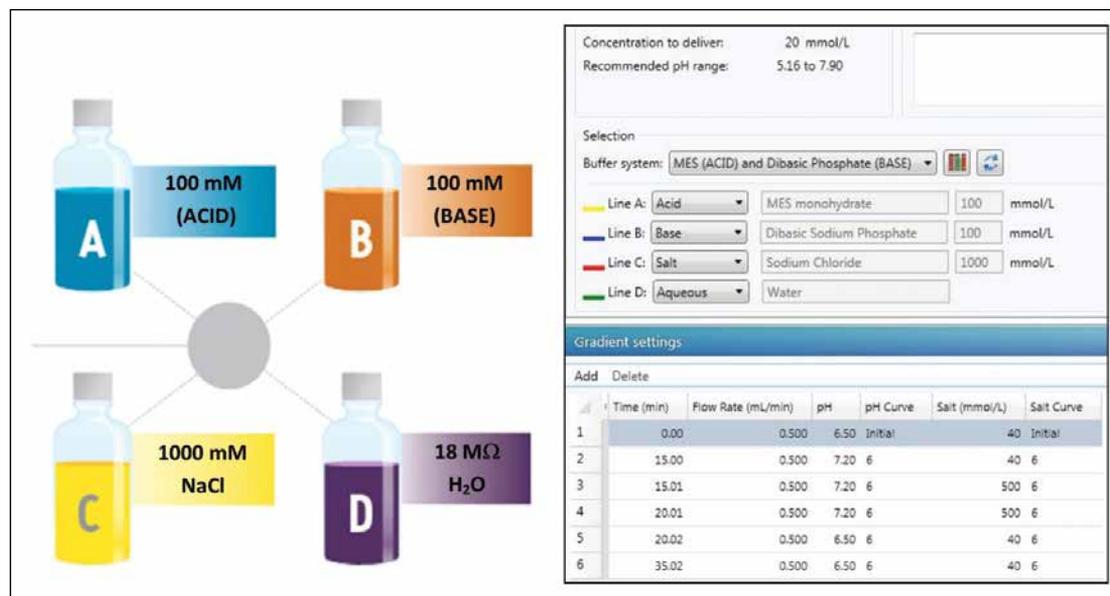


Figure 2. An example of the intuitive interface for programming pH or salt gradients rendered by the Auto•Blend Plus Technology. The software algorithm takes in the specified values and automatically calculates the percentage of acid and base required to deliver the desired pH gradient range and ionic strength.

Figure 2 shows a pH gradient table that Auto•Blend Plus Technology is programmed to generate using pure solutions and concentrated stocks on a quaternary solvent management system. The gradient table presents an easy-to-use interface to the end user, where the gradient is expressed directly in terms of pH and ionic strength. The software algorithm can independently control pH or ionic strength enabling the analyst to generate a variety of gradient conditions including constant pH with varying ionic strength, vice versa, or change pH and ionic strength simultaneously. With Auto•Blend Plus, the software automatically calculates the percentage of acid and base required for each pump stroke to deliver the specified pH using the chosen buffer system. Auto•Blend Plus allows for multiple buffer compositions to be mixed and evaluated from a single set of pure components, reducing cost and time in method development.

Increasing productivity with custom buffer systems

Buffer System Details (Read-Only)

Buffer System

Buffer system name: MES (ACID) and Dibasic Phosphate (BASE)

Buffer concentration to deliver: 20 mmol/L

Acid concentration: MES monohydrate 100 mmol/L

Base concentration: Dibasic Sodium Phosphate 100 mmol/L

Salt concentration: Sodium Chloride 1000 mmol/L

Aqueous concentration: Water

Refresh solvent names: [Refresh]

Comment: pH gradient

pH Calibration:

pH Calibration: pKa 7 Empirical Data Enforce validation of concentration

Add Delete Clear

	% Acid	% Base	% Salt	% Aqueous	pH
1	18.0	2.0	0.0	80.0	5.16
2	10.0	10.0	0.0	80.0	6.53
3	2.0	18.0	0.0	80.0	7.90
4	18.0	2.0	25.0	55.0	5.15
5	10.0	10.0	25.0	55.0	6.35
6	2.0	18.0	25.0	55.0	7.56
7	18.0	2.0	50.0	30.0	5.19
8	10.0	10.0	50.0	30.0	6.35
9	2.0	18.0	50.0	30.0	7.56

OK Cancel

Figure 3. An example of the empirical table used by Auto•Blend Plus. Nine buffer mixture standards were used to create the exemplary reference table from which Auto•Blend Plus is based to automatically deliver a pH range over 5.2 to 7.9.

Traditional ion exchange chromatography employs buffers comprised of the same molecular species such as MES buffer (pH 5.5 to 6.7), phosphate buffer (pH 6.7 to 7.6), and HEPES buffer (pH 7.6 to 8.2). The limited working pH ranges of these individual buffer systems prolong the method development process for a separation based on pH gradients since multiple buffers need to be prepared and tested to optimize the separation performance over the entire pH range.

Auto•Blend Plus Technology allows for the preparation of custom buffers with an extended working pH range through the use of the empirical calibration table as shown in Figure 3. For this work, a 100 mM solution of 2-ethanesulfonic acid monohydrate (MES monohydrate) was prepared as the acidic reservoir and a 100 mM solution of sodium phosphate dibasic was prepared as the basic reservoir as illustrated in Figure 2. The empirical table was constructed from nine buffer mixture standards prepared from the concentrated stocks. The pH value was measured with a pH meter and entered into the table as shown in Figure 3. The use of MES monohydrate and sodium phosphate dibasic as an ion exchange buffer system gives an extended working pH range of 5.2 to 7.9, allowing for a larger set of experimental parameters to be tested from a single set of buffers.

The ability to generate extended pH ranges from concentrated stock buffers via Auto•Blend Plus without the need for multiple buffer systems makes it ideal for increasing productivity and reducing method development costs.

Linear pH gradients with Auto•Blend Plus

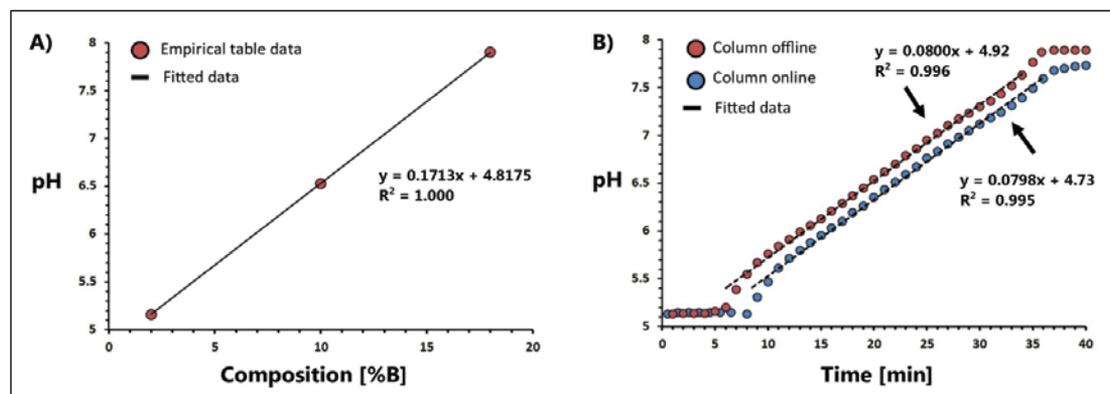


Figure 4. Using the A) empirical table data, Auto•Blend Plus was programmed to generate a 30 minute gradient from 5 to 35 minutes with a pH value ranging from 5.20 to 7.9. B) The generated gradient was evaluated for pH linearity in the presence or absence of the IEX column in the LC system. Each point in the figure was acquired using an on-line pH meter for the buffer composition (gradient) generated by Auto•Blend Plus.

The fidelity of a pH gradient generated by Auto•Blend Plus was assessed for its ability to produce a designed linear pH gradient over an extended pH range using a selection of buffer systems. For illustrative purposes, a plot of pH versus composition %B (% Base) was constructed from the empirical table data shown in Figure 3 to assess pH response linearity for the selected buffer system. From Figure 4A it can be seen that the empirical data for the chosen buffer system has a linear response over a pH range of 5.2 to 7.9. Auto•Blend Plus was programmed to generate a 30-minute gradient over a pH range of 5.2 to 7.9 starting at the 5-minute mark using the empirical table data from Figure 3. The programmed gradient was evaluated with or without the Waters Protein-Pak Hi Res SP, a strong cation exchange column, online so the impact of column effects on the variation of pH linearity could also be assessed.

Similar to a method from previous work⁶ the mobile phase pH was monitored in-line using a GE Healthcare monitor pH/C-900 with pH being manually recorded in 1 minute intervals. From Figure 4B it can be seen that Auto•Blend Plus is capable of delivering a highly linear pH gradient over the gradient time window using the extended pH buffer system. The close agreement of the slopes of the fitted data on both plots indicates the column had no significant effect on the linearity of the pH gradient other than a small time delay due to the additional volume introduced by the column. The ability to deliver an extended linear pH gradient using custom buffer selections makes Auto•Blend Plus well suited for flexible method development.

Automation for pre-screening experimental method parameters

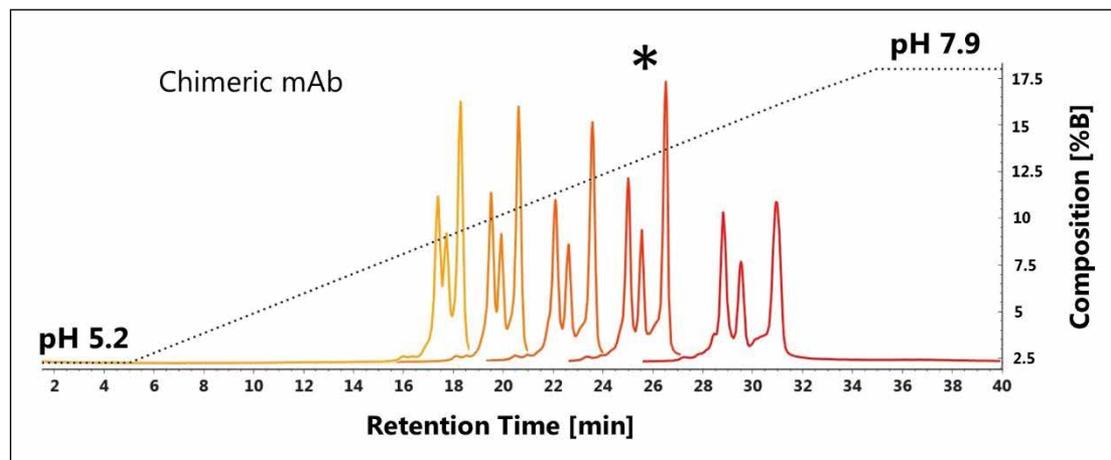


Figure 5. Optimization of ionic strength in the separation of lysine charge variants of a chimeric monoclonal antibody. From right to left, the ionic strength tested for each chromatogram was 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM NaCl. The ionic strength of 40 mM (denoted by the asterisks) provides the highest resolution between the main lysine variant peaks.

Method development of IEX separations often involves a time-consuming trial and error procedure because a successful separation cannot be readily predicted. The iterative process involves preparing multiple buffers at a specific pH and ionic strength, followed by testing each buffer system for separation performance until an adequate separation is achieved. The ability of Auto•Blend Plus Technology to blend multiple buffer compositions from a single set of concentrated stocks allows for evaluation of many experimental parameters in a highly efficient manner.

For example, it is well known that ionic strength affects separation performance, and that it should be evaluated in the optimization process. Figure 5 shows how Auto•Blend Plus Technology was used to evaluate the impact of ionic strength on a mAb charge separation with the extended pH buffer range prepared earlier. From the concentrated stocks the ionic strength (line C) was increased in 20 mM intervals for each chromatographic trace shown in Figure 5, starting with 20 mM on the right side of the plots.

Using the chromatographic peak that represents the charge variant of the monoclonal antibody containing two C-terminal Lysine residues as the investigative target (see Figure 6), we systematically evaluated the impact of ionic strength on the charge separation performance during our experiments. Resolution for the +2 Lys peak was reported as 2.66, 2.70, 2.33, 1.77, and 1.28 corresponding to the ionic strengths of 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM, respectively. This automated evaluation process renders an efficient and consistent way to find the optimized ionic strength for the pH gradient slope, and demonstrates that Auto•Blend Plus is an integrated software solution designed to streamline method development.

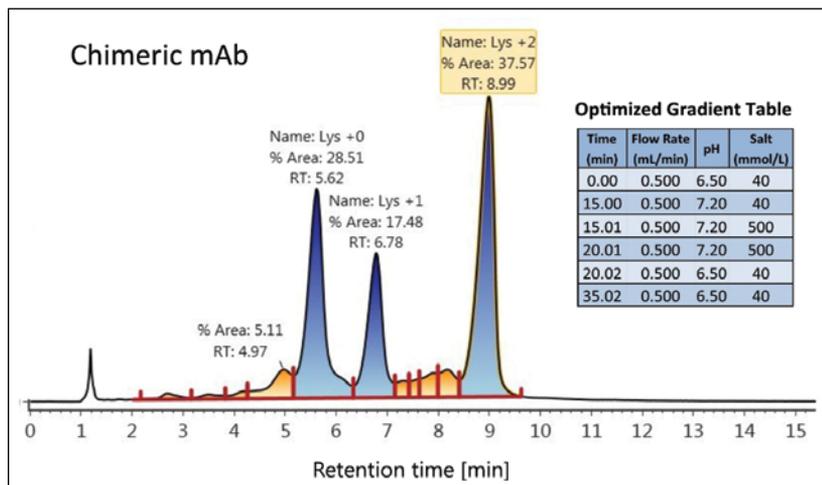


Figure 6. An optimized high throughput pH gradient separation of a chimeric monoclonal antibody in under 10 minutes. The pH gradient was optimized from pH 6.50 to 7.20 with ionic strength constant at 40 mM NaCl. Identification and integration of the C-terminal lysine variants were performed using tools within UNIFI Software and automatically displayed in the chromatogram window.

Integration with advanced informatics for automated data acquisition, processing, and reporting

The Waters Biopharmaceutical Platform Solution with UNIFI provides a highly efficient analytical platform solution for routine characterization of biotherapeutics. Auto•Blend Plus Technology combines with other informatics tools within UNIFI Software to deliver an integrated workflow to perform charge-based separation and optimization using pH gradients. This workflow possesses the capability to automatically acquire data, process the data and provide a report, further enhancing and streamlining the method development process. Utilizing the informatics tools from UNIFI, further optimization of the pH gradient was performed.

Examination of the separation results from Figure 5 suggests that the pH gradient range that would effectively deliver the same separation performance observed at 40 mM NaCl would only require a portion of the 40-minute run time. Since the pH gradient curve generated by Auto•Blend Plus Technology follows a predictable mathematical equation, the pH value at any point along the gradient can be readily calculated and changed so equivalent separation performance can be achieved with shorter analysis time.

Using this methodology, the separation achieved in Figure 5 at an ionic strength of 40 mM was further optimized with the elution of the full charge variant profile of the chimeric monoclonal antibody within 10 minutes as shown in Figure 6. Relative peak area, retention time, and component name of the chromatographic peaks of interest were automatically calculated and labeled by the UNIFI Software. Quick delivery of the information by UNIFI to evaluate the separation performance is time-saving and promotes the method development process.

CONCLUSIONS

The development of methods for the analysis of biopharmaceutical charge heterogeneity profiles is a time-consuming process that requires methods that can be automated, quickly adapted, and readily deployed to meet the demands of the biopharmaceutical industry. The combination of Auto•Blend Plus Technology with the ACQUITY UPLC H-Class System improves workflow efficiency by allowing multiple buffer compositions to be tested from a single set of pure components. The flexibility to work with pH or salt gradients, combined with the ability to automate, makes Auto•Blend Plus Technology a powerful tool for increasing productivity and reducing development costs.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Simultaneous Determination of Molecular Size, Concentration, and Impurity Composition of Biotherapeutics with SEC and the Biopharmaceutical Platform Solution with UNIFI

Robert Birdsall, Thomas E. Wheat, Henry Shion, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Efficient and integrated workflow for running batched samples to maximize analytical information content of biotherapeutics.
- Increased productivity through the automation of an analytical workflow including data acquisition, processing, and reporting.
- Increased productivity through automated assessment of protein aggregates.

WATERS SOLUTIONS

Biopharmaceutical Platform Solution
with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV)
Detector with 5-mm titanium flow cell

Auto•Blend Plus™ Technology

ACQUITY UPLC Protein BEH SEC Column,
200Å, 1.7-µm

KEY WORDS

Size exclusion chromatography (SEC),
monoclonal antibody (mAb), bioseparation,
method development, quantification

INTRODUCTION

Size exclusion chromatography (SEC) is often used to assess the size distribution of molecular species for therapeutic proteins in a solution (*e.g.* protein clips, aggregates, etc.). The non-denaturing buffers commonly employed in SEC allow for the characterization of proteins in their native state. In addition to measuring molecular size, peak areas from SEC can be readily used in the relative and absolute quantitation of biological samples for increased productivity. As such, this technique has been particularly useful in the biotechnology industry for detecting and quantifying protein aggregation of biotherapeutics.

Protein aggregation in biotherapeutics have been linked to potential loss of therapeutic efficacy as well as unwanted immunogenic responses.^{1,2} Controlling factors that contribute to aggregate formation, for example, protein misfolding during expression stages,³ protein denaturation during purification processes,¹ and high protein concentration during formulation,⁴ has been an area of continuing interest in the pharmaceutical industry.

Increasing demand from regulatory bodies to provide detailed information about the quantity and nature of aggregates in biotherapeutics, combined with rising development costs and a demanding work environment, require cost-effective solutions that have minimum impact on productivity. Efficient workflows that seamlessly combine characterization and quantitation information for biotherapeutics are highly desirable.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class System with Auto•Blend Plus Technology
Detector:	ACQUITY UPLC TUV Absorption
Wavelength:	220 nm
Vials:	Total recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	ACQUITY UPLC Protein BEH SEC, 200Å, 1.7-µm, 4.6 x 150 mm (p/n 186005225)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	2 µL
Flow rate:	0.150 mL/min
Mobile phase A:	100 mM sodium phosphate monobasic monohydrate (NaH ₂ PO ₄)
Mobile phase B:	100 mM sodium phosphate dibasic (Na ₂ HPO ₄)
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H ₂ O
Autoblend Plus	
Method:	Isocratic (150 mM NaCl in 20 mM phosphate buffer; pH 7.4)

Informatics for data collection and processing

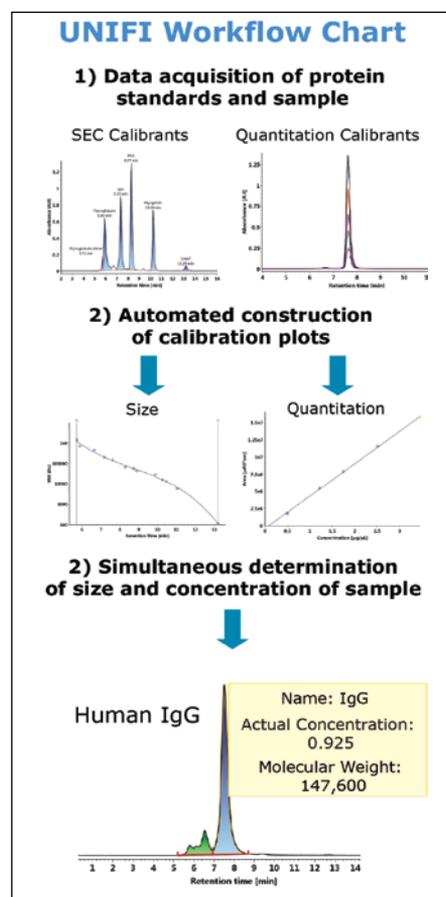
UNIFI Scientific Information System, v1.6

The Waters Biopharmaceutical Platform solution with UNIFI is developed to streamline the analytical workflow to increase the productivity in the characterization of biotherapeutic samples. The ability to perform SEC using conditions that minimally perturb aggregate composition make it ideal in the assessment and communication of multiple attributes of biotherapeutics such as size, aggregate composition, and concentration. Through the use of calibrated standards, tools within the UNIFI Scientific Information System can simultaneously determine the molecular size (apparent molecular weight) and the concentration of chromatographically resolved species in a biotherapeutic sample in the same analysis.

The objective of this application note is to demonstrate the ability to determine molecular weight and amount of the constituents of an antibody sample using UNIFI informatics. A purified antibody from human serum was used as a model protein to test the application.

Protocol

A Waters ACQUITY UPLC Protein BEH SEC Column was conditioned as outlined by the manufacturer. Waters BEH200 SEC protein standards (p/n 186006518) and BEH125 SEC protein standards (p/n 186006519) were prepared in 1 mL and 0.2 mL of 18 MΩ water, respectively. Apoferritin (p/n A3660), β-amylase (p/n A8781), carbonic anhydrase (p/n C7025), insulin (p/n I0516), sodium phosphate monobasic monohydrate (p/n S3522), sodium phosphate dibasic (p/n S5136), and sodium chloride (S5886) were purchased from Sigma Aldrich. The Waters Glycoworks control standard (p/n 186007033; purified human IgG) was used as an “unknown” and prepared at a concentration of 1 µg/µL as per the labeled amount using 18 MΩ water. Apoferritin, β-amylase, carbonic anhydrase, and insulin were prepared at concentrations of 10 µg/µL, 2.9 µg/µL, 1.5 µg/µL, and 5.0 µg/µL, respectively. The Waters mAb mass check standard (p/n 186006552) used for quantification was prepared at concentrations of 0.49 µg/µL, 1.22 µg/µL, 1.74 µg/µL, 2.51 µg/µL, and 3.46 µg/µL in 18 MΩ water.



RESULTS AND DISCUSSION

Integrated informatics tools for the construction of calibration plots

Size exclusion chromatography is often employed by the pharmaceutical industry for the assessment of aggregate content in biotherapeutic samples. In principle, the elution time of a protein in a SEC separation is determined by how much of the intra-particle pore volume is accessible to the protein.⁶ In practice, this separation mechanism prescribes that protein species will elute in order of decreasing hydrodynamic radius. This chromatographic behavior is illustrated by the lower left panel of Figure 1, where Waters SEC protein standards (calibrants) are separated using an ACQUITY UPLC Protein BEH SEC Column (200Å, 1.7-µm, 4.6 x 150 mm).

Using the known molecular weight of the calibrants defined in the component manager as shown in the top panel of Figure 1, the built-in UNIFI informatics tool automatically constructs a size calibration plot (log MW vs. RT) for the standards as shown in the lower right panel of Figure 1. Proteins used in this optimized size calibration plot include thyroglobulin dimer, thyroglobulin, apoferritin, β-amylase, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, ribonuclease A, insulin, and uracil. The calibration plot can be constructed using the logarithmic scale of MW of protein standards as y-axis plotted against either the elution time or the elution volume (x-axis). The data is automatically fitted with a linear or a higher-order polynomial equation to acquire a calibration curve as shown in Figure 1.



Figure 1. Waters BEH200 SEC protein standards (p/n 186006518) are separated (bottom left panel) using an ACQUITY UPLC Protein BEH SEC Column (p/n186005225). A separation calibration plot of log MW versus retention time (lower right panel) is automatically constructed using UNIFI informatics tools from a set of defined protein standards (top panel).

For disparate measurements such as apparent molecular weight (elution time vs. molecular weight) and concentration (area vs. amount), individual assays that generate targeted data sets are usually required. UNIFI informatics allows for the incorporation of multiple calibration plots that can be applied to a single data stream for the measurement of such dissimilar attributes as size and concentration.

Figure 2 illustrates how UNIFI constructs a concentration calibration curve in the same analysis as the apparent molecular weight measurement using a Waters mAb mass check standard as a calibrant for proof of principle. Using the mAb standard, 200 μL of 18M Ω water was added to the 1 mg of lyophilized protein to generate a stock calibrant solution with a concentration of 5.00 $\mu\text{g}/\mu\text{L}$. From the stock calibrant, five standard samples were prepared at concentrations of 0.493 $\mu\text{g}/\mu\text{L}$, 1.22 $\mu\text{g}/\mu\text{L}$, 1.74 $\mu\text{g}/\mu\text{L}$, 2.51 $\mu\text{g}/\mu\text{L}$, and 3.46 $\mu\text{g}/\mu\text{L}$. Three replicates of the five standard samples were performed in a serial fashion with a constant volume (2 μL) injected on an ACQUITY UPLC Protein BEH SEC Column (200 \AA , 1.7- μm , 4.6 x 150 mm). Using the defined concentrations as indicated by their concentration level in the component summary window of Figure 2, UNIFI automatically constructs the concentration calibration plot as shown in the bottom right panel of Figure 2.

The ability to automate the construction of multiple calibration plots and apply them in a single data stream to discern uniquely disparate critical quality attributes makes the Waters Biopharmaceutical Platform Solution with UNIFI a preferred system for increasing productivity during the method development process.

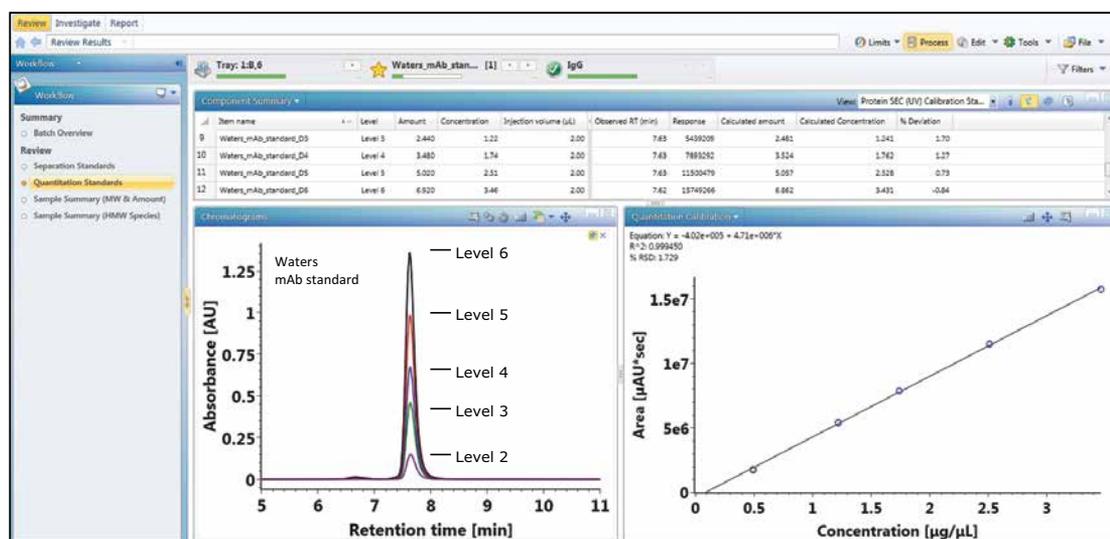


Figure 2. The Waters mAb mass check standard was injected at five different concentration levels (lower left panel). Using the supplied concentrations as indicated by their level in the component summary panel, UNIFI informatics automatically constructs a calibration plot of peak area versus sample concentration (bottom right panel).

Simultaneous determination of apparent molecular weight and concentration of purified human IgG from a single injection

The experimental results for the calibration plots show that as an integrated platform, UNIFI is fully capable of determining the apparent molecular weight and concentration of biotherapeutics analyzed in the same sample set as the protein standards. A purified human IgG sample (p/n 186007033) was analyzed using the method to demonstrate the capability of the platform to generate such information for an “unknown” sample. The lyophilized sample was reconstituted in 100 μL of 18 M Ω water to a concentration of 1 $\mu\text{g}/\mu\text{L}$. Three replicate injections of the sample were analyzed within the same sample set of the calibration standards as shown in the bottom left pane of Figure 3. For each run, 2 μL of the sample was injected.

At the end of the analysis workflow, UNIFI automatically reports the calculated concentration ($\mu\text{g}/\mu\text{L}$), amount (μg), and molecular weight (Da) of the parent peak or monomer peak of the human IgG sample as shown in the upper component summary pane of Figure 3. Using the data from the component summary pane, the mean concentration and apparent molecular weight of the human IgG parent peak were calculated to be 0.93 $\mu\text{g}/\mu\text{L} \pm 0.01 \mu\text{g}/\mu\text{L}$ and 147,600 Da ± 100 Da, respectively. The ability to automatically determine apparent molecular weight and concentration of a sample within a single injection confirms that Waters Biopharmaceutical Platform with UNIFI is an integrated solution for increasing productivity and maximizing characterization content for the analysis of biotherapeutics.

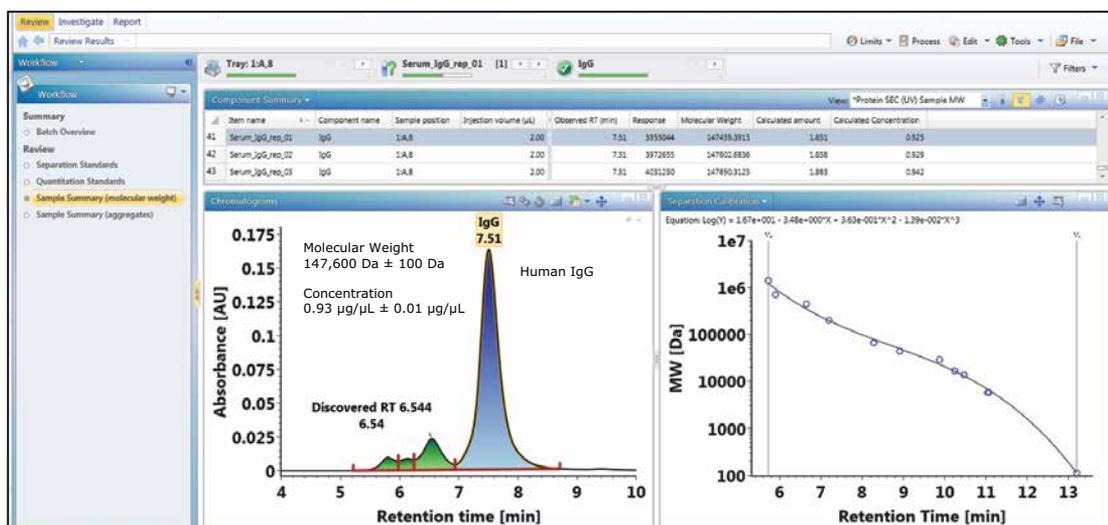


Figure 3. UNIFI reports the concentration, amount, and molecular weight (top pane) of a human IgG parent peak (lower left pane) using the calibration plots constructed from reference standards (lower right pane).

Bioinformatics tools for automated reporting of SEC characterization of biotherapeutics

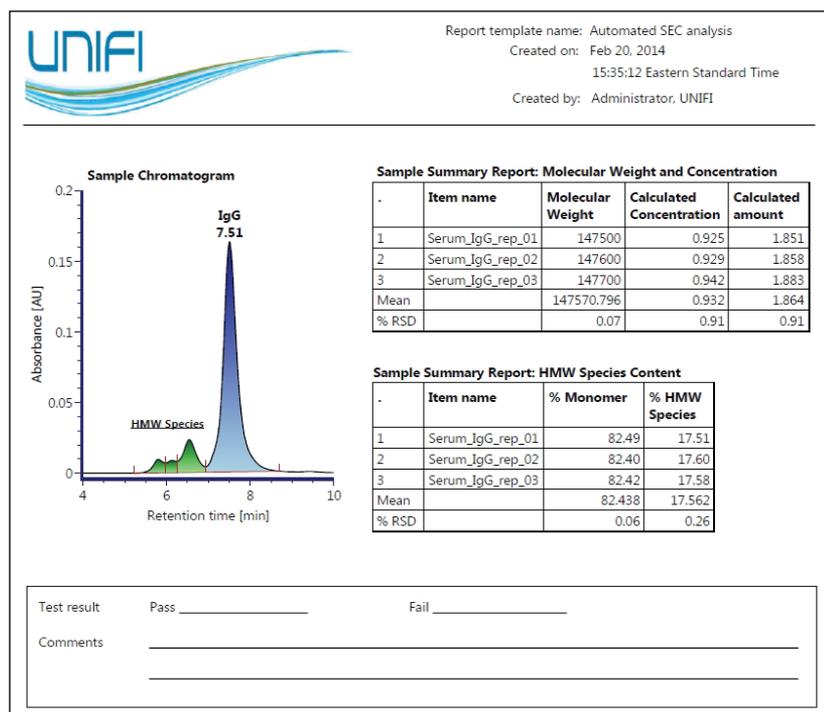


Figure 4. A report template example created by UNIFI. The calculated molecular weight, concentration, amount, as well as the relative higher molecular weight species content is shown in the report for the IgG sample.

UNIFI integrates strong reporting functionalities with the ability to generate meaningful analytical measurements to form a seamless informatics workflow. These informatics tools allow for custom reports to be automatically generated for the efficient communication and cataloging of analytical results. Report templates can be readily constructed and customized for assessment of analysis results.

Figure 4 is an example of a report template designed for SEC characterization of biotherapeutics such as monoclonal antibodies. Using the results for the purified IgG sample from Figure 3, a summary report on the apparent molecular weight, concentration, and relative amount of the parent peak (monomer) and corresponding statistical evaluation (e.g. mean and % R.S.D) is generated after data acquisition and processing. In addition, pertinent biotherapeutic information on the level of relative aggregation in each sample is also assessed and reported as a percentage of monomer and the percentage of higher molecular weight (HMW) species (collectively).

The flexibility to design custom report templates based on analysis needs makes the Biopharmaceutical Platform Solution with UNIFI a powerful integrated system for the acquisition, processing, and reporting of analysis results.

CONCLUSIONS

Assessing and controlling aggregate content in therapeutic proteins is a critical component in the manufacturing process. Increasing sample complexity coupled with the rising research and development costs highlight the need for more efficient analytical methods that are readily deployable in therapeutic protein characterization. Waters' Biopharmaceutical Platform Solution with UNIFI offers answers to these challenging problems.

The efficient built-in workflow aided by the UNIFI's data acquisition, processing, and reporting capabilities enable simultaneous determination and reporting of the apparent molecular weight, concentration, and aggregate composition of biotherapeutic proteins. This process, which is fully automated, makes the Biopharmaceutical Platform Solution with UNIFI ideal for increasing productivity through efficient method deployment for biotherapeutic characterization.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 508 478 2000
F: 508 872 1990
www.waters.com