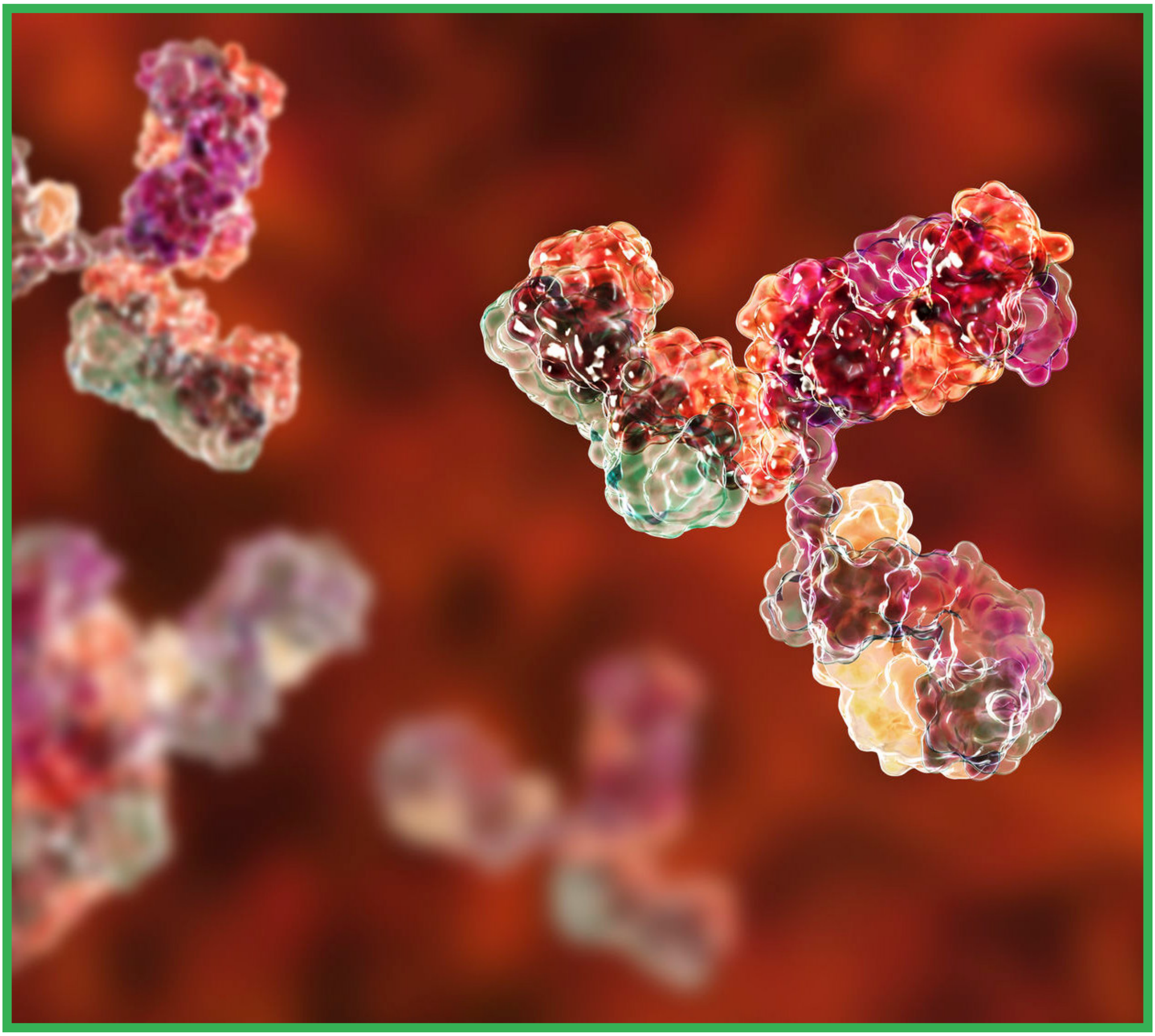
Mass Detection: The Key to Faster Biopharma Analysis

Generate mass data quickly and unleash greater productivity in your laboratory



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Introduction

As the biopharmaceutical industry continues to grow and expand into new and ever more complex modalities, biopharmaceutical developers and manufacturers are in urgent need of analytical solutions that can streamline development, accelerate time-to-market and enable greater productivity.

Mass spectrometry (MS) is arguably the most important analytical tool in the biopharma developer's tool chest. Its ability to directly analyze and determine the mass, structure and sequence of biomolecules (mAbs, proteins, peptides, nucleic acids; conjugated versions of all) makes it a unique detection technology. Due to its historic size, cost and complexity, however, deployment of this tool is often limited to core MS labs, run by highly trained MS experts who aim to serve the analytical needs of all departments in the organization.

As the pipeline for biological drug products continues to increase, so does the need for MS data to support their characterization and ongoing development, and increasingly, core MS labs are becoming bottlenecks within their respective organizations.

Adding to this demand is a growing desire to make even greater use of MS throughout development and manufacturing and in QC

Contents

as well. For example, Quality by Design (QbD) approaches to method and process development, and critical quality attribute (CQA) control strategies widely being adopted across the industry, rely on the ability to effectively monitor quality attributes across a wide range of conditions in order to build product and process understanding. Being able to leverage the power of MS to carry out tasks such as peak tracking and multi-attribute monitoring (MAM), whereby multiple product attributes are measured/ monitored in a single analysis, can not only accelerate development timelines, but also lead to more robust and sustainable methods and processes.

Adopting a QbD approach to method and process development is consequently seen as one of the best ways to improve quality, lower risk and achieve greater productivity — but these efforts have been hampered by limited access to mass spectrometry tools and an inability to generate mass data quickly.

Mass for the Masses

To address this industry dynamic and enable companies to unleash greater productivity, Waters has been working to make mass spectrometry instruments smaller, easier to use and more accessible to analytical scientists throughout the organization.

In 2013, Waters launched the ACQUITY QDa Mass Detector; a first-of-its-kind single quadrupole mass detector designed specifically for liquid chromatographers/ analytical scientists who are non-MS experts. With a highly compact design, it can be added to a liquid chromatography system as easily as an optical detector and runs under the company's compliance-ready chromatography data software, Empower.

The ACQUITY QDa. The compact footprint of the ACQUITY QDa allows for seamless integration into existing instrument stacks. The "plug and play" approach allows for the implementation of complementary orthogonal detection techniques with minimal effort into a single integrated informatics platform.

Building upon that innovation, in 2019 Waters launched the BioAccord LC-MS System that includes the ACQUITY RDa Mass Detector, a first-of-its-kind high-resolution time-of-flight (TOF) mass detector, also designed for liquid chromatographers/ analytical scientists who are non-MS experts. This Smart-MS™ enabled LC-MS system includes an intelligent health system that maximizes uptime and gives scientists throughout the organization the ability to generate highly robust and reproducible high-resolution mass data. Running under UNIFI - Waters' modern compliance-ready LC-MS software - the BioAccord System supports intact mass analysis, glycan analysis, and peptide mapping/monitoring workflows.

A resource for greater productivity

This eBook contains key application notes that demonstrate the capability and performance of both the ACQUITY QDa mass detector and the BioAccord System with the ACQUITY RDa mass detector for routine biopharmaceutical analyses and monitoring applications. Links to additional application notes and other reference material (peer-reviewed publications, videos, webinars, etc.) are provided as well. Together, this compendium of materials is intended as a resource for anyone in biopharmaceutical development, manufacturing and QC who wishes to make greater use of mass data, and who seeks to accelerate time-to-market and fuel greater productivity within their respective organizations.

The eBook is divided into two sections:

- **Section 1: The ACQUITY QDa mass detector and Empower**
In this section, we include key application notes and links to additional content that demonstrate how the ACQUITY QDa with Empower is being applied today, and the value it can bring to routine biopharmaceutical analysis and monitoring applications.

- **Section 2: The BioAccord System with the ACQUITY RDa mass detector & UNIFI**
In this section, we include key application notes and links to additional content that demonstrate the three primary biopharmaceutical analysis workflows that can be run by non-MS experts using the BioAccord System, including 1) Intact/subunit analysis, 2) released glycan analysis, and 3) peptide mapping/multi-attribute monitoring.

Dr. Henry Shion, Principal Scientist, Waters Corporation, explains how the BioAccord System meets researchers' needs and current challenges

ACQUITY QDa Mass Detector

“Waters provides the tools we need to do our jobs, enabling us to do our work well.”

Ease of use: ★★★★★ **After sales service:** ★★★★★ **Value for money:** ★★★★★

Rating: 5 ★★★★★

Application Area: Pharmaceutical R&D

“The ACQUITY QDa coupled with an ACQUITY UPLC system is keeping us in touch with the future. It helps reduce analysis time and increases productivity. The ACQUITY QDa is easy to use, requiring less user expertise. We’ve found it useful in our continuous expansion of findings in drug discovery research.”

David Lewis, Chiesi Ltd

The ACQUITY QDa is a compact, robust, and easy-to-use single quadrupole mass detector that has been purposefully designed to support routine analysis and monitoring applications throughout pharma/biopharmaceutical development, manufacturing and QC.

It can be easily added to Waters HPLC/UHPLC/UPLC chromatography systems and operates under Empower chromatography data software (CDS) to provide a fully integrated compliance-ready solution for LC-MS data capture, processing and reporting.

Key biopharmaceutical applications include:

- Monitoring peptides from protein digests to confirm product ID/peak tracking
- Quality control of synthetic peptides and oligonucleotides
- Released N-glycan monitoring
- Relative quantification of peptide variants / post-translational modifications (PTMs)
- Analysis and monitoring of formulation components
- Monitoring multiple product and process attributes for design-of-experiment (DOE) studies in support of quality-by-design (QbD) initiatives

[APPLICATION NOTE]

Increasing Specificity and Sensitivity in Routine Peptide Analyses Using Mass Detection with the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Simple add-on to existing Empower® Software based GMP compliant workflows
- Supplement optical data with mass data for routine peptide assays for biotherapeutics
- Increased assay specificity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable UV (TUV) Detector

ACQUITY UPLC Peptide CSH™ Column

Empower 3 Software

KEY WORDS

Peptides, specificity, mass detection

INTRODUCTION

Peptide analyses are frequently used during protein-based biotherapeutics development, to assess critical quality attributes (CQAs) of candidate molecules.[1,2] Throughout the development process, many optical based assays, often developed from mass spectrometric based characterization, are used to assess product identity, purity, and monitor CQAs.

While optical detection provides a level of assurance, often there is a need to add an orthogonal detection technique, such as mass detection, to improve specificity, expand detection limits, and increase confidence about peak homogeneity.[3]

The objective of this application note is to demonstrate that the ACQUITY QDa Detector (Figure 1) provides a simple and cost-effective solution for the acquisition of optical and MS-based data, for increased specificity in routine peptide monitoring assays associated with monoclonal antibodies (mAbs), while strengthening confidence in the biotherapeutic production environment.

Figure 1. The ACQUITY QDa Detector. The compact footprint of the ACQUITY QDa Detector allows for convenient integration into laboratories, for improving productivity and strengthening process control and quality assurance in the biotherapeutic production environment.

EXPERIMENTAL

An ACQUITY UPLC Peptide CSH Column was conditioned as outlined by the column care and use manual. Chemical reagents were purchased from Sigma Aldrich and used as received. Sequence grade modified trypsin from Promega was used to prepare a digest of trastuzumab (reduced and alkylated) at a concentration of 0.5 mg/mL, as outlined by the manufacturers' protocol.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	215 nm
Vials:	Total recovery vial, 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	ACQUITY UPLC Peptide CSH C_{18}, 130Å, 1.7 µm, 2.1 mm x 100 mm (p/n 186006937)
Column temp.:	65 °C
Sample temp.:	10 °C
Injection volume:	8 µL
Mobile phase A:	H_2O, 0.1% FA
Mobile phase B:	Acetonitrile, 0.1% FA
Mobile phase C:	H_2O
Mobile phase D:	H_2O
Gradient table:	

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	99	1	0	0
3.00	0.200	99	1	0	0
120.00	0.200	67	33	0	0
127.00	0.200	20	80	0	0
130.00	0.200	20	80	0	0
131.00	0.200	99	1	0	0
140.00	0.200	99	1	0	0

ACQUITY TUV Detector settings

Sample rate:	20 Hz
Detector λ:	215 nm
Filter TC:	normal

ACQUITY QDa Detector settings

Sample rate:	2 points/sec
Mass range:	350–1250 Da
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

Data management

Empower 3 SR2 Chromatography Data Software (CDS) with mass analysis

RESULTS AND DISCUSSION

Optical based assays are often used in the biotherapeutic production environment to assess CQAs that impact product efficacy and safety. For biotherapeutics, such as mAbs, monitoring peptides that contain complementary determining region (CDR) sequences are critical in assuring product identity, and to ensure safety standards.[4,5] Optical based techniques that are easily deployed and universally accessible across sites are often used in the development of peptide map profile assays.

As shown in Figure 2A, a chromatographic region containing critical peptides such as the CDR peptide (L3) and its associated deamidated form (L3D) are eluting with non-related neighboring peptides.[5] These factors, combined with baseline noise associated with the use of formic acid, raise the possibility of peak heterogeneity, which can impact the accurate assessment of CQAs. Optimization of such peptide map profiles may require an iterative process where parameters such as gradient, column, temperature, solvent, and ion-pairing agents are systematically changed and evaluated to produce robust separations and ensure peak homogeneity.

With the addition of the ACQUITY QDa Detector into existing workflows, accurate assessment of CQAs can be made with minimal impact on productivity, or the need to modify chromatographic methods.

Using the Empower CDS Software, optical data (Figure 2A) was analyzed to generate an extracted ion chromatogram (XIC) profile (Figure 2B) using mass spectral information acquired with the ACQUITY QDa Detector. From the XIC profile, multiple species were confirmed to be coeluting with the critical pair of interest, demonstrating the ACQUITY QDa Detector's ability to enable rapid assessment of peak homogeneity through the addition of mass detection.

Difficult separations that contain partial or perfectly coeluting peptide species can require extensive optimization and often result in marginal improvements in separation efficiency and subsequent quantification. The ACQUITY QDa Detector features the ability to perform Single-Ion-Recording (SIR) for maximum specificity and sensitivity in routine assays such as peptide mapping profiles.

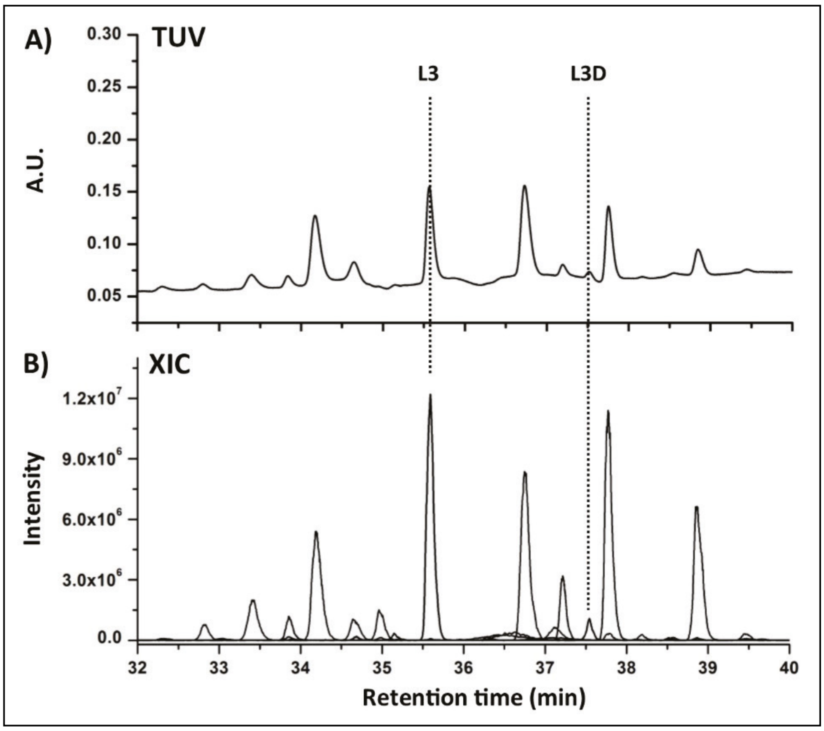
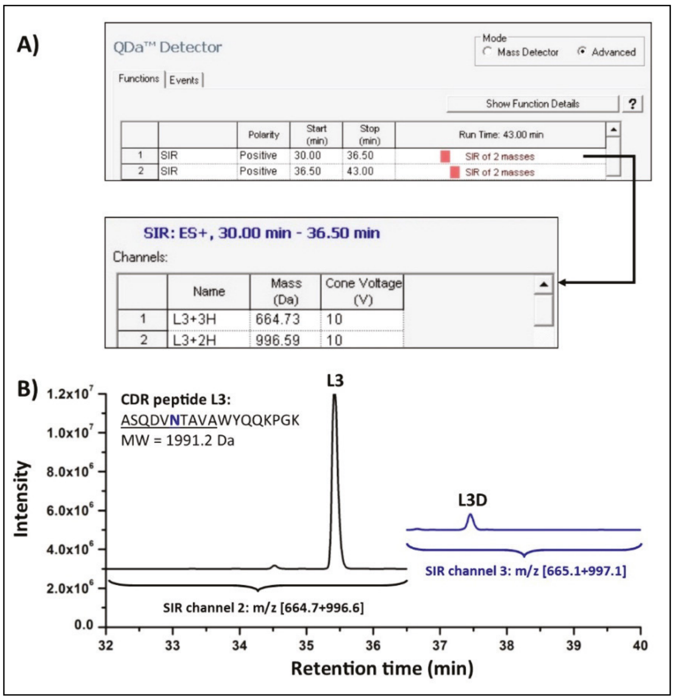


Figure 2. Peptide mapping with the ACQUITY QDa Detector. Using an ACQUITY UPLC Peptide CSH C_{18} Column, 130Å, 1.7 µm, 2.1 mm x 100 mm. A peptide map of trastuzumab was acquired using A) optical and B) mass spectral detectors simultaneously. Unique masses identified from the mass spectra collected for each peak were used to generate an XIC, confirming multiple species were co-eluting in the chromatographic region containing the CDR peptide L3 and its deamidated form L3D.

For peptides with characterized elution times, such as the L3 peptide species containing a critical CDR sequence, timed SIR events can be entered under the advanced option through the instrument method setup screen within the instrument method. As shown in Figure 3A, two timed SIR events were programmed to acquire the +2 and +3 charge state for the non-deamidated (L3), and main deamidated (L3D) form of the L3 peptide species.

Through the use of SIRs, only ions exhibiting the mass of interest are recorded as shown in Figure 3B, resulting in two chromatograms that exhibits high signal to noise ratio (SNR), and are free of co-eluting species (Figure 2) that could otherwise impact the accuracy of quantification, when using optical based techniques.

Determining the relative amount of each peptide form across two data channels is addressed through the use of an inter-channel calculation processing feature of Empower CDS Software, as shown in the workflow of Figure 4.



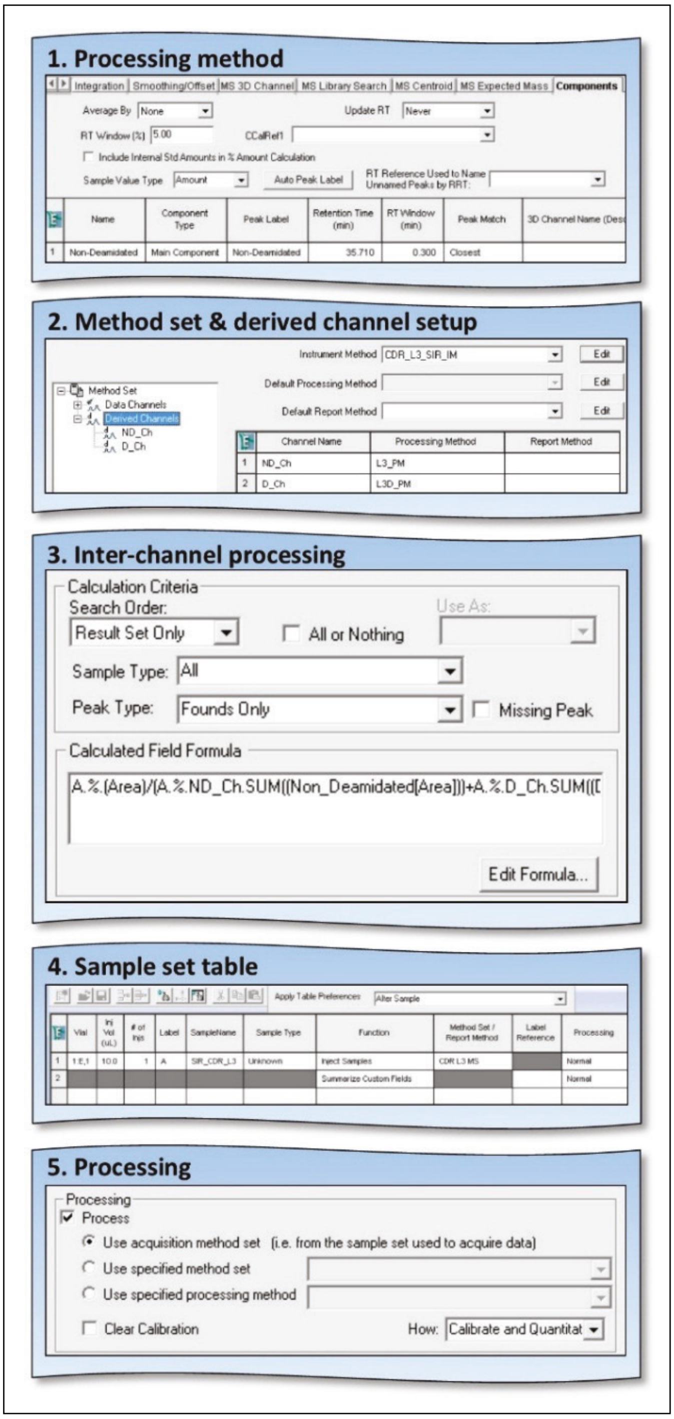
		Polarity	Start (min)	Stop (min)	Run Time: 43.00 min
1	SIR	Positive	30.00	36.50	SIR of 2 masses
2	SIR	Positive	36.50	43.00	SIR of 2 masses

SIR: ES+, 30.00 min - 36.50 min

Channels:

	Name	Mass (Da)	Cone Voltage (V)
1	L3+3H	664.73	10
2	L3+2H	996.59	10

Figure 3. SIR with the ACQUITY QDa Detector. A) Timed SIRs were programmed to acquire non-deamidated (m/z 664.73, m/z 996.59) and deamidated (m/z 665.05, m/z 997.08) masses from 30.00 – 36.50 min and 36.50–43.00 min, respectively for the L3 peptide containing a CDR sequence (underlined). B) Resulting ion chromatograms for each species were summed using a derived channel and plotted with a y-axis offset of 3 x 10^6 (non-deamidated form) and 5 x 10^6 (deamidated form) to contrast the two unique data channels.


1. Processing method

Average By: None
Update RT: Never
RT Window (%): 5.00
CCalRef1:
☐ Include Internal Std Amounts in % Amount Calculation
Sample Value Type: Amount
RT Reference Used to Name Unnamed Peaks by RRT:

	Name	Component Type	Peak Label	Retention Time (min)	RT Window (min)	Peak Match	3D Channel Name (Des
1	Non-Deamidated	Main Component	Non-Deamidated	35.710	0.300	Closest	

2. Method set & derived channel setup

Instrument Method: CDR_L3_SIR_IM
Default Processing Method:
Default Report Method:

	Channel Name	Processing Method	Report Method
1	ND_Ch	L3_PM	
2	D_Ch	L3D_PM	

3. Inter-channel processing

Calculation Criteria
Search Order: Result Set Only
☐ All or Nothing
Use As:
Sample Type: All
Peak Type: Founds Only
☐ Missing Peak

Calculated Field Formula

A.%.(Area)/(A.%.ND_Ch.SUM((Non_Deamidated[Area]))+A.%.D_Ch.SUM(([

4. Sample set table

Apply Table Preferences: Alter Sample

	Vial	Inj Vol (uL)	# of Injs	Label	SampleName	Sample Type	Function	Method Set / Report Method	Label Reference	Processing
1	1:E,1	10.0	1	A	SIR_CDR_L3	Unknown	Inject Samples	CDR L3 MS		Normal
2							Summarize Custom Fields			Normal

5. Processing

Processing
☑ Process
● Use acquisition method set (i.e. from the sample set used to acquire data)
○ Use specified method set
○ Use specified processing method
☐ Clear Calibration
How: Calibrate and Quantitat

Figure 4. Inter-channel calculation workflow. A generic workflow for determining relative abundance of peaks between two data channels using the inter-channel calculations feature of Empower Software is shown for CDR sequence containing peptide L3.

As an example, the CDR peptide species L3 is used to illustrate the process of inter-channel quantification. As shown in step 1 of Figure 4, a unique processing method for each species form should be created, and include an integration window spanning the peak of interest with the individual component listed in the component table, along with its expected retention time for identification.

In this example the non-deamidated component of L3 is listed in the component table. Once processing methods are defined for each peptide species form, a method is set-up where derived channels are created (Figure 4, step 2), corresponding to the SIR channels from the instrument method. In this example the derived channels ND_Ch and D_Ch correspond to the non-deamidated and deamidated SIR channels, respectively, used for data acquisition in Figure 3A. Upon creation, the individual processing methods defined in step 1 are assigned to the derived channels.

An inter-channel calculation is then set-up using custom fields using the standardized syntax: Label.Injection.Channel.Summary Function(Field)

For this study, relative percent was calculated using the algebraic expression

$$\left(\frac{\text{(Area)}}{\text{Area1} + \text{Area2}}\right) \times 100$$

using the following formulas

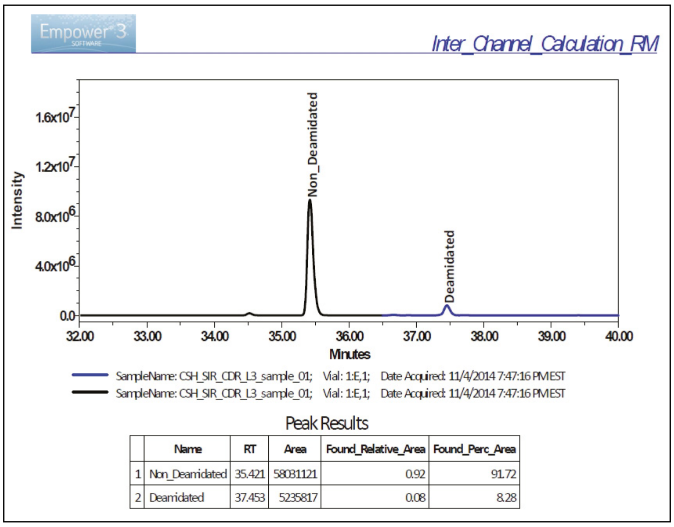
Area: A.%.(Area)

Area1 (non-deamidated area):
(A.%.ND_Ch.SUM((Non_Deamidated[Area]))

Area2 (deamidated area): A.%.D_Ch.SUM((Deamidated[Area]))

which were combined in the Calculated Field Formula window as shown in Figure 4, step 3.

Upon entering the inter-channel calculation, a sample set can be created to process the data (Figure 4, step 4). In this example, a sample set was created using the injection named SIR_CDR_L3 and labeled as (A) with the method CDR_L3_MS selected. The summarize custom fields function is selected as shown in Figure 4, step 4, to facilitate the inter-channel quantification. Processing the sample set using the acquisition method set as shown in Figure 4, step 5 will generate a result set containing the two derived channels. Using the preview/publisher option with both derived channels selected allows for the ability to design custom reports for the review of inter-channel processed data, as shown in Figure 5.



SampleName: CSH_SIR_CDR_L3_sample_01; Vial: 1:E,1; Date Acquired: 11/4/2014 7:47:16 PM EST
SampleName: CSH_SIR_CDR_L3_sample_01; Vial: 1:E,1; Date Acquired: 11/4/2014 7:47:16 PM EST

Peak Results

	Name	RT	Area	Found_Relative_Area	Found_Perc_Area
1	Non_Deamidated	35.421	58031121	0.92	91.72
2	Deamidated	37.453	5235817	0.08	8.28

Figure 5. Reporting results using inter-channel calculations. Integration, identification, and relative quantification of the non-deamidated (black trace) and deamidated (blue trace) L3 peptide species using SIRs was performed using the inter-channel calculation feature of Empower CDS Software. A report template was created for the review of processed data.

Data acquired using timed SIR channels can be overlayed for easy review, while individual metrics for identified peaks are displayed along with the inter-channel calculations and shown in the last two columns of the associated data tables, as shown in Figure 5.

For this study, the CDR containing peptide L3 was calculated to contain 8.28% deamidation. From this data, it can be seen that the ACQUITY QDa Detector, in combination with Empower 3 CDS Software, affords improved productivity in development of routine peptide monitoring assays in the biotherapeutic environment.

CONCLUSION

Developing efficient methods for the accurate assessment of CQAs that impact product efficacy and safety are highly desirable in the biopharmaceutical production environment. This study has demonstrated orthogonal detection techniques, such as mass detection, can be readily adapted to existing workflows for increased specificity and sensitivity in routine monitoring assays.

The ACQUITY QDa Detector in conjunction with Empower 3 CDS Software affords a means for cost-effective mass detection, with increased productivity, and confidence in the development and analysis of routine monitoring assays.

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[APPLICATION NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

LC-UV-Based Synthetic Peptide Impurity Tracking and Reporting with Compliant-Ready Empower 3 Software

Brooke M. Koshel, Robert E. Birdsall, and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Integrated tracking and reporting of synthetic peptide impurities using compliant-ready Empower® 3 Software
- Customizable acceptance criteria thresholds based on ICH guidelines and USP standards
- Integrated reporting of multiple results to readily assess method acceptance criteria

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY UPLC Peptide CSH C_{18} Column

Empower 3 Chromatography Data Software

KEYWORDS

Synthetic peptide, Empower, impurities, impurity tracking, compliance

INTRODUCTION

The number of peptides in pre-clinical and clinical trials has seen steady growth over the past decade. Where development was previously hindered by factors such as efficient clearance rates and short half-lives, advances in formulation and alternative delivery systems have contributed to a resurgence.[1,2] Peptides make up a unique class of pharmaceuticals not readily classified as small molecules or biologics. This becomes especially important in a regulatory framework. The United States Food and Drug Agency (FDA) has revised the definition of a biological product to include a protein (except any chemically synthesized peptide), where the term protein refers to a "defined sequence that is greater than 40 amino acids in size."[3] A chemically synthesized polypeptide must be made completely by chemical synthesis and be less than 100 amino acids in size.[3] Effective March 2020, a protein will require submission of an application for a biological product. With this in mind, pursuing a synthetic peptide manufacturing strategy over recombinant strategies may become more appealing in an effort to reduce costs and deliver products to market more quickly.

Analytical characterization and quality control of synthetic peptide products falls into one of four test categories: identification, assay, impurities, and specific tests. Impurities can result from the manufacturing process or from degradation during manufacturing or storage, and are typically determined by HPLC.[4-6] Treatment of the HPLC data can suffer from a variety of user-induced pitfalls. Peak area integration, for example, can often be subjective without defined processing methods in place. Also, it is not uncommon to export results to external software for processing, which can introduce transcription errors or incorrect calculations, but can also create an added burden to maintain compliance. Informatics suites that support impurity profiling workflows and offer integrated data analysis options are highly desirable, both for eliminating user error and ensuring compliance.

In this work, a UPLC-UV-based method is used for determining product purity in accordance with the International Council for Harmonisation (ICH) and United States Pharmacopeia (USP) functionalities built into Empower 3 Chromatography Data Software (CDS). The peptide used for this study was eledoisin, which is a biologically active peptide that acts as a vasodilator, and is thus used as a clinically relevant model system. Through establishing system suitability and impurity limits within the software, standards or samples not meeting acceptance criteria can be flagged and reported. By developing processing and reporting methods, future data can be handled in a relatively automated fashion.

EXPERIMENTAL

The synthetic peptide eledoisin (pE-PSKDAFIGLM-amide) was purchased from New England Peptide Inc. (Gardner, MA) at ≥95% purity by HPLC percent area. A stock solution of 2 mg/mL eledoisin in water was further diluted to a working concentration of 0.4 mg/mL. A Waters® ACQUITY UPLC Peptide CSH C_{18} 130 Å, 1.7 µm Column was selected for this study based on the high peak capacity separations it can provide for peptides in mobile phases with formic acid ion-pairing.[7]

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System
Detector:	ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector
Wavelength:	215 nm
Vials:	LCMS Certified Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial (p/n 600000750cv)
Column:	ACQUITY UPLC Peptide CSH C_{18} 130 Å, 1.7 µm, 2.1 mm x 100 mm (p/n 186006937)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection vol.:	5 µL
Mobile Phase A:	H_2O with 0.1% (v/v) FA
Mobile Phase B:	Acetonitrile with 0.1% (v/v) FA

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	%C	%D
Initial	0.200	85.0	15.0	0.0	0.0
2.00	0.200	85.0	15.0	0.0	0.0
22.00	0.200	55.0	45.0	0.0	0.0
22.01	0.200	15.0	85.0	0.0	0.0
24.00	0.200	15.0	85.0	0.0	0.0
24.01	0.200	85.0	15.0	0.0	0.0
30.00	0.200	85.0	15.0	0.0	0.0

Data management

Empower 3 CDS, SR2

RESULTS AND DISCUSSION

VERIFYING ASSAY AND INSTRUMENT PERFORMANCE USING EMPOWER 3 SYSTEM SUITABILITY OPTION

Empower 3 Software can be readily tailored to meet user-defined needs. By deploying the system suitability application, the user can verify that both instrument and method requirements are met. To verify performance, a system suitability standard can be used to assess criteria aligned with the FDA.[8] In practice, this standard and the associated criteria would undergo careful examination to determine what parameters are appropriate. For our study, more general criteria will be put into place for demonstrative purposes. We recognize the importance of assuring data quality, but we will also assume successful instrument qualification and method validation.[9] Because the manufacturing process used to produce synthetic peptides allows for some variation in the final product, a specifically prepared system suitability standard is used. For this study, the Waters® MassPREP™ Peptide Mixture is used as the system suitability standard, with enolase T35 being used to determine suitability.

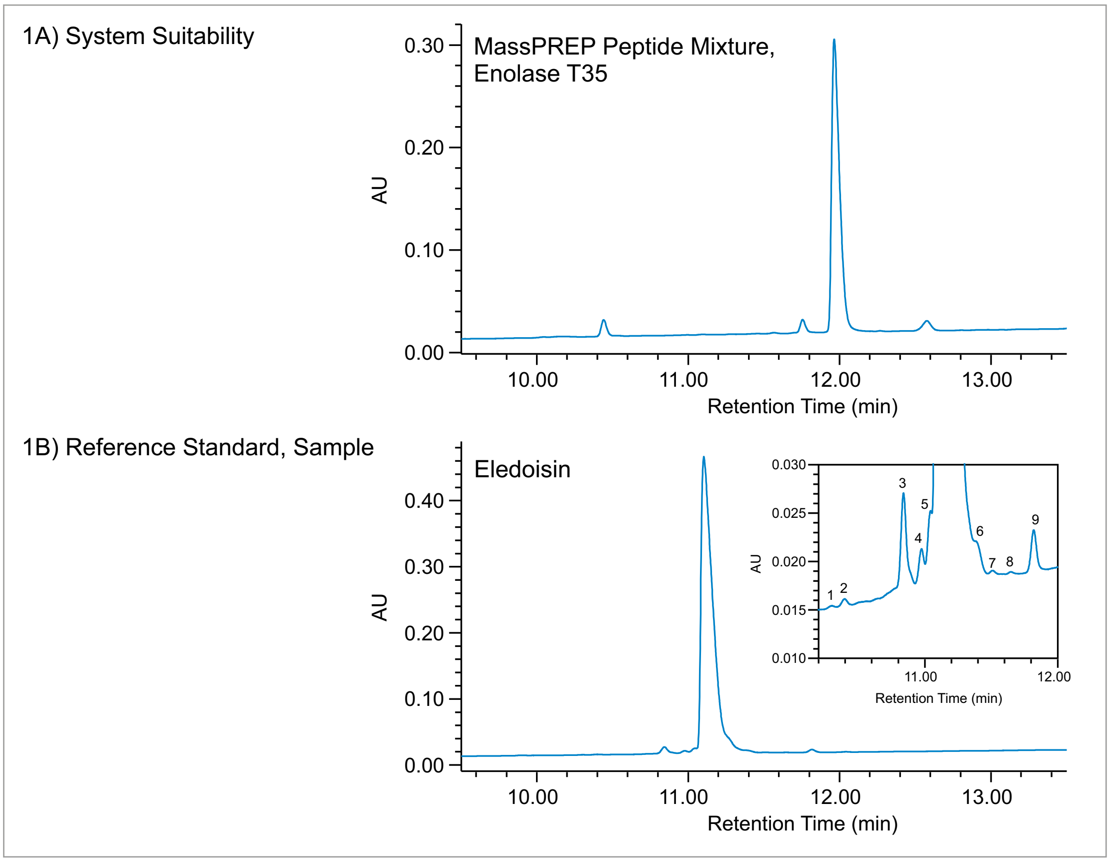
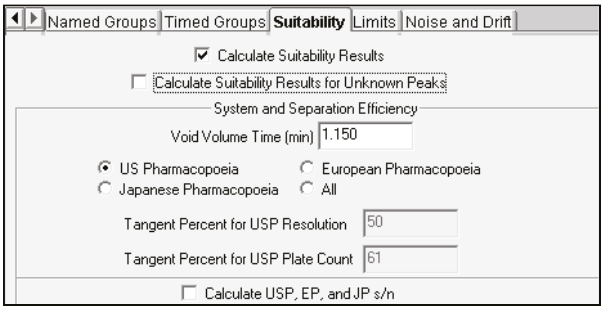


Figure 1. System Suitability and Reference Standard chromatograms. 1A) Waters MassPREP Peptide Mixture was used as a system suitability solution, where enolase T35 was used to assess if suitability criteria were met. Additional peaks in the chromatogram are peptide fragments that are typical of the sample used. 1B) Eledoisin reference standard solution. Inset shows impurity peaks later used to assess if acceptance criteria is met. Chromatogram is representative of both reference standard solution and sample solution.

A sample set was created with five injections of the system suitability sample at the beginning of the run. The chromatogram for the system suitability standard is shown in Figure 1. To calculate suitability results, this feature must be enabled within the processing method under the Suitability tab (Figure 2A). Suitability results can be calculated according to the United States, European, or Japanese Pharmacopeia. For our example, results are calculated according to the USP. Calculating signal to noise (s/n) in this case is not enabled, as we will select different acceptance criteria parameters. Should the user wish to use s/n as a suitability parameter, detector noise and drift must be enabled in the Noise and Drift tab. Instead, USP Tailing is used with an upper limit set to 2.0, which is selected in the Limits tab (Figure 2B). If peak tailing meets this criterion, the USP Tailing field in the Review window appears in unmodified text (Figure 2C). Should peak tailing exceed this value, it will be flagged in the Review window and appear in red text.

Capacity factor, resolution, and theoretical plate number are also often used to establish acceptance criteria, but in this case, the default guidelines are less pertinent to our UPLC method.[10] A second acceptance parameter will require peak area of the five injections RSD ≤1%, which is in accordance with USP <621>.[11] The mean, standard deviation, and RSD can be added as summary calculations and displayed through Empower reporting. By using a component summary, minimum and maximum limits for each of these fields can be incorporated. A final report will be generated after establishing impurity limits for reference standard and sample assessment.

2A) Suitability Tab



☑ Calculate Suitability Results
☐ Calculate Suitability Results for Unknown Peaks

System and Separation Efficiency

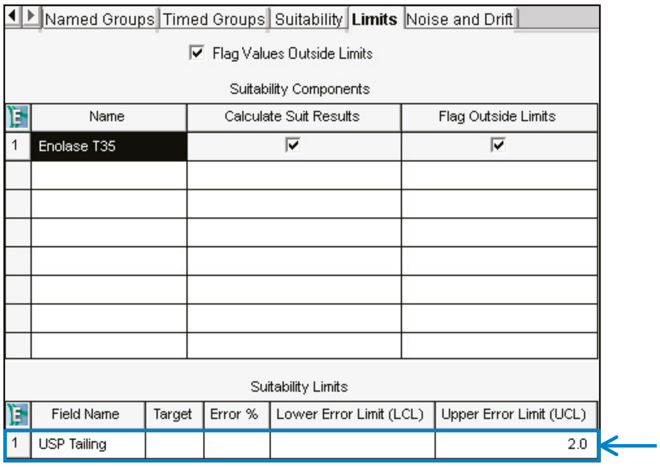
Void Volume Time (min) 1.150

● US Pharmacopoeia ○ European Pharmacopoeia
○ Japanese Pharmacopoeia ○ All

Tangent Percent for USP Resolution 50
Tangent Percent for USP Plate Count 61

☐ Calculate USP, EP, and JP s/n

2B) Limits Tab



☑ Flag Values Outside Limits

Suitability Components

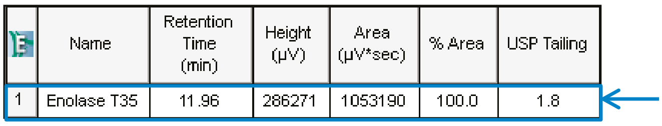
	Name	Calculate Suit Results	Flag Outside Limits
1	Enolase T35	☑	☑

Suitability Limits

	Field Name	Target	Error %	Lower Error Limit (LCL)	Upper Error Limit (UCL)
1	USP Tailing				2.0

Assigned limit

2C) Review Window



	Name	Retention Time (min)	Height (µV)	Area (µV*sec)	% Area	USP Tailing
1	Enolase T35	11.96	286271	1053190	100.0	1.8

Meets criteria

Figure 2. Processing method parameters: calculating system suitability and setting limits. 2A) Suitability tab. Suitability package must be installed and enabled to allow suitability results to be calculated. User must enter an appropriate void volume time, which is needed for capacity factor and selectivity calculations. Results can be calculated according to the United States, European, or Japanese Pharmacopeia. User may also enable s/n calculations if needed. 2B) Limits tab. The Limits tab is used to select which peak(s) is used as a suitability component. The user can then select which parameters are to be used to assess suitability, in this case, USP Tailing is selected with an upper limit of 2.0. 2C) Review window from a single injection of the system suitability solution. A USP Tailing value of 1.8 meets acceptance criteria. Should peak tailing exceed 2.0, this field would appear in red text.

SETTING MAXIMUM ALLOWED VALUES FOR IMPURITY SCREENING IN A REFERENCE STANDARD SOLUTION AND SAMPLE SOLUTION USING EMPOWER 3 IMPURITY TAB

Because chemical manufacture of synthetic peptides does not always produce the same impurities from batch to batch, impurities cannot always be easily identified based on relative retention time alone. For this reason, the following acceptance criteria for standard and sample analysis will be used:

Any individual impurity: Not more than (NMT) 1.5%

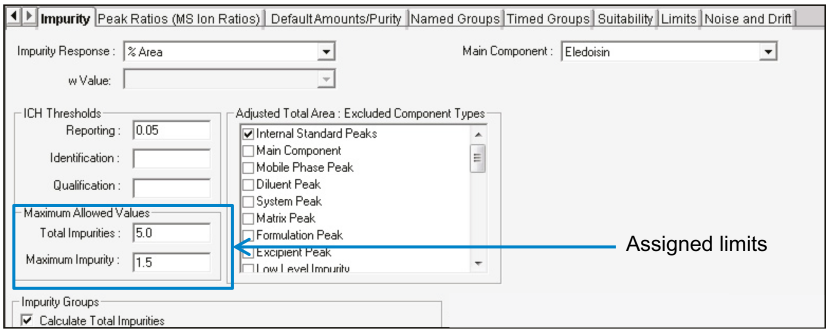
Total impurities: NMT 5.0%

In practice, more tightly defined acceptance criteria may be used, but these limits are based on the purity of the eledoisin sample. It is also possible to qualify impurities to loosen criteria in a case where a known impurity has been characterized and is known to not be harmful.

Criteria can be built into the processing method through the Impurity tab (Figure 3A). The Impurity Response should be set to % Area with eledoisin selected as the Main Component. The Maximum Allowed Values are those shown above.

A chromatogram of the eledoisin reference standard is shown in Figure 1B, which is also representative of what a chromatogram of the eledoisin sample would look like. Figure 3B in the Review Window identifies nine impurities that will be reported in the final Empower report. From the view of this chromatogram, Impurity 1 has 0.03% area and is identified as being Below Reporting Threshold in the ICH Threshold field and is not included in Total Impurities. Impurity 3 is below the individual impurity limit for the reference standard, but the sample exceeds 1.5%. Because this value exceeds the maximum allowed value, it is highlighted in red text. Total Impurities is reported as 4.2, which meets the acceptance criteria.

3A) Impurity Tab



Impurity Response : % Area
Main Component : Eledoisin
w Value:

ICH Thresholds
Reporting : 0.05
Identification :
Qualification :

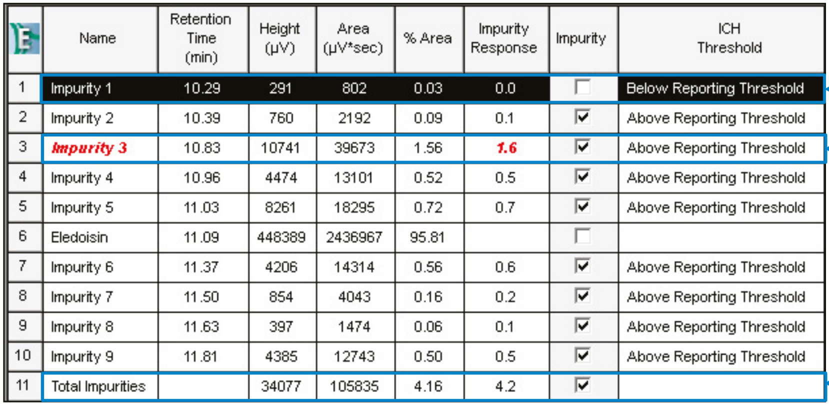
Maximum Allowed Values
Total Impurities : 5.0
Maximum Impurity : 1.5

Adjusted Total Area : Excluded Component Types
☑ Internal Standard Peaks
☐ Main Component
☐ Mobile Phase Peak
☐ Diluent Peak
☐ System Peak
☐ Matrix Peak
☐ Formulation Peak
☐ Excipient Peak
☐ Low Level Impurity

Impurity Groups
☑ Calculate Total Impurities

Assigned limits

3B) Review Window



	Name	Retention Time (min)	Height (µV)	Area (µV*sec)	% Area	Impurity Response	Impurity	ICH Threshold
1	Impurity 1	10.29	291	802	0.03	0.0	☐	Below Reporting Threshold
2	Impurity 2	10.39	760	2192	0.09	0.1	☑	Above Reporting Threshold
3	***Impurity 3***	10.83	10741	39673	1.56	***1.6***	☑	Above Reporting Threshold
4	Impurity 4	10.96	4474	13101	0.52	0.5	☑	Above Reporting Threshold
5	Impurity 5	11.03	8261	18295	0.72	0.7	☑	Above Reporting Threshold
6	Eledoisin	11.09	448389	2436967	95.81		☐	
7	Impurity 6	11.37	4206	14314	0.56	0.6	☑	Above Reporting Threshold
8	Impurity 7	11.50	854	4043	0.16	0.2	☑	Above Reporting Threshold
9	Impurity 8	11.63	397	1474	0.06	0.1	☑	Above Reporting Threshold
10	Impurity 9	11.81	4385	12743	0.50	0.5	☑	Above Reporting Threshold
11	Total Impurities		34077	105835	4.16	4.2	☑	

Below Reporting Threshold

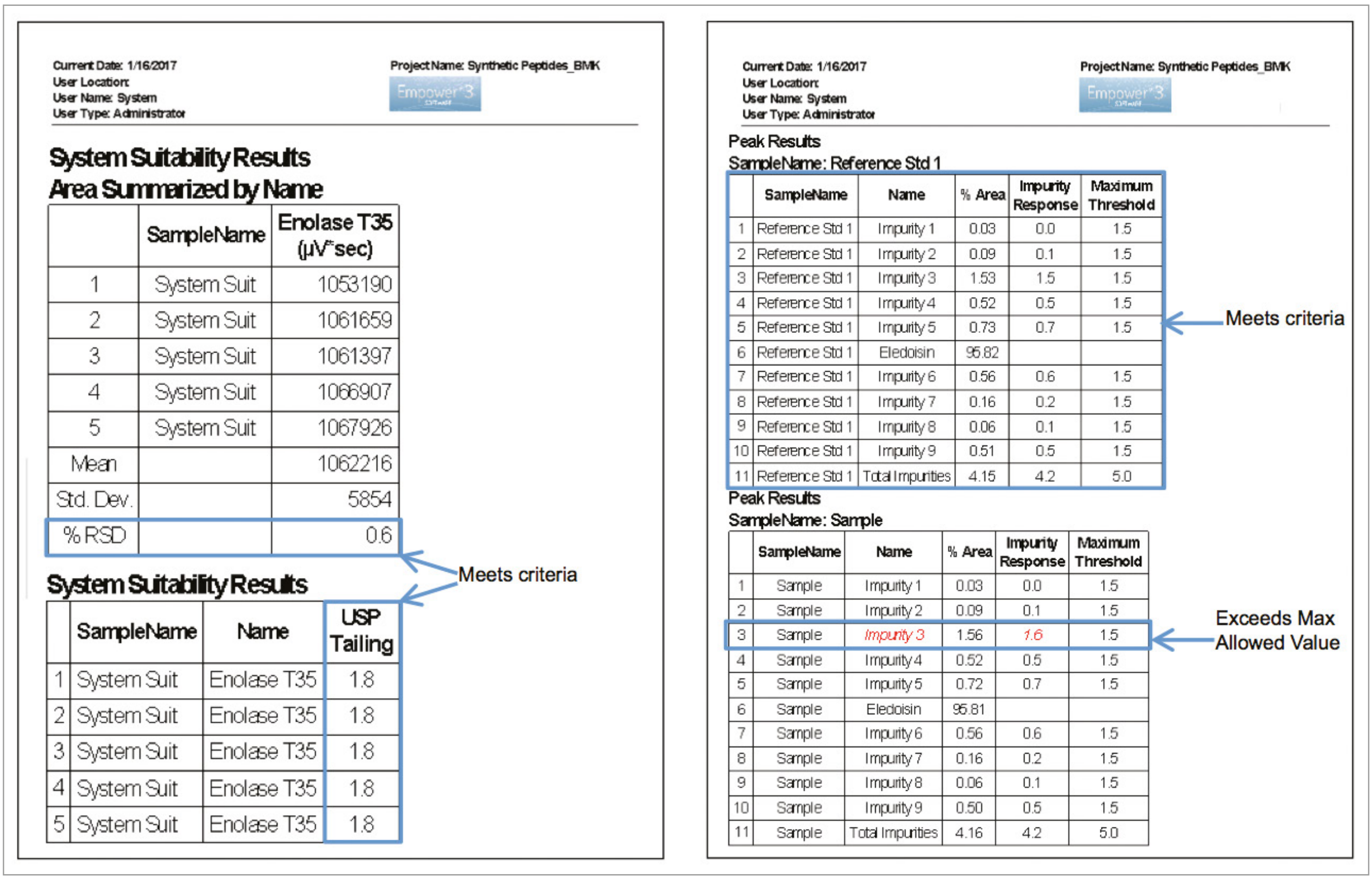
Exceeds Max Allowed Value

Meets criteria

Figure 3. Processing method parameters: setting maximum allowed values for impurity screening of reference standard solution and sample solution. 3A) Impurity tab. Impurity response is determined as peak area percent. ICH Thresholds may be entered, in this case, a reporting limit of 0.05 is used. From the acceptance criteria, any individual impurity is to be NMT 1.5%, and the total impurities must be NMT 5.0% These values are entered into the Maximum Allowed Values fields. The user also has the option of excluding component types from the total area if needed. 3B) Review window from a single injection of the sample solution. From the ICH Threshold field, peaks below the reporting threshold are noted and not included in the total area. Because Impurity 3 (from Figure 1B) exceeds the maximum allowed value for an individual impurity, the value is flagged in red.

REPORTING OF SYSTEM SUITABILITY, REFERENCE STANDARD SOLUTION AND SAMPLE SOLUTION PEAK RESULTS USING EMPOWER 3 SOFTWARE

Empower 3 reporting can be customized to display data based on a user's needs. For this example, system suitability results and individual peak tables for the Reference Standard and Sample will be reported to readily highlight any criteria that do not meet specification. Final reporting can be seen in Figure 4. System suitability results show that %RSD for peak area of the five injections is 0.6%, which meets the 1.0% criteria requirement. The acceptance criterion for USP Tailing was set at NMT 2.0, and for each of the five injections peak tailing was 1.8. Tables of peak results can be seen for both the reference standard solution and the sample solution. The reference standard meets criteria for both the total number of impurities as well as the limit of any individual impurity. Impurity 3 in the sample solution exceeds the individual impurity limit and is flagged in red text. Empower 3 Software allows data to be reported in a clear and efficient fashion so that any precautionary actions necessary can be carried out in a more timely manner.



Current Date: 1/16/2017
User Location:
User Name: System
User Type: Administrator

Project Name: Synthetic Peptides_BMK

System Suitability Results
Area Summarized by Name

	SampleName	Enolase T35 (µV*sec)
1	System Suit	1053190
2	System Suit	1061659
3	System Suit	1061397
4	System Suit	1066907
5	System Suit	1067926
Mean		1062216
Std. Dev.		5854
% RSD		0.6

System Suitability Results

	SampleName	Name	USP Tailing
1	System Suit	Enolase T35	1.8
2	System Suit	Enolase T35	1.8
3	System Suit	Enolase T35	1.8
4	System Suit	Enolase T35	1.8
5	System Suit	Enolase T35	1.8

Meets criteria

Current Date: 1/16/2017
User Location:
User Name: System
User Type: Administrator

Project Name: Synthetic Peptides_BMK

Peak Results
SampleName: Reference Std 1

	SampleName	Name	% Area	Impurity Response	Maximum Threshold
1	Reference Std 1	Impurity 1	0.03	0.0	1.5
2	Reference Std 1	Impurity 2	0.09	0.1	1.5
3	Reference Std 1	Impurity 3	1.53	1.5	1.5
4	Reference Std 1	Impurity 4	0.52	0.5	1.5
5	Reference Std 1	Impurity 5	0.73	0.7	1.5
6	Reference Std 1	Eledoisin	95.82		
7	Reference Std 1	Impurity 6	0.56	0.6	1.5
8	Reference Std 1	Impurity 7	0.16	0.2	1.5
9	Reference Std 1	Impurity 8	0.06	0.1	1.5
10	Reference Std 1	Impurity 9	0.51	0.5	1.5
11	Reference Std 1	Total Impurities	4.15	4.2	5.0

Meets criteria

Peak Results
SampleName: Sample

	SampleName	Name	% Area	Impurity Response	Maximum Threshold
1	Sample	Impurity 1	0.03	0.0	1.5
2	Sample	Impurity 2	0.09	0.1	1.5
3	Sample	*Impurity 3*	1.56	*1.6*	1.5
4	Sample	Impurity 4	0.52	0.5	1.5
5	Sample	Impurity 5	0.72	0.7	1.5
6	Sample	Eledoisin	95.81		
7	Sample	Impurity 6	0.56	0.6	1.5
8	Sample	Impurity 7	0.16	0.2	1.5
9	Sample	Impurity 8	0.06	0.1	1.5
10	Sample	Impurity 9	0.50	0.5	1.5
11	Sample	Total Impurities	4.16	4.2	5.0

Exceeds Max Allowed Value

Figure 4. Empower reporting. System Suitability results are summarized to show that both %RSD and USP Tailing criteria are met. The maximum allowable %RSD is recorded in the components summary of the reporting method, and will appear in red text if it is outside of the accepted criteria. All five injections of the System Suitability solution are also shown to meet the USP Tailing requirement of a 2.0 upper limit. Peak results can be summarized to contain data of the user's choice. Here, % Area, Impurity Response, and the Maximum Threshold are shown. Results are compared for a reference standard solution of eledoisin and a sample solution of eledoisin. The reference standard solution meets both the individual impurity requirement (NMT 1.5%) and the total impurities requirement (NMT 5.0%). The sample solution, however, contains a peak that is outside of the maximum allowed value, which appears in red text.

CONCLUSIONS

This work demonstrates how Empower 3 Software can be easily employed for synthetic peptide impurity tracking in a compliant-ready workflow. Eledoisin was used as a model peptide to demonstrate how acceptance criteria can be used to assess reference sample material and a sample solution. Sample material was identified as having an impurity above the maximum allowed value for individual impurities to demonstrate failure to meet acceptance criteria. Empower reporting can then be used to retrieve and report data. In summary, this work demonstrates how Empower 3 Software offers integrated functionality for processing and reporting synthetic peptide data, thus enabling the assessment of product quality in an efficient, accurate, and compliant-ready manner.

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[APPLICATION NOTE]

Improving Glycan Profiling in Process Development Using Empower Spectral Library

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Highly robust spectral matching algorithm for putative identification of unknown glycans and co-elutions
- User created library for greater flexibility and peak identification accuracy
- Integrated workflow to improve productivity and confidence in glycan analysis

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class Bio System

ACQUITY QDa™ Mass Detector

ACQUITY UPLC Fluorescence (FLR) Detector

ACQUITY BEH Glycan Amide Column

Empower™ 3 Chromatography Data Software (CDS)

KEYWORDS

Released glycans, Glycoworks *Rapi*Fluor-MS™ N-Glycan Kit, ACQUITY QDa Mass Detector

INTRODUCTION

Glycosylation is one of the most common and complex post-translational modifications of monoclonal antibodies (mAbs). Due to its direct correlation to drug efficacy, glycosylation has been recognized as a critical quality attribute (CQA) of mAb-based therapeutics.[1] To ensure product safety and efficacy, biopharmaceutical companies invest a significant amount of resource in the selection and control of cell line glycosylation profiles. As biotherapeutic pipelines continue to expand, deploying methods that can assess glycosylation profiles accurately and consistently become increasingly important for efficient product development and quality control. As an orthogonal detection technique to LC-UV or LC-fluorescence (FLR), MS detection is being increasingly adopted in process development and manufacturing environments, offering an opportunity to improve confidence and productivity in the assessment of product quality attributes throughout the product life cycle.

The ACQUITY QDa Mass Detector requires minimal maintenance and can be readily adapted to existing LC-UV or LC-FLR workflows as a fit-for-purpose detector to address the changing needs of the biopharmaceutical industry.[2] In this study we will demonstrate an LC-FLR-MS based workflow using an MS enabled spectral library matching algorithm to improve productivity and confidence of glycan profiling. As a proof of concept, a library will be created using *Rapi*Fluor-MS (RFMS) labeled glycan standard and then used to address challenging glycan analyses in process development.

EXPERIMENTAL

Sample preparation

*Rapi*fluor-MS labeled glycan standard was diluted in 40 µL 9/10/21 Water/DMF/ACN solvent with 2 µL injected for creating the library. N-glycans from mAb standard were released and labeled using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176004082).[3] A volume of 10 µL sample was injected for each analysis.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY FLR, $\lambda_{excitation}$ = 265 nm, $\lambda_{emission}$ = 425 nm ACQUITY QDa (performance model)
LC column:	ACQUITY UPLC Glycan BEH Amide 130 Å, 1.7 µm, 2.1 × 150 mm
Column temp.:	60 °C
Sample vial:	12 × 32 mm glass vial, total recovery (p/n 600000750cv)
Mobile phase A:	H_2O, 50 mM Ammonium formate, pH = 4.4
Mobile phase B:	Acetonitrile
Informatics:	Empower 3, Feature Release 4

Gradient table:

Time (min)	Flow rate (min)	%A	%B
Initial	0.400	25.0	75.0
35.00	0.400	46.0	54.0
36.50	0.200	100.0	0.0
39.50	0.200	100.0	0.0
43.10	0.200	25.0	75.0
47.60	0.400	25.0	75.0
55.00	0.400	25.0	75.0

ACQUITY QDa Mass Detector settings

Mass range:	500–1250 *m/z*
Mode:	ESI+
Collection mode:	Centroid
Sample rate:	2 points/sec
Cone voltage:	15 V
Probe temp.:	400 °C
Capillary voltage:	1.5 kV

RESULTS AND DISCUSSION

SPECTRAL LIBRARY ENHANCED WORKFLOWS

Integration of MS with LC-UV or LC-FLR workflows allows analysts to access MS spectral information that can be used to expedite process development and manufacturing workflows. In the case of the former, MS spectral libraries, as a repository of 3D MS spectral data, can be used as a spectral "fingerprint" for automated glycan identification or confirmation (Figure 1).

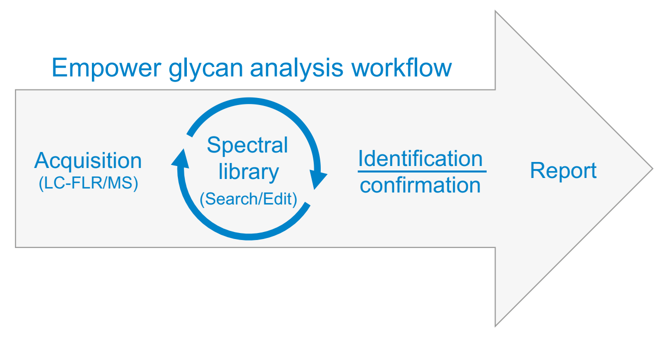


Figure 1. Workflow of using Empower spectral library for glycan analysis.

In practice, a spectral library is created using LC-MS data of pre-characterized glycan standard(s) or reference material. To demonstrate the benefits of a spectral library enhanced glycan workflow within Empower, the *Rapi*Fluor-MS labeled Glycan Performance Test Standard was used as a surrogate model of pure standard to create a library. As shown in Figure 2, 18 peaks were separated and integrated using a 55 minute method. The library functionality within Empower offers the flexibility of adding the MS spectra, along with an identifying label, to pre-existing libraries or to newly created libraries using the "Library" pull-down menu as shown in Figure 2. In addition to being flexible, once created, libraries are universal within the Empower CDS allowing library access to all projects for spectral matching of unknown glycan samples.

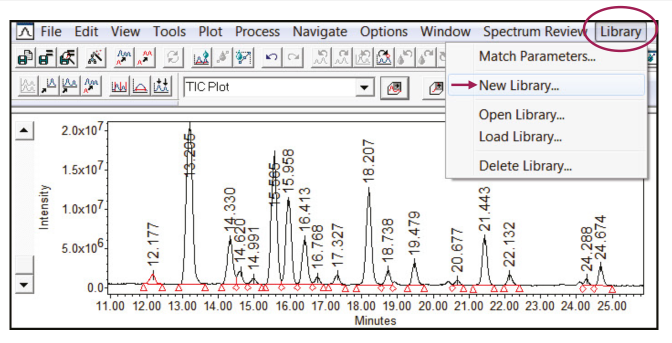
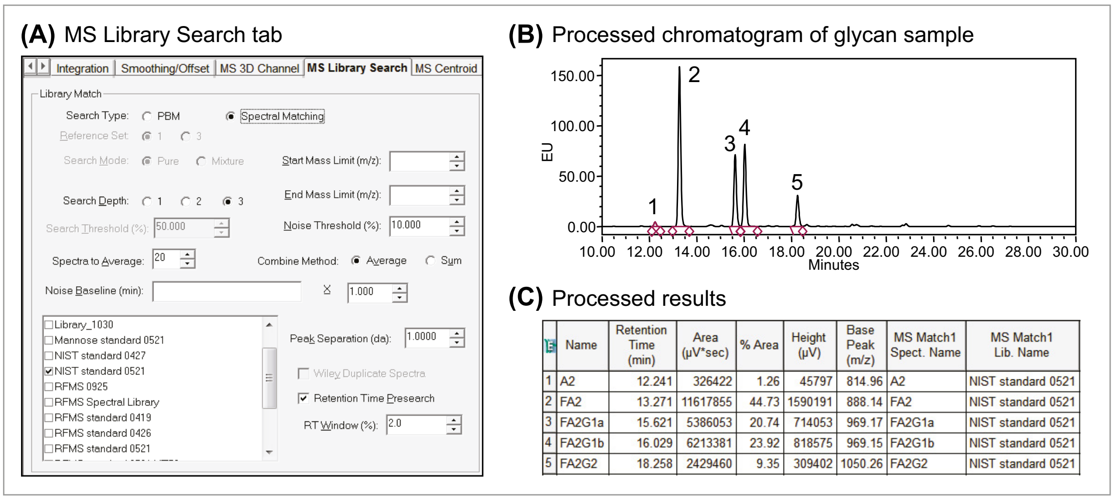


Figure 2. Creating a spectral library using RFMS glycan standard.

As an integrated function of the processing method, multiple parameters such as Noise Threshold and Retention Time Pre-search (Figure 3A) are available in the MS Library Search tab for method optimization. The Retention Time Pre-search filter is of particular use when the sample and library are separated using the same conditions as in the case of platform or screening methods. In this instance the Retention Time Pre-search restricts the spectral search within a RT window around the target peak for increased spectral matching accuracy. This can be beneficial when dealing with challenging MS spectra associated with isomers or in source fragmentations. In addition to offering multiple processing parameters for flexible method development, the MS Library Search tab also supports the use of multiple libraries during the matching process. This allows users to create discreet libraries under different conditions, an ideal scenario when dealing with multiple product lines with unique separation conditions or legacy methods that can't be readily altered. Once optimized, the spectral library enhanced workflow can be used for putative identity or confirmation of glycan peaks as shown in Figure 3. In this example a spectral library based on the RFMS glycan standard was used with a 2% retention time filter and a noise threshold of 10% (Figure 3A) to confirm the identity of released N-glycans from the Waters mAb standard (Figure 3B) that were labeled with *Rapi*Fluor-MS. As shown in Figure 3C, the best match based on the spectral profile is listed in the "MS Match 1 Spect. Name" column in Peak Result table.



(A) MS Library Search tab

Search Type: ○ PBM ● Spectral Matching
Reference Set: ● 1 ○ 3
Search Mode: ● Pure ○ Mixture
Start Mass Limit (m/z):
End Mass Limit (m/z):
Search Depth: ○ 1 ○ 2 ● 3
Search Threshold (%): 50.000
Noise Threshold (%): 10.000
Spectra to Average: 20
Combine Method: ● Average ○ Sum
Noise Baseline (min): X 1.000

- ☐ Library_1030
- ☐ Mannose standard 0521
- ☐ NIST standard 0427
- ☑ NIST standard 0521
- ☐ RFMS 0925
- ☐ RFMS Spectral Library
- ☐ RFMS standard 0419
- ☐ RFMS standard 0426
- ☐ RFMS standard 0521

Peak Separation (da): 1.0000
☐ Wiley Duplicate Spectra
☑ Retention Time Presearch
RT Window (%): 2.0

(B) Processed chromatogram of glycan sample

(C) Processed results

	Name	Retention Time (min)	Area (µV*sec)	% Area	Height (µV)	Base Peak (m/z)	MS Match1 Spect. Name	MS Match1 Lib. Name
1	A2	12.241	326422	1.26	45797	814.96	A2	NIST standard 0521
2	FA2	13.271	11617855	44.73	1590191	888.14	FA2	NIST standard 0521
3	FA2G1a	15.621	5386053	20.74	714053	969.17	FA2G1a	NIST standard 0521
4	FA2G1b	16.029	6213381	23.92	818575	969.15	FA2G1b	NIST standard 0521
5	FA2G2	18.258	2429460	9.35	309402	1050.26	FA2G2	NIST standard 0521

Figure 3. Library search of glycan samples. (A) MS Library Search tab in Empower processing method. (B) Processed chromatogram of glycan samples. (C) Processed results in Peak Results table.

CASE STUDIES: IDENTIFICATION OF UNKNOWN GLYCANS AND CO-ELUTION

Developing control strategies of glycosylation profiles during the process development phase generally entails evaluating a significant number of parameters to determine optimal conditions with minimal risk. Unknown or new peaks that appear during this phase often reflect process changes and require identification to assess their impact on product quality and safety. In this study, we demonstrate how a spectral library enhanced workflow can be used to expedite the identification process. Representative chromatograms for the reference material and a process development sample acquired with the same platform method are shown in Figure 4A. In this example, two additional peaks were observed in the glycan sample compared to the reference material. With MS data acquired on the ACQUITY QDa, the spectral library was used to provide putative identification of the new peaks. As shown in the results table of Figure 4B, the two blank spaces for Peak 1 and Peak 4 in the "Name" column, which represent the unknown peaks, were tentatively assigned as FA1 and Man5 within the "MS Match1 Spect. Name" column. Cross-referencing the spectral match with the calculated Glucose Unit (GU) as shown in the table provides orthogonal information that can be used to increase confidence in peak assignment and streamline investigations into process deviations. To this end, incorporation of spectral library enhanced workflows offers the means to readily assess and respond to process changes that can potentially impact product safety and efficacy.

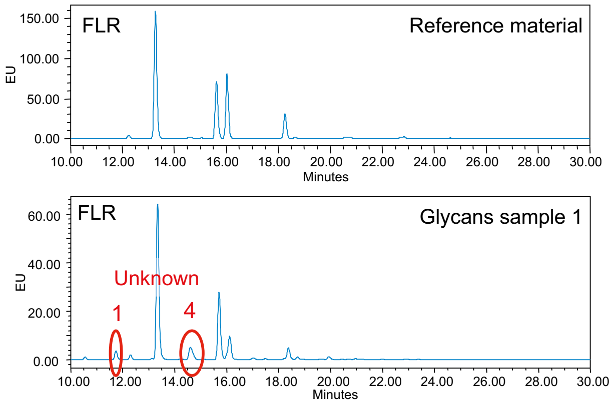
In addition to new peaks, co-elution can also occur in glycan separation and impact the quantitation accuracy as it may be misinterpreted as a pure peak in an FLR chromatogram.

In Empower, potential co-elutions can be investigated via the Purity View in the Mass Analysis window or in a semi-automated fashion using the spectral library functionality. The MS spectral library functionality within Empower offers the ability to report the best three matches of one MS spectrum via an iterative spectral search algorithm. As shown in the zoom-in chromatograms in Figure 5A, the FLR peak appears to be a single species and was assigned as Man5 in the "Name" column (Figure 5B) based on the retention time. However, MS spectra showed that there might be two species of glycans present within the peak (Figure 5A MS inset). Using the iterative search function the spectral library was able to identify two species within the single peak. As shown in Figure 5B, the dominant peak was identified in the first match as Man5 while the less abundant peak was detected and identified in the second match as FA1G1, indicating a co-elution occurred. In this aspect MS spectral library improves the product knowledge and assay confidence that can benefit glycan profiling in process development or screening workflows.

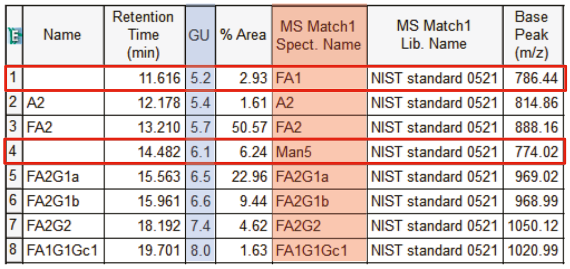
REPORTING

Empower is equipped with the ability to generate reports to simplify the review, sharing, and filing of acquired data. As part of the workflow, spectral matching data can be reported along with other data processing results in an automated fashion. To demonstrate the reporting function of Empower, a report was generated for the library searching results previously shown in Figure 4 using a pre-existing template. As shown in Figure 6, the report was formatted to display Sample Information, Fluorescence Chromatogram, and Peak Results table. All results can be customized to include desired information to meet different laboratory requirements.

(A) Separation of sample containing unknown glycans



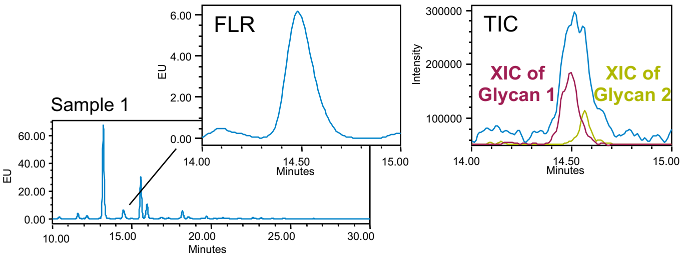
(B) Spectral Library match results



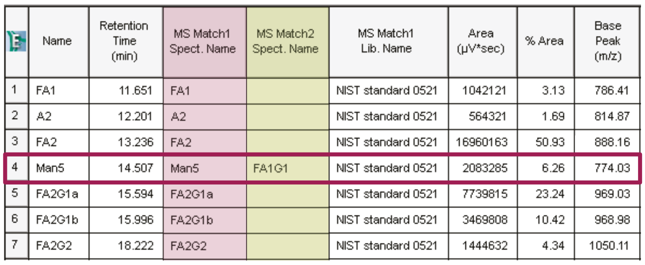
	Name	Retention Time (min)	GU	% Area	MS Match1 Spect. Name	MS Match1 Lib. Name	Base Peak (m/z)
1		11.616	5.2	2.93	FA1	NIST standard 0521	786.44
2	A2	12.178	5.4	1.61	A2	NIST standard 0521	814.86
3	FA2	13.210	5.7	50.57	FA2	NIST standard 0521	888.16
4		14.482	6.1	6.24	Man5	NIST standard 0521	774.02
5	FA2G1a	15.563	6.5	22.96	FA2G1a	NIST standard 0521	969.02
6	FA2G1b	15.961	6.6	9.44	FA2G1b	NIST standard 0521	968.99
7	FA2G2	18.192	7.4	4.62	FA2G2	NIST standard 0521	1050.12
8	FA1G1Gc1	19.701	8.0	1.63	FA1G1Gc1	NIST standard 0521	1020.99

Figure 4. Identification of unknown glycans. (A) Separation of reference material and glycan sample containing unknown glycans. (B) Peak results table. The spectral matching results were summarized in peak results table, showing the putative identities of unknown peaks (color-coded in red). GU value (color-coded in blue) can also be calculated in the same workflow to provide additional information.

(A) Separation of sample containing co-eluted peaks

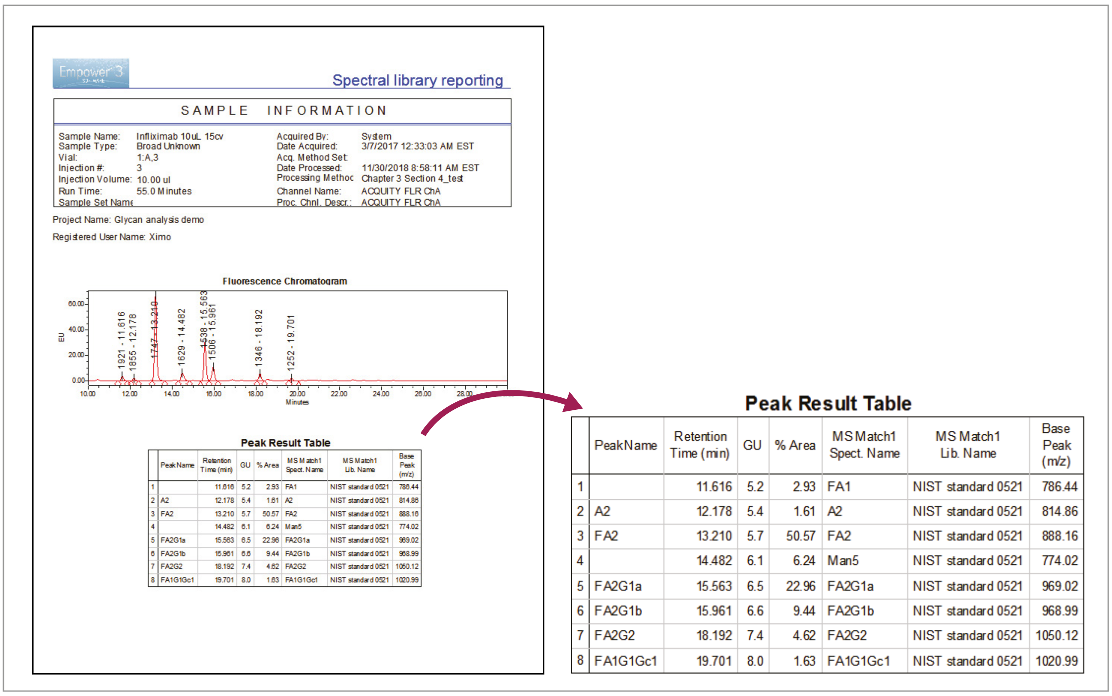


(B) Match results **Glycan 1** **Glycan 2**



	Name	Retention Time (min)	MS Match1 Spect. Name	MS Match2 Spect. Name	MS Match1 Lib. Name	Area (µV*sec)	% Area	Base Peak (m/z)
1	FA1	11.651	FA1		NIST standard 0521	1042121	3.13	786.41
2	A2	12.201	A2		NIST standard 0521	564321	1.69	814.87
3	FA2	13.236	FA2		NIST standard 0521	16960163	50.93	888.16
4	Man5	14.507	Man5	FA1G1	NIST standard 0521	2083285	6.26	774.03
5	FA2G1a	15.594	FA2G1a		NIST standard 0521	7739815	23.24	969.03
6	FA2G1b	15.996	FA2G1b		NIST standard 0521	3469808	10.42	968.98
7	FA2G2	18.222	FA2G2		NIST standard 0521	1444632	4.34	1050.11

Figure 5. identification of co-elution supported by orthogonal detection. (A) Separation of sample containing co-eluted peaks that cannot be discovered in fluorescence data. (B) Peak Results table. With the dominant peak being identified as the first match (color-coded in red), the less abundant peak was detected and identified as the second match (color-coded in green).



Spectral library reporting

SAMPLE INFORMATION

Sample Name:	Infliximab 10uL 15cv	Acquired By:	System
Sample Type:	Broad Unknown	Date Acquired:	3/7/2017 12:33:03 AM EST
Vial:	1:A,3	Acq. Method Set:	
Injection #:	3	Date Processed:	11/30/2018 8:58:11 AM EST
Injection Volume:	10.00 ul	Processing Methoc	Chapter 3 Section 4_test
Run Time:	55.0 Minutes	Channel Name:	ACQUITY FLR ChA
Sample Set Name		Proc. Chnl. Descr.:	ACQUITY FLR ChA

Project Name: Glycan analysis demo

Registered User Name: Ximo

Peak Result Table

	PeakName	Retention Time (min)	GU	% Area	MS Match1 Spect. Name	MS Match1 Lib. Name	Base Peak (m/z)
1		11.616	5.2	2.93	FA1	NIST standard 0521	786.44
2	A2	12.178	5.4	1.61	A2	NIST standard 0521	814.86
3	FA2	13.210	5.7	50.57	FA2	NIST standard 0521	888.16
4		14.482	6.1	6.24	Man5	NIST standard 0521	774.02
5	FA2G1a	15.563	6.5	22.96	FA2G1a	NIST standard 0521	969.02
6	FA2G1b	15.961	6.6	9.44	FA2G1b	NIST standard 0521	968.99
7	FA2G2	18.192	7.4	4.62	FA2G2	NIST standard 0521	1050.12
8	FA1G1Gc1	19.701	8.0	1.63	FA1G1Gc1	NIST standard 0521	1020.99

Figure 6. With compliant-ready Empower CDS, data can be processed and reported in an automated manner.

CONCLUSIONS

In conclusion, this work demonstrated the application of the MS Spectral Library functionality within Empower for analysis of RFMS labeled glycans. In combination with the ACQUITY QDa mass detector, MS spectral library enhanced workflows can be readily deployed to assist in the identification of unknown peaks and co-elutions for increased confidence and productivity in data interpretation of released N-Glycan analyses. With the reporting capability of Empower, the workflow can be completed in an automated manner and is ideal for released glycan analysis in process development.

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3. Lauber, M. A. et. al. Rapid Preparation of Released N-glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection. *Anal Chem.* 87:5401–5409. 2015.

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[APPLICATION NOTE]

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Monitoring Multiple Attributes in a Single Assay Using the ACQUITY QDa Detector for Product Confirmation and Process Monitoring of Product Quality Attributes

Brooke M. Koshel, Robert E. Birdsall, and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Multiple attribute monitoring through a single acquisition to enable both product confirmation and routine screening of post-translational modifications
- Empower® Software enables an automated and compliant workflow for data acquisition, processing, and reporting of multiple product quality attributes

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Tunable Ultraviolet (TUV) Detector

ACQUITY QDa® Detector

ACQUITY UPLC Peptide CSH C_{18} Column

Empower 3 Chromatography Data Software

KEYWORDS

Monoclonal antibody, mAb, multi-attribute monitoring, MAM, product quality attributes, PQA, ACQUITY QDa Detector, peptide map

INTRODUCTION

The concept of using a single LC-MS-based analytical method to monitor multiple product quality attributes (PQAs) is a strategy that has started to gain momentum in the biopharmaceutical industry. The idea behind this concept is that a single LC-MS method can be used to assess a product's important quality attributes simultaneously, as opposed to running a panel of optically-based chromatographic methods, which are unable to assess product attributes at a molecular level. The motivation for extending mass spectrometry (MS) beyond characterization into all stages of development and even quality control, is largely due to the complexity of protein-based therapeutics compared to small molecule drugs.[1] Replacing conventional chromatographic methods with a single, more sophisticated LC-MS method ultimately provides greater product and process understanding, which is required to support Quality by Design (QbD) regulatory submissions. Implementation of QbD is encouraged by regulatory agencies as a way of improving product quality and patient safety by offering a systematic and proactive approach to product development.[2,3]

It has recently been shown that a method for identity testing which monitors the complementarity determining region (CDR) peptides of a monoclonal antibody (mAb) has been developed and validated using the ACQUITY QDa Detector.[4] Using this work as the foundation of our study, we consider the need for a single test to be used for both product confirmation and also for monitoring a number of pre-characterized post-translational modifications (PTMs) using the ACQUITY QDa Detector, which provides a cost-effective solution for incorporating mass data into analysis. To align with the published work, a sample of trastuzumab and Waters Intact mAb Standard can be checked against the CDR peptides of a trastuzumab reference standard to verify method specificity through retention time and mass determination. Because a drug product's critical quality attributes (CQAs) would need to be independently determined, the current study is meant to serve as a proof of concept for providing a strategy for identifying multiple attributes within a single study using the ACQUITY QDa Detector with compliant-ready chromatographic data software such as Empower.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV ACQUITY QDa Detector (Performance Model)
Absorption wavelength:	215 nm
Column:	ACQUITY UPLC Peptide CSH C_{18} 130 Å, 1.7 µm, 2.1 mm x 100 mm (P/N 186006937)
Column temp.:	65 °C
Mobile phase A:	H_2O with 0.1% (v/v) formic acid
Mobile phase B:	Acetonitrile with 0.1% (v/v) formic acid
Sample temp.:	10 °C
Injection volume:	10 µL

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	%C	%D
Initial	0.200	97	3	0	0
3.00	0.200	97	3	0	0
120.00	0.200	67	33	0	0
127.00	0.200	20	80	0	0
130.00	0.200	20	80	0	0
131.00	0.200	97	3	0	0
150.00	0.200	97	3	0	0

Detector settings

Sampling rate:	2 Hz
Mass range:	350–1250 Da
Ionization mode:	ESI+, centroid
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

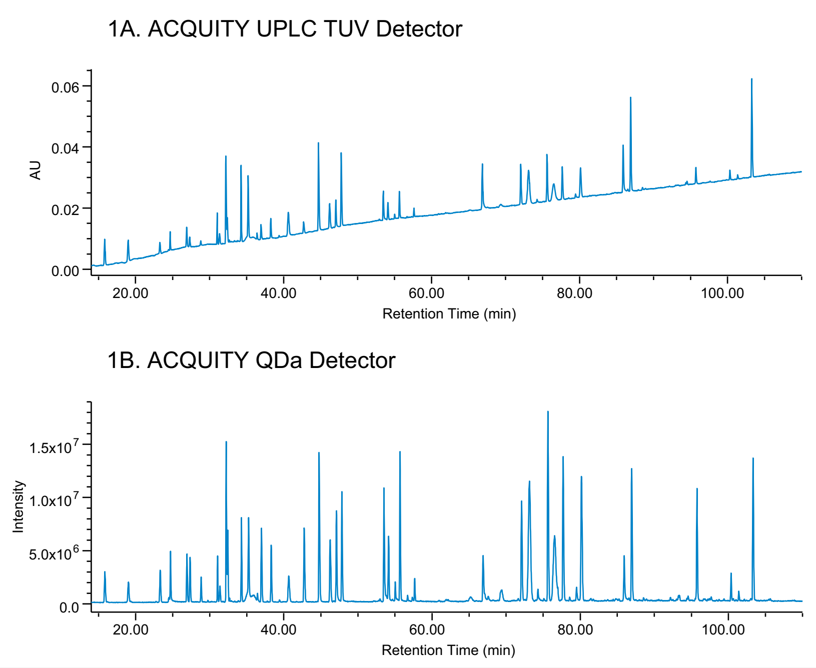
Data management

Empower 3 CDS, SR2

RESULTS AND DISCUSSION

MONITORING MULTIPLE ATTRIBUTES USING EXTRACTED ION CHROMATOGRAMS FOR IDENTITY CONFIRMATION

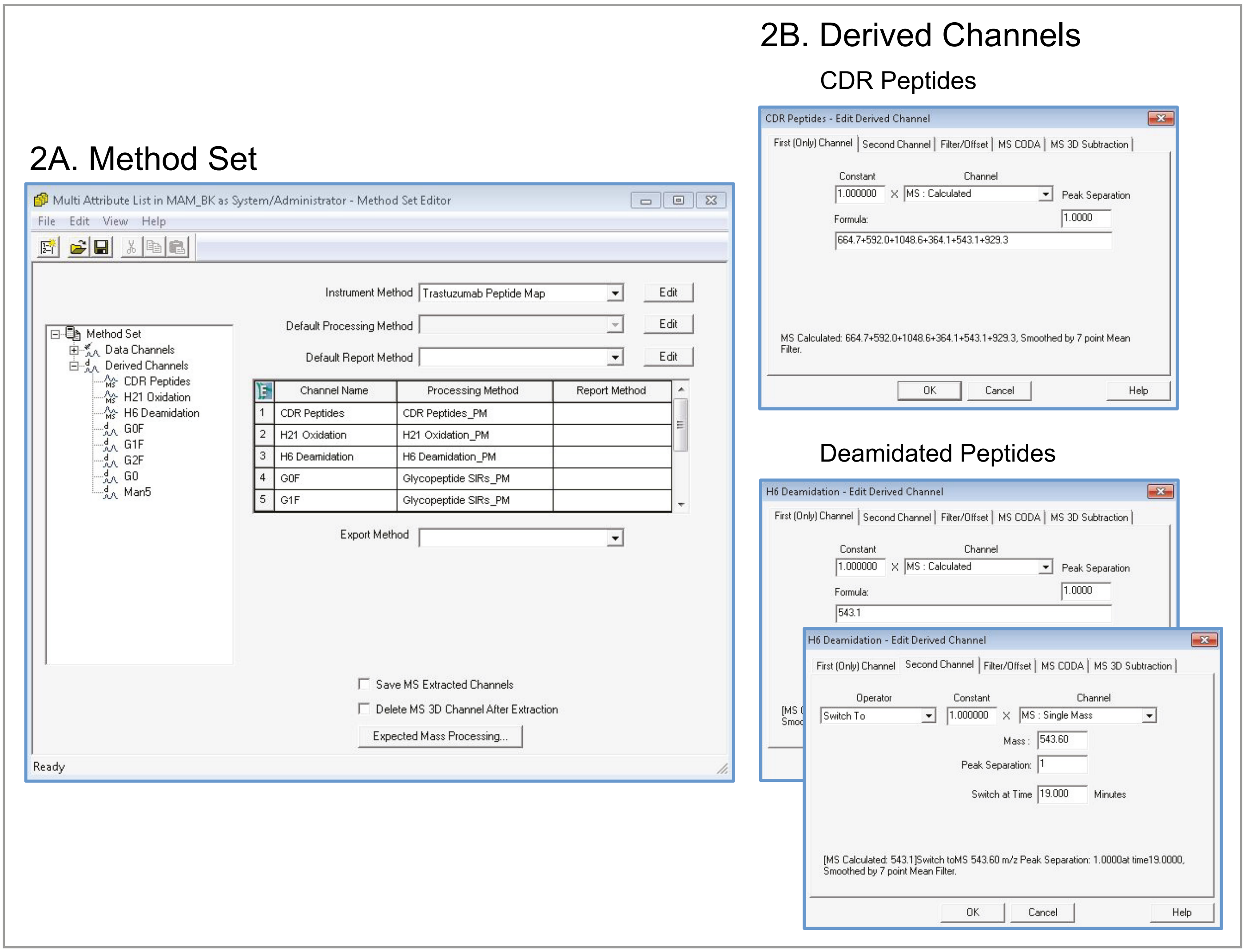
The variable region of an antibody contains CDR peptides that are unique to that specific antibody, which allows these peptides to be used for identification purposes. To begin the evaluation of the ACQUITY QDa Detector for reporting multiple attributes, we began by collecting a peptide map of trastuzumab. A trypsin digest of reduced and alkylated trastuzumab was prepared and injected at a final concentration of approximately 0.5 mg/mL without further dilution. The peptide mapping method described above was used with the ACQUITY QDa Detector set to collect a full scan so that extracted ion chromatograms (XICs) could be used to identify the attributes of interest. Figure 1 shows a strong correlation between the optical trace and the corresponding mass data. This data suggests that the ACQUITY QDa Detector provides an effective way to incorporate mass measurements into an LC-UV based peptide map assay.


1A. ACQUITY UPLC TUV Detector

1B. ACQUITY QDa Detector

Figure 1. Peptide map detection. 1A) Optical detection of a trypsin digest of trastuzumab. 1B) Corresponding ACQUITY QDa data.

The CDR peptides can be used to confirm the identity of a given antibody by using XICs to extract the *m/z* of each CDR peptide. To avoid manual integration of each of the respective XIC channels, derived channels can be used. The process for creating derived channels has been previously described.[5] In brief, a method set can be created to contain a derived channel containing the *m/z* of each of the CDR peptides. This will essentially extract the *m/z* of each of the desired peptides into a single channel. By linking the derived channel to a processing method with associated retention time and component labels, the result is a single channel containing each of the CDR peptides which are now identified according to mass and retention time. Figure 2A shows a screen capture from Empower that illustrates how a method set can be created to contain derived channels, where a new derived channel can be created for each of the attributes of interest. In the case of the CDR peptides, the mass of six CDR peptides can be entered into the Formula field of the derived channel as shown in Figure 2B. In this example, a single dominant charge state is used to identify each of the CDR peptides, but the user could sum over additional charge states if desired.



2A. Method Set

Multi Attribute List in MAM_BK as System/Administrator - Method Set Editor

Instrument Method: Trastuzumab Peptide Map

Default Processing Method:

Default Report Method:

	Channel Name	Processing Method	Report Method
1	CDR Peptides	CDR Peptides_PM	
2	H21 Oxidation	H21 Oxidation_PM	
3	H6 Deamidation	H6 Deamidation_PM	
4	G0F	Glycopeptide SIRs_PM	
5	G1F	Glycopeptide SIRs_PM	

Export Method:

☐ Save MS Extracted Channels

☐ Delete MS 3D Channel After Extraction

Expected Mass Processing...

2B. Derived Channels

CDR Peptides

CDR Peptides - Edit Derived Channel

Constant: 1.000000 × Channel: MS : Calculated

Peak Separation: 1.0000

Formula: 664.7+592.0+1048.6+364.1+543.1+929.3

MS Calculated: 664.7+592.0+1048.6+364.1+543.1+929.3, Smoothed by 7 point Mean Filter.

Deamidated Peptides

H6 Deamidation - Edit Derived Channel

Constant: 1.000000 × Channel: MS : Calculated

Peak Separation: 1.0000

Formula: 543.1

H6 Deamidation - Edit Derived Channel

Operator: Switch To

Constant: 1.000000 × Channel: MS : Single Mass

Mass : 543.60

Peak Separation: 1

Switch at Time 19.000 Minutes

[MS Calculated: 543.1]Switch toMS 543.60 m/z Peak Separation: 1.0000at time19.0000, Smoothed by 7 point Mean Filter.

Figure 2. Empower screen captures. 2A) A method set containing derived channels used to determine if previously characterized attributes are present in a sample. The attributes of interest in this case are CDR peptides, an oxidized peptide, a deamidated peptide, and glycopeptides. Each attribute has an independent processing method associated with it. Attributes to be quantitated using XICs can be monitored through a single derived channel for that attribute, while attributes to be quantitated using SIRs must be monitored through individual channels and quantitated through custom calculations. 2B) Examples of derived channels. To create a derived channel for CDR peptides, the m/z of each peptide can be entered into the formula field. Six CDR peptides are monitored in this example. For the deamidated peptide, a time switch can be entered to switch from one m/z to another at a given time. This avoids signal overlap from using XICs having only a small difference in mass between the native and modified peptides.

The average mass for each of the CDR peptides is reported in Table 1 as well as the calculated *m/z* values used for this study. This table also contains mass information for additional attributes to be monitored as discussed below. The chromatogram resulting from the derived channel can be seen in Figure 3A. In this figure, XICs of each of the six CDR peptides are clearly identified, which confirms sample identity.

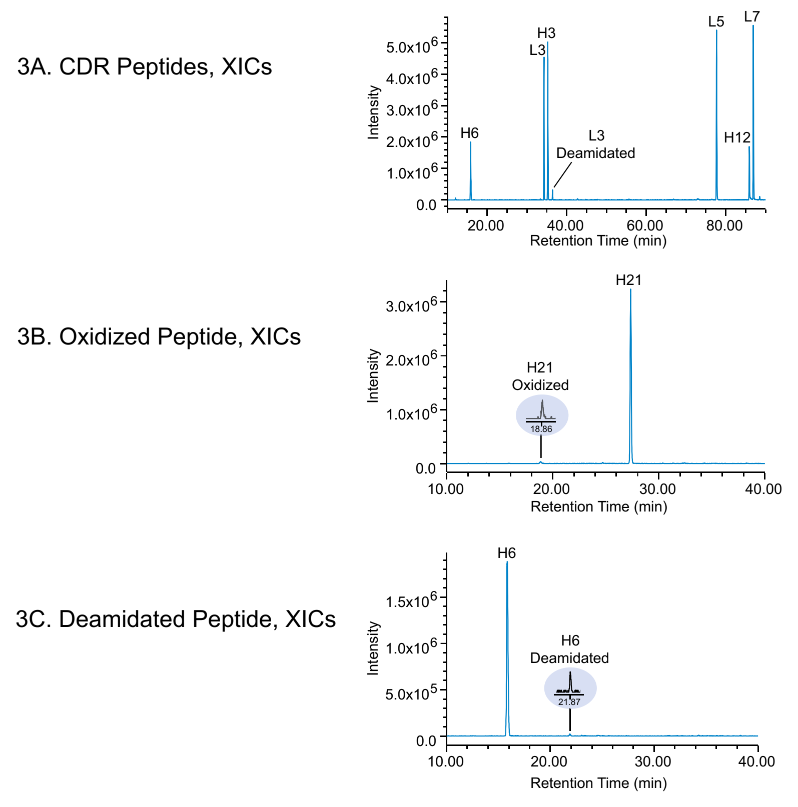
MONITORING MULTIPLE ATTRIBUTES USING EXTRACTED ION CHROMATOGRAMS FOR PROCESS MONITORING

A similar approach can be used to quantify chemical modifications, such as deamidation or oxidation. Process changes during manufacturing or storage conditions can affect the rate of modification, which could potentially impact antibody activity or antigen binding.[6] In this example, we consider asparagine deamidation and methionine oxidation. Again, we make the assumption that these modifications would have been characterized using high resolution MS and determined to be important quality attributes.

Because the mass difference of the oxidation modification is large, this modification can be tracked in derived channels in a similar manner as the CDR peptides. The resulting XICs can be seen in Figure 3B. The deamidation event, however, shows a much smaller difference in mass between the native and deamidated peptides, so the data must be treated in a different way to reliably determine the relative abundance of each of the peptides. In this case, because the peaks are chromatographically resolved, a derived channel can be used that switches from one calculated mass to another at a time established by the user. This process is more clearly illustrated in Figure 2B. From the Empower screen captures, the mass to be monitored switches from *m/z*=543.1 Da (native peptide, z=2) to 543.6 Da (deamidated peptide, z=2) at 19 minutes. Using a second channel avoids signal overlap from the native and deamidated species. The XICs of the native and deamidated peptide can be seen in Figure 3C.

Peptide	Identification/ Modification	Average mass (Da)	Charge state	Calculated (*m/z*)
L3	CDR	1991.17	$[M+3H]^{+3}$	664.7
L5	CDR	1773.04	$[M+3H]^{+3}$	592.0
L7	CDR	4190.48	$[M+4H]^{+4}$	1048.6
H3	CDR	1089.21	$[M+3H]^{+3}$	364.1
H6	CDR	1084.18	$[M+2H]^{+2}$	543.1
H6	Deamidation	1085.17	$[M+2H]^{+2}$	543.6
H12	CDR	2785.01	$[M+3H]^{+3}$	929.3
H21	Native	834.43	$[M+2H]^{+2}$	418.2
H21	Oxidation	850.42	$[M+2H]^{+2}$	426.2
H25	G0F	2634.53	$[M+3H]^{+3}$	879.2
H25	G1F	2796.67	$[M+3H]^{+3}$	933.2
H25	G2F	2958.81	$[M+3H]^{+3}$	987.3
H25	G0	2488.39	$[M+3H]^{+3}$	830.5
H25	G1	2650.53	$[M+3H]^{+3}$	884.5
H25	Man5	2406.28	$[M+3H]^{+3}$	803.1

Table 1. Peptide information for reported attributes.


3A. CDR Peptides, XICs

3B. Oxidized Peptide, XICs

3C. Deamidated Peptide, XICs

Figure 3. XICs used to identify attributes of interest. Peak labels "H" and "L" refer to heavy chain and light chain peptides, respectively. 3A) The CDR peptides unique to trastuzumab. 3B) A native peptide and its oxidized form. 3C) A native peptide and its deamidated form. Note that the native peptide, H6, is also a CDR peptide. This peptide can function to establish identity but can also be used along with its deamidated form to quantitate the percent modification independent of the CDR channel. Insets in 3B. and 3C. show a 10X zoomed in image of the oxidized and deamidated peptides, respectively. Signal-to-noise is well above minimum requirements to reliably quantitate these low level modifications.

MONITORING MULTIPLE ATTRIBUTES USING SELECTED ION RECORDING FOR PROCESS MONITORING

When additional specificity and sensitivity are needed for peak monitoring, selected ion recording (SIR) can be used. By incorporating SIRs, a single *m/z* is selected and passed through to the detector. To demonstrate the utility of SIRs, the dominant charge state of the five most abundant glycopeptides (G0F, G1F, G2F, G0, and Man5) was previously determined. Figure 4 shows an overlay of the five SIR channels.

For monitoring glycopeptides, the additional sensitivity afforded by the SIRs is required for accurate quantitation. Because each SIR is associated with its own channel, custom calculations can be used to calculate the relative abundance of each glycopeptide.

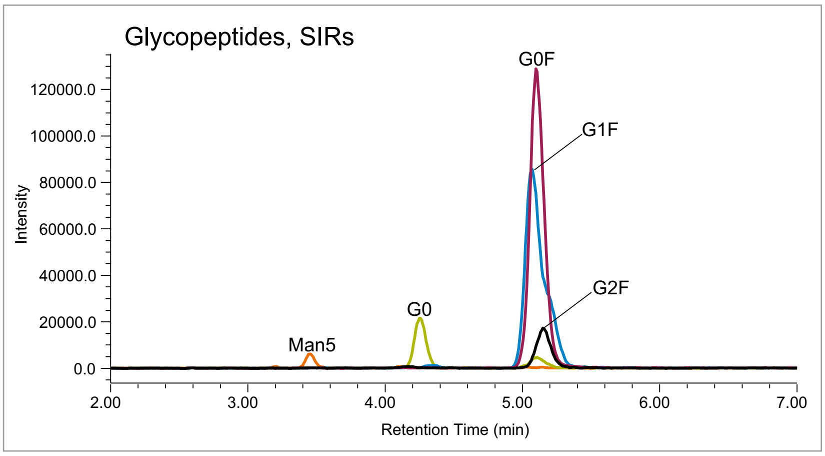
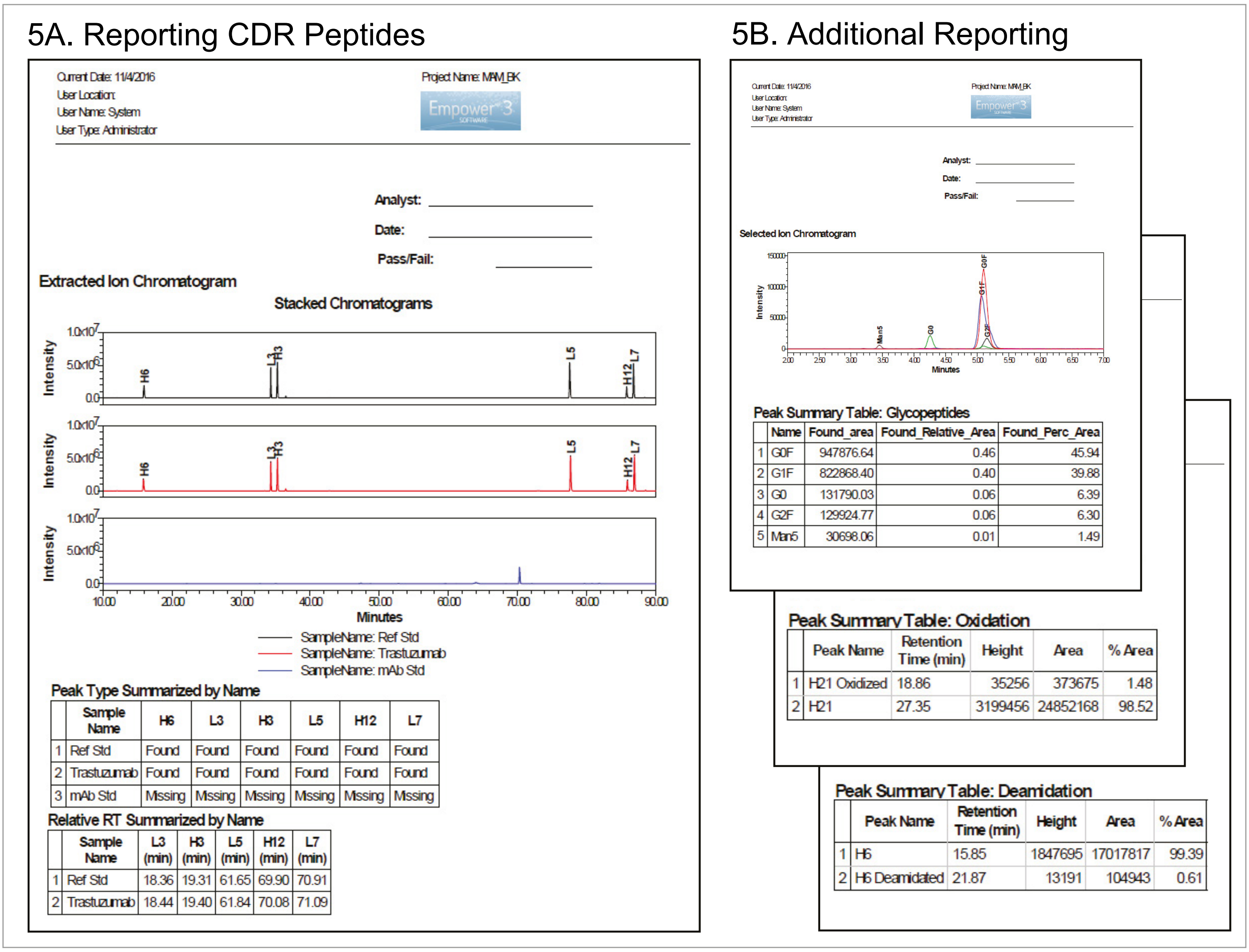


Figure 4. Overlay of five SIR channels used for determining relative abundance of five glycopeptides. Because each SIR is collected in an individual channel, custom calculations must be used to automate the process of determining relative abundance of each glycopeptide.

EMPOWER SOFTWARE ENABLES AUTOMATED REPORTING OF MULTIPLE ATTRIBUTES

We have just shown how a method set can be created to contain derived channels used to monitor attributes of interest, each of which can have an independent processing method associated with it. This same method set can be used to associate independent reporting methods for each of the attributes of interest, which aids to further automate the monitoring process. Should a user wish to import all results into a single report, Empower can accommodate this functionality as well. Figure 5 shows screen captures of Empower reports generated to monitor each of the attributes previously discussed.



5A. Reporting CDR Peptides

Current Date: 11/4/2016
User Location:
User Name: System
User Type: Administrator
Project Name: MAM_BK

Analyst: ____________
Date: ____________
Pass/Fail: ____________

SampleName: Ref Std
SampleName: Trastuzumab
SampleName: mAb Std

Peak Type Summarized by Name

	Sample Name	H6	L3	H3	L5	H12	L7
1	Ref Std	Found	Found	Found	Found	Found	Found
2	Trastuzumab	Found	Found	Found	Found	Found	Found
3	mAb Std	Missing	Missing	Missing	Missing	Missing	Missing

Relative RT Summarized by Name

	Sample Name	L3 (min)	H3 (min)	L5 (min)	H12 (min)	L7 (min)
1	Ref Std	18.36	19.31	61.65	69.90	70.91
2	Trastuzumab	18.44	19.40	61.84	70.08	71.09

5B. Additional Reporting

Current Date: 11/4/2016
User Location:
User Name: System
User Type: Administrator
Project Name: MAM_BK

Analyst: ____________
Date: ____________
Pass/Fail: ____________

Peak Summary Table: Glycopeptides

	Name	Found_area	Found_Relative_Area	Found_Perc_Area
1	G0F	947876.64	0.46	45.94
2	G1F	822868.40	0.40	39.88
3	G0	131790.03	0.06	6.39
4	G2F	129924.77	0.06	6.30
5	Man5	30698.06	0.01	1.49

Peak Summary Table: Oxidation

	Peak Name	Retention Time (min)	Height	Area	% Area
1	H21 Oxidized	18.86	35256	373675	1.48
2	H21	27.35	3199456	24852168	98.52

Peak Summary Table: Deamidation

	Peak Name	Retention Time (min)	Height	Area	% Area
1	H6	15.85	1847695	17017817	99.39
2	H6 Deamidated	21.87	13191	104943	0.61

Figure 5. Empower reporting. 5A) Comparing a reference standard, a sample of trastuzumab, and an intact mAb standard (negative control). Peak tables summarize whether the peaks of interest were detected and the relative retention time for each CDR peptide. In lieu of an internal standard, H6 was used to calculate relative retention time. 5B) Additional reporting for a sample of trastuzumab. Each of the items reported is the result of designing a processing method and derived channel for that specific attribute. In reporting relative abundance of the glycopeptides, Found_area, Found_Relative_Area, and Found_Perc_Area are defined by the custom calculations functionality in Empower. These fields are created so that peak area from individual channels can be reported relative to one another.

CONCLUSIONS

The ACQUITY QDa Detector provides an efficient and cost-effective solution for monitoring important product attributes in a development or QC environment when characterization has been previously carried out using a high resolution MS instrument. This application note demonstrates that CDR peptides, oxidized and deamidated peptides, and glycopeptides can be identified, quantitated, and reported from a single acquisition using the derived channel and inter-channel calculation functionalities in the Empower software. The list of attributes to be monitored would need to be determined for each respective antibody, but could be readily expanded to meet the individual user's needs.

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BioAccord System

“21 CFR-compliant software and user-friendly operations”

Ease of use: ★★★★★ **After sales service:** ★★★★★ **Value for money:** ★★★★★

Rating: 5 ★★★★★

Application Area: Proteins and peptides

“Easy operation and cutting-edge technology. The instrument is nicely designed and meets expectations.”

Swarup Prabhune, Cipla Ltd.

The BioAccord System is a fully integrated, compliance-ready LC-MS system for biopharmaceutical applications. With SmartMS technology at its core, it provides a new level of user experience through simple, consistent, automated setup for every user. At the heart of the system is the ACQUITY RDa Mass Detector, a first-of-its-kind, small-footprint high-resolution time-of-flight (TOF) mass detector, designed specifically for liquid chromatographers/ analytical scientists who are MS novice users.

The BioAccord System enables efficient accurate mass profiling and monitoring of quality attributes (CQAs) at the protein, peptide, and released glycan level, and supports all phases of drug development, process development and monitoring, and QC release testing as well. The system is optimized for ease-of-use and delivers unparalleled robustness and reproducibility for each application/ workflow, providing consistent high-quality results day in and day out that enable accelerated development and enhanced productivity overall.

[APPLICATION NOTE]

Waters
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Enabling Routine and Reproducible Intact Mass Analysis When Data Integrity Matters

Henry Shion, Ying Qing Yu, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

To demonstrate how the BioAccord™ System, a compact high-performance LC-MS platform, streamlines data acquisition, processing, and reporting for intact mass analysis. This is achieved through workflow automation, increased user accessibility, and performance standardization, all of which should enable more labs to generate information-rich results to make better analytical decisions more efficiently.

WATERS SOLUTIONS

Waters™ BioAccord™ System for Proteins and Peptides

KEYWORDS

BioAccord, ACQUITY UPLC, I-Class PLUS, TUV, ACQUITY RDa Detector, SmartMS, UNIFI, intact mass analysis, subunit analysis, humanized mAb mass check standards, mAb subunit standard

INTRODUCTION

Mass spectrometry (MS) analysis of biotherapeutic proteins at the intact and subunit level is conducted throughout the product development life cycle. These analyses provide information to confirm sequence integrity and product variations. The increased utility and accessibility of this technology for intact mass analysis has led to its widespread use in support of regulatory filings for innovator and biosimilar molecules.[1] Despite the widespread practice of intact protein mass analysis, experienced MS users are typically required to manage the instrument operation, data processing, and interpretation. The desire to expand intact mass capability later into development and ultimately into a QC role requires even greater accessibility to non-expert MS users to generate high-quality results produced routinely by experienced analysts.

The benefits of powerful MS capabilities in regulated labs have been realized by many companies.[2-3] Challenges (perceived and real) that have slowed adoption of MS by analysts more familiar with optical detection methods are attributable to the inherent complexities of MS technology and relatively recent availability of compliant-ready informatics platforms capable of converting raw mass spectrometry data into product quality attribute results.

Driven by increasing industry demand for a robust system for biotherapeutic late development and quality organizations, the BioAccord System (Figure 1) was purposefully designed to offer operational modes that have been simplified and optimized to deliver automated, accurate, and reproducible mass measurements for proteins, peptides, and glycans.

Figure 1. The BioAccord System comprised of an ACQUITY UPLC I-Class PLUS configured with an optical detector (TUV/FLR) coupled in-line to the ACQUITY RDa Detector.

The BioAccord System is a compact, high-performance LC-MS platform integrating an ACQUITY RDa Detector with the ACQUITY UPLC I-Class PLUS under control by the UNIFI Scientific Informatics System. The integrated nature of this UNIFI controlled system streamlines data acquisition, processing, and reporting using method-based, workflow-driven processes. A built-in mass calibration reference standard for automated instrument calibration and guided instrument and method setup combines to achieve the goals of workflow automation, increased user accessibility, and performance standardization.

This application note describes the performance characteristics of the BioAccord System for intact mass analysis of an intact monoclonal antibody and IdeS generated subunits. The data shows the high quality, reproducible intact mass data generated by the BioAccord System, with a reduced burden of data processing and management for the user, enabling more labs to generate information-rich results to make better analytical decisions more efficiently.

EXPERIMENTAL

Sample preparation

Humanized mAb mass check standard (P/N 186009152) and mAb Subunit Standard (P/N 186008927) were used for this study. For intact mass analysis, 400 µL of LC-MS grade water was added to the sample vial (contains 80 µg of intact mAb material) to produce a solution of 0.2 µg/µL before injection (2 µL). For subunit analysis, 250 µL of water was added to the sample vial (contains 25 µg of subunit mAb material) to produce a solution of 0.1 µg/µL before injection (2 µL).

Intact mass analysis: LC-MS method setup

Column:	ACQUITY UPLC Protein BEH C4 300Å, 1.7 µm, 2.1 mm × 50 mm (P/N 186004495)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid (or 0.1% TFA, data shown in Figure 6)
Mobile phase B:	Acetonitrile with 0.1% formic acid (or 0.1% TFA, data shown in Figure 6)
Optical detection:	UV 280 nm

LC gradient table for intact mAb analysis:

	Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
1	0.00	0.40	95.0	5.0	Initial
2	1.00	0.40	95.0	5.0	6
3	3.50	0.40	15.0	85.0	6
4	3.70	0.40	15.0	85.0	6
5	4.00	0.40	5.0	95.0	6
6	4.50	0.40	15.0	85.0	6
7	5.00	0.40	95.0	5.0	6
8	5.50	0.40	95.0	5.0	6
9	7.00	0.40	95.0	5.0	6

Total run time: 7.0 min

MS conditions: Intact mass analysis

Acquisition settings

Mode:	Full scan
Mass range:	High (400–7000 *m/z*)
Polarity:	(+)
Scan rate:	2 Hz
Cone voltage:	Custom (70 V) (or 150 V with 0.1% TFA in the mobile phase)
Capillary voltage:	Custom (1.50 kV)
Desolvation temp.:	Custom (550 °C)

Subunit mass analysis: LC-MS method setup

Column:	BioResolve™ RP mAb Polyphenyl 450Å, 2.7 µm, 2.1 mm × 50 mm (P/N 186008944)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Optical detection:	UV 280 nm

**Waters humanized mAb mass check standard (P/N 186009152) is a stabilized and lyophilized format of NIST Reference Material 8671 (NIST mAb) .*

LC gradient table for intact mAb analysis:

	Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
1	0.00	0.30	80	20	6
2	10.00	0.30	60	40	6
3	10.30	0.30	20	80	6
4	11.30	0.30	20	80	6
5	11.60	0.30	80	20	6
6	15.00	0.30	80	20	6

Total run time:	15.0 min

MS conditions: Subunit analysis

Acquisition settings

Mode:	Full scan
Mass range:	High (400–7000 *m/z*)
Polarity:	(+)
Scan rate:	2 Hz
Cone voltage:	Custom (30 V)
Capillary voltage:	Custom (1.00 V)
Desolvation temp.:	Custom (450 °C)

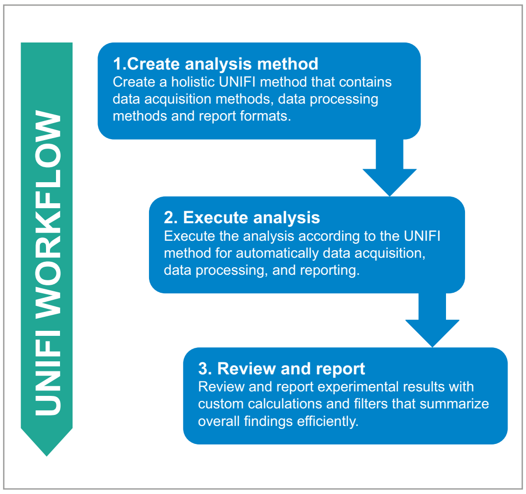
RESULTS AND DISCUSSION

INTACT MASS ANALYSIS WORKFLOW SETUP WITHIN THE BIOACCORD SYSTEM

Under the control of the UNIFI software platform, the BioAccord System streamlines the complete intact protein mass analysis process, from acquisition method creation, data processing to final reporting, by automating and standardizing a common analytical workflow (Figure 2). Special attention has been paid to reduce the complexity of the ACQUITY RDa Detector instrument operation into four standardized modes:

1. Positive lower mass analysis (up to *m/z* = 2000 data acquisition, suitable for peptide mapping)
2. Positive high mass analysis (up to *m/z* = 7000, suitable for intact mass and subunit analysis)
3. Negative lower mass analysis (up to *m/z* = 2000)
4. Negative higher mass analysis (up to *m/z* = 5000).

This standardization of operation modes together with a simple and intuitive user interface, automated optimization of advanced tuning parameters, and the pre-programmed calibration checks facilitate the integration of the system into standard operating procedures and ensure consistency between different operators and different laboratories. Figure 3 shows the screen capture of the ACQUITY RDa Detector instrument control window, where typical settings for intact data acquisition are displayed.

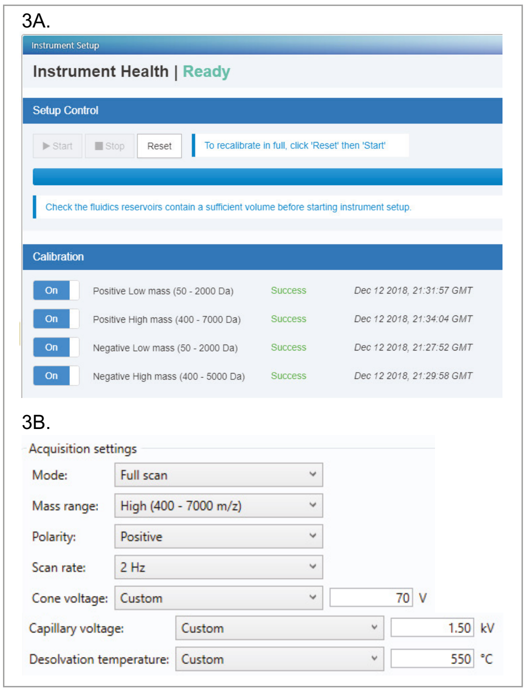


1.Create analysis method
Create a holistic UNIFI method that contains data acquisition methods, data processing methods and report formats.

2. Execute analysis
Execute the analysis according to the UNIFI method for automatically data acquisition, data processing, and reporting.

3. Review and report
Review and report experimental results with custom calculations and filters that summarize overall findings efficiently.

Figure 2. Automated and standardized intact mass workflow.



Instrument Health | Ready

To recalibrate in full, click 'Reset' then 'Start'

Check the fluidics reservoirs contain a sufficient volume before starting instrument setup.

On	Positive Low mass (50 - 2000 Da)	Success	Dec 12 2018, 21:31:57 GMT
On	Positive High mass (400 - 7000 Da)	Success	Dec 12 2018, 21:34:04 GMT
On	Negative Low mass (50 - 2000 Da)	Success	Dec 12 2018, 21:27:52 GMT
On	Negative High mass (400 - 5000 Da)	Success	Dec 12 2018, 21:29:58 GMT

Acquisition settings

Mode:	Full scan		
Mass range:	High (400 - 7000 m/z)		
Polarity:	Positive		
Scan rate:	2 Hz		
Cone voltage:	Custom	70	V
Capillary voltage:	Custom	1.50	kV
Desolvation temperature:	Custom	550	°C

Figure 3. (A) ACQUITY RDa Detector MS source controlling parameters and method setup for intact mass analysis; (B). ACQUITY RDa Detector MS data acquisition parameters for intact mass analysis with mobile phases containing 0.1% formic acid.

INTACT MAB ANALYSIS WITH THE BIOACCORD SYSTEM

The full MS spectrum obtained from 400 ng humanized mAb mass check standard (P/N 186009152) applied to a 2.1 mm × 50 mm ACQUITY BEH Column is displayed in Figure 4. The mass spectrum, acquired over *m/z* 400–7000 shows the typical charge distribution observed for a large protein. The spectrum from four high abundant charge states (from 51+ to 54+) spanning *m/z* range from 2730 to 2950, represented in the zoomed figure (Figure 4B), shows the five most abundant glycoforms of the intact antibody and the consistency in the relative abundance of glycoforms across the charge states.

The intact masses of these five most abundant glycoforms and a series of less abundant glycoforms were obtained after the deconvolution of the full MS spectrum (Figure 4C). The assignment of the peaks was based on the in-silico calculation from protein sequence, including bi-antennary glycan structures common to a humanized mAb. The deconvoluted spectrum (Figure 4C) shows a very similar pattern for the relative abundance of the major glycoforms compared to the raw spectrum.

An automated LC-MS analysis of 13 injections of humanized mAb mass check standard was acquired, processed, and reported (Figure 5). Data, representative of a simple method development set, were used to assess the extent of product glyco-variation and analytical reproducibility. The average relative abundances of the five major glycoforms identified by the software for 13 injections are displayed in the trending plot (Figure 5A). Tight %RSD values below 2.5% indicate that the MaxEnt1 processed data is of equivalent quality to that generated by experienced users of LC-MS platforms.

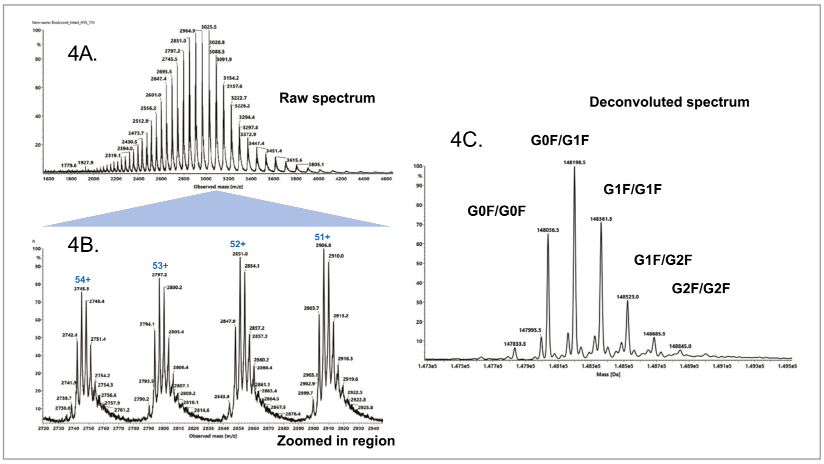
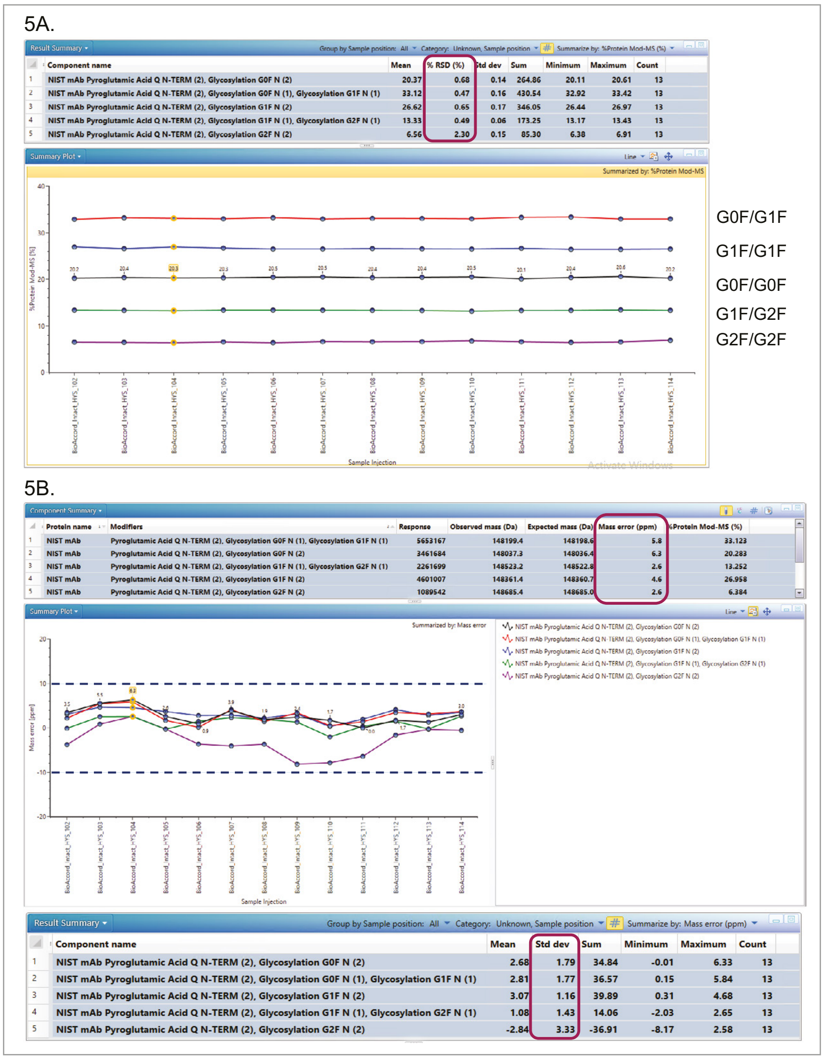


Figure 4. Intact mass spectrum from mobile phases with 0.1% FA.



5A.

Result Summary — Group by Sample position: All — Category: Unknown, Sample position — Summarize by: %Protein Mod-MS (%)

	Component name	Mean	% RSD (%)	Std dev	Sum	Minimum	Maximum	Count
1	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (2)	20.37	0.68	0.14	264.86	20.11	20.61	13
2	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (1), Glycosylation G1F N (1)	33.12	0.47	0.16	430.54	32.92	33.42	13
3	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (2)	26.62	0.65	0.17	346.05	26.44	26.97	13
4	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (1), Glycosylation G2F N (1)	13.33	0.49	0.06	173.25	13.17	13.43	13
5	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G2F N (2)	6.56	2.30	0.15	85.30	6.38	6.91	13

5B.

Component Summary

	Protein name	Modifiers	Response	Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	%Protein Mod-MS (%)
1	NIST mAb	Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (1), Glycosylation G1F N (1)	5653167	148199.4	148198.6	5.8	33.123
2	NIST mAb	Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (2)	3461684	148037.3	148036.4	6.3	20.283
3	NIST mAb	Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (1), Glycosylation G2F N (1)	2261699	148523.2	148522.8	2.6	13.252
4	NIST mAb	Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (2)	4601007	148361.4	148360.7	4.6	26.958
5	NIST mAb	Pyroglutamic Acid Q N-TERM (2), Glycosylation G2F N (2)	1089542	148685.4	148685.0	2.6	6.384

NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (2)
NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (1), Glycosylation G1F N (1)
NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (2)
NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (1), Glycosylation G2F N (1)
NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G2F N (2)

Result Summary — Group by Sample position: All — Category: Unknown, Sample position — Summarize by: Mass error (ppm)

	Component name	Mean	Std dev	Sum	Minimum	Maximum	Count
1	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (2)	2.68	1.79	34.84	-0.01	6.33	13
2	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G0F N (1), Glycosylation G1F N (1)	2.81	1.77	36.57	0.15	5.84	13
3	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (2)	3.07	1.16	39.89	0.31	4.68	13
4	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G1F N (1), Glycosylation G2F N (1)	1.08	1.43	14.06	-2.03	2.65	13
5	NIST mAb Pyroglutamic Acid Q N-TERM (2), Glycosylation G2F N (2)	-2.84	3.33	-36.91	-8.17	2.58	13

Figure 5A. Spectrum quality and data consistency are two key factors of successful intact mass analysis. Relative quantitation of major glycoforms of the humanized mAb standard is displayed here. Across 13 injections, we were able to obtain consistent relative percentage of glycoforms. The RSD% is less than 2.5% as highlighted in red. 5B Automatically calculated humanized mAb standard experiment results displayed in the UNIFI review panel. Good mass accuracy is achieved for individual injection and as well as the whole dataset .

Intact mass analysis of the humanized mAb mass check standard (Figure 5B) includes a component table for the top five major glycoforms by MS response, the observed and expected (theoretical) masses, the calculated ppm mass error, and relative abundance of the respective glycoforms in each injection. The summary plot below shows the mass errors of the five major glycoforms identified in 13 injections in one simple display.

Trifluoroacetic acid (TFA) is commonly used as a mobile phase additive for reversed-phase LC (RP-LC) separations of proteins and peptides. TFA works as an ion-pairing agent to improve the chromatographic peak shape of proteins by minimizing the interaction between the protein functional groups with residual silanol groups on silica LC particles. While TFA can interfere, reduce the MS signal, lowering absolute MS sensitivity of the analysis, TFA has positive effects on quantification by UV detection. As such, TFA has been widely used as an additive in the laboratories traditionally associated with optical-based assays that would likely be the basis for future LC-MS methods.

The LC-MS spectrum of intact humanized mAb mass check standard using mobile phases with 0.1% TFA shows greater spread of the *m/z* charge envelope from *m/z* of 2000 to 6500, in comparison to mobile phases containing 0.1% formic acid. Higher sample consumption (~5x) overcomes the TFA ion-suppression effects commonly observed during electrospray ionization process to generate data equivalent to a formic acid based analysis. Higher cone voltages (150 volts vs. 60 volts) prove more effective for declustering any TFA adducts, producing high-quality spectral data (Figure 6B) from the BioAccord System, comparable to the 0.1% FA data (Figure 4B).

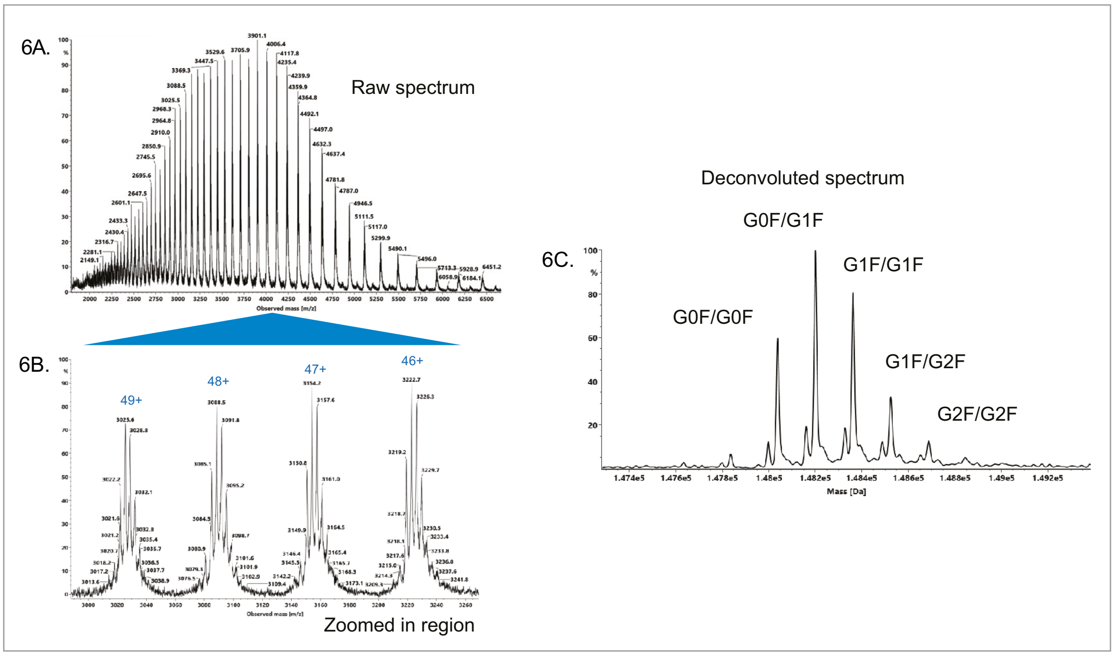
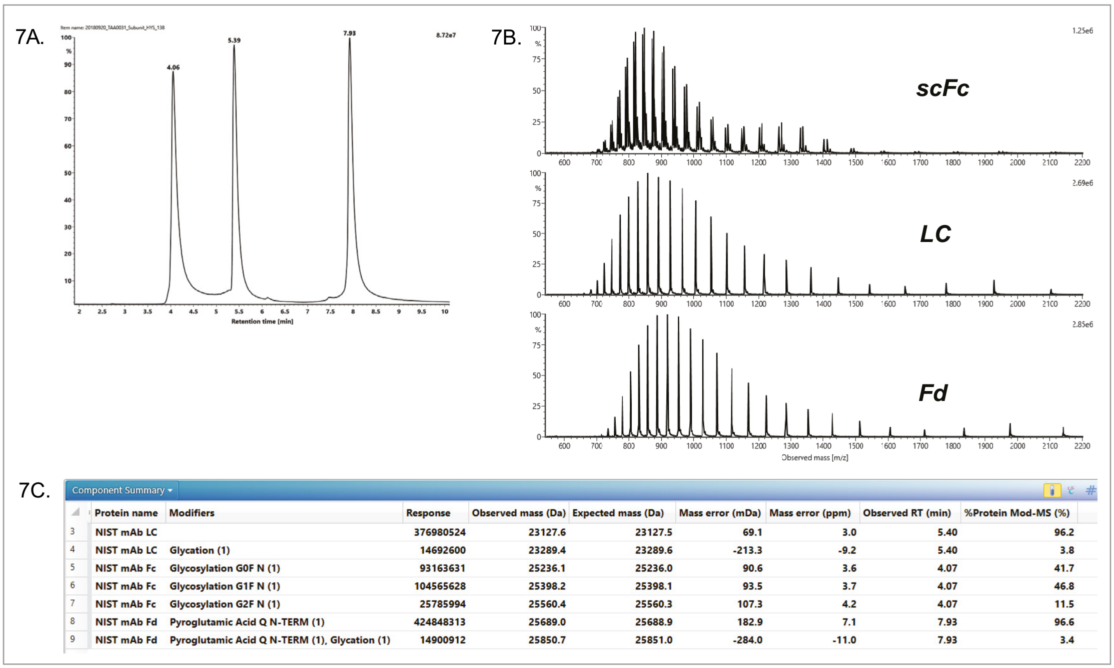


Figure 6. Intact mass spectrum from mobile phases with 0.1% TFA.

mAb SUBUNIT STANDARD (scFc, LC, AND Fd) ANALYSIS WITH THE BIOACCORD SYSTEM

Subunit analysis (Figure 7A–B) was performed using mAb Subunit Standard (P/N 186008927) The TIC chromatogram (Figure 7A) contained three resolved chromatographic peaks (scFc, LC, and Fd) with reported retention times of 4.06, 5.39, and 7.93 min respectively. Combined raw spectra (Figure 7B) corresponding to each peak of humanized mAb mass check standard (200 ng on column) subunits: scFc. LC and Fd exhibit high s/n, multiple charged spectral envelopes for the subunits that were automatically processed into deconvoluted results (Figure 7C). The component summary table shows the identified LC, scFc, and Fd subunits with their respective MS response, the mass after deconvolution, the calculated mass error, the retention time, and the percentage of modification on the subunit. The review panel also shows the automatically identified and labeled major glycoforms from the scFc after deconvolution in the component plot.



	Protein name	Modifiers	Response	Observed mass (Da)	Expected mass (Da)	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	%Protein Mod-MS (%)
3	NIST mAb LC		376980524	23127.6	23127.5	69.1	3.0	5.40	96.2
4	NIST mAb LC	Glycation (1)	14692600	23289.4	23289.6	-213.3	-9.2	5.40	3.8
5	NIST mAb Fc	Glycosylation G0F N (1)	93163631	25236.1	25236.0	90.6	3.6	4.07	41.7
6	NIST mAb Fc	Glycosylation G1F N (1)	104565628	25398.2	25398.1	93.5	3.7	4.07	46.8
7	NIST mAb Fc	Glycosylation G2F N (1)	25785994	25560.4	25560.3	107.3	4.2	4.07	11.5
8	NIST mAb Fd	Pyroglutamic Acid Q N-TERM (1)	424848313	25689.0	25688.9	182.9	7.1	7.93	96.6
9	NIST mAb Fd	Pyroglutamic Acid Q N-TERM (1), Glycation (1)	14900912	25850.7	25851.0	-284.0	-11.0	7.93	3.4

Figure 7. TIC, combined raw spectra and major peaks for the humanized mAb standard (200 ng on column) IdeS digested subunits scFc, LC, and Fd.

CONCLUSIONS

In this study, the BioAccord System demonstrated exceptional performance from a new compact design mass detector, workflow automation, and simplified system interaction capability via the UNIFI Informatics platform. intact and subunit mAb mass data generated by this system facilitated automated humanized mAb peak assignment and relative glycoform abundance determination, typical of method validation or routine operation sample sets. The ability to deploy this system in both regulated and non-regulated environments should facilitate rapid method development and simplified downstream transfer of these methods to late development and quality organizations challenged to make more informed analytical decisions faster.

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[APPLICATION NOTE]

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Establishing an Integrated Peptide Attribute Profiling and Monitoring Workflow for Improved Productivity and Confidence

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- SmartMS™ system setup, calibration, and system status monitoring
- Automated software for data acquisition, processing, and reporting
- Scientific library feature for creating user-specific peptide attribute libraries for recurrent use
- Routine peptide level product quality attribute identification and monitoring using one instrument-informatics platform

WATERS SOLUTIONS

BioAccord System

ACQUITY UPLC™ I-Class PLUS

ACQUITY UPLC Tunable UV Detector

UNIFI™ Scientific Information System

KEYWORDS

BioAccord System, SmartMS, ACQUITY RDa Detector, peptide attributes, routine peptide monitoring, PTM characterization, scientific library, UNIFI Scientific Information System

INTRODUCTION

Biotherapeutics undergo rigorous characterization and monitoring during their development and manufacturing to establish product quality and safety for regulatory compliance.[1,2] High-performance LC-MS technology, often utilized for the characterization of a protein's chemical and post-translational modifications at the peptide level, has now been expanded beyond the conventional realm to attribute monitoring across different stages of the product life cycle. Routine monitoring of these quality attributes requires fit-for-purpose analytical platforms that are robust, easy to operate, and readily deployable in laboratories across an organization. The BioAccord System is a new, compact, high-performance, LC-MS system purposefully designed to meet such requirements. It consists of an ACQUITY UPLC I-Class PLUS, ACQUITY RDa Detector featuring SmartMS Technology, compliance-ready UNIFI Software, and consumables all tailored to assist biopharmaceutical applications. The UNIFI Scientific Information System provides integrated, analytical workflow methods with automated data acquisition, processing and reporting capabilities allowing peptide monitoring assays to be readily performed and documented for a single or large batch of samples. In this application note, we demonstrate peptide mapping and monitoring capabilities of the BioAccord System using forced degradation[3,4] study of a monoclonal antibody (mAb) as a case study.

EXPERIMENTAL

Preparation of forced degradation samples

Heat stress: An aliquot of NISTmAb reference material (NIST RM 8671, Gaithersburg, USA) heated at 37 °C for one week.

Oxidative stress: Two aliquots of NISTmAb reference material was incubated with 0.01% and 3% H_2O_2 respectively at room temperature for 24 h.

Peptide samples: Intact mAb samples were reduced and alkylated with 0.5 M DTT and iodoacetamide solutions before buffer exchanging into 0.1 M tris (pH 7.6) using NAP- 5 size-exclusion columns (GE Healthcare Life Sciences, Pittsburgh, USA). The samples were digested with trypsin (Promega, Madison, USA) for 4 h at 37 °C (20:1 protein to enzyme) followed by acidification with 10% formic acid.

LC conditions

System:	ACQUITY UPLC I-Class PLUS
Detection:	ACQUITY TUV
Vials:	LCGC Certified Clear Glass 12 × 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 1 mL (p/n: 186000385C)
Column:	ACQUITY UPLC CSH C_{18}, 130 Å, 1.7 µm, 1 × 100 mm (p/n: 186006937)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	5 µL
Flow rate:	0.2 mL/min
Mobile phase A:	0.1% formic acid in H_2O
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	1% B over 1 min, 1%–65% B over 50 min, 35%–85% B over 6 min, 85% B for 4 min, 85%–1% B over 5 min and 1% B for 13 min

MS conditions

System:	ACQUITY RDa Detector
Ionization mode:	ESI positive
Acquisition mode:	Full MS scans with CID fragmentation
Acquisition range:	*m/z* 50–2000
Capillary voltage:	1.2 kV
Collision energy:	60–120 V (low/high energy ramping)
Cone voltage:	30 V
Desolvation energy:	350 °C
Intelligent data capture:	On

Data management

Informatics software:	UNIFI Scientific Information System v. 1.9.4

RESULTS AND DISCUSSION

FROM PEPTIDE ATTRIBUTE IDENTIFICATION TO ROUTINE MONITORING

This application note highlights the utilization of the BioAccord System for routine peptide quality attribute profiling and monitoring. This includes: identification of peptides and their modifications using peptide mapping data, archiving a selected list of potential quality attributes in a secured user-defined custom library using UNIFI scientific library feature followed by targeted peptide monitoring (Figure 1). During routine monitoring of peptide attributes, the custom library items are retrieved and utilized in the accurate mass screening method for targeted CQA monitoring and relative quantification. To enhance usability, the accurate mass screening method can be programmed to notify the user of any attributes exhibiting unusual modification levels through a thresholding feature (the limit check). Both peptide mapping and accurate mass screening described below are compliance-ready methods integrated in the same informatics platform with the library feature to provide the user with a continuous workflow from peptide attribute profiling to monitoring. The overall analysis is further elaborated using trastuzumab test case data.

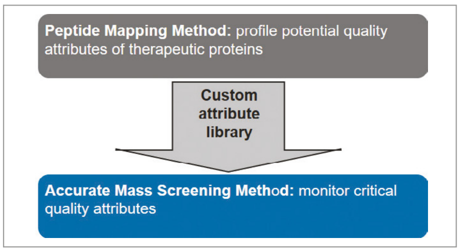
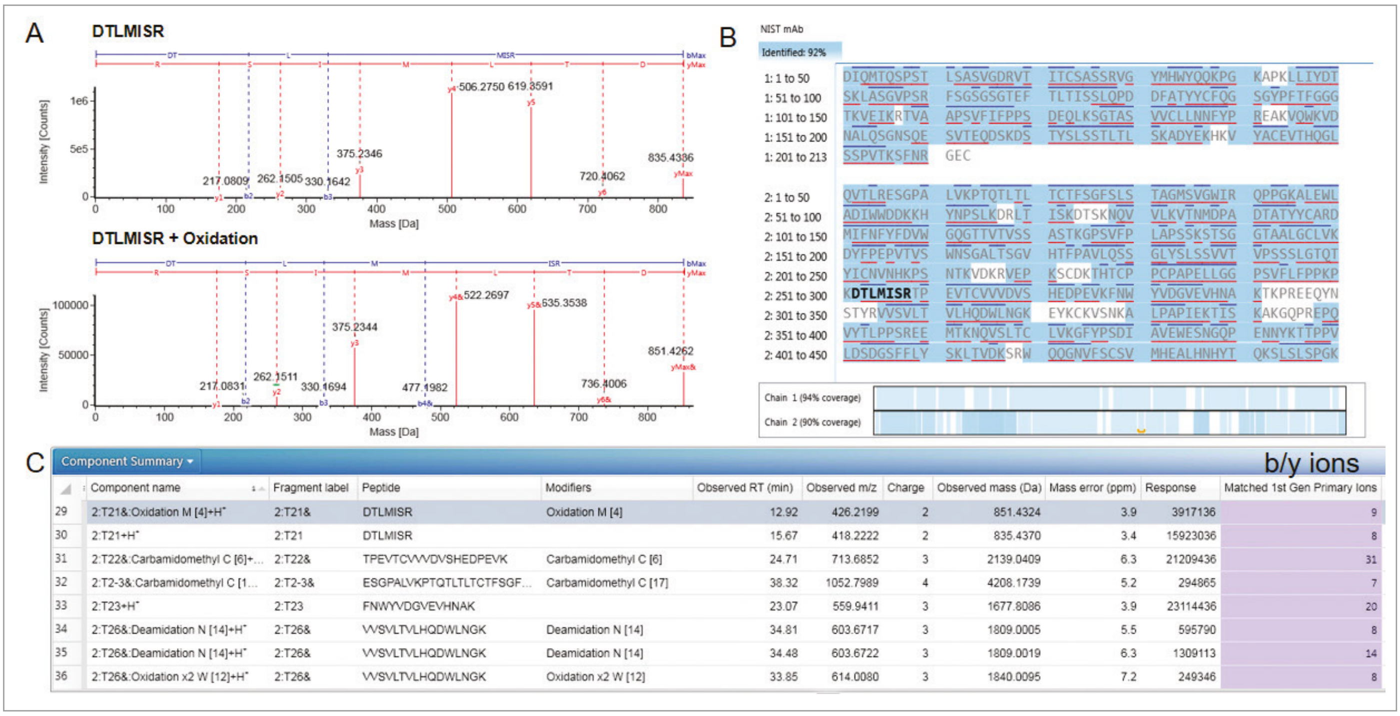


Figure 1. Schematic representation of workflow methods from peptide attribute identification to routine monitoring.

Step 1. Peptide mapping workflow for potential quality attribute identification

In brief, the mAb sample aliquots were subjected to elevated temperature and chemical oxidation followed by tryptic digestion as stated in the experimental section. The data acquisition was performed using the BioAccord System with alternative MS scans, first with no collision energy and then at elevated collision energy to generate informative fragment ions in data independent acquisition (DIA) mode using an energy ramp from 60–120 V. The acquired data were processed in the peptide mapping method to confirm peptides and their modifications based on the accurate mass, and each assignment was further verified using primary fragment ions for high confidence peptide attribute profiling. The annotated fragmentation spectra for unmodified and modified HC:T21 (Heavy Chain, tryptic peptide number 21 from N-terminus) are shown in Figure 2A. The sequence coverage in Figure 2B includes all peptide sequences verified with ±10 ppm mass accuracy and containing a minimum of 3 b/y fragment ions per peptide. The control and stressed NISTmAb samples reported over 90% sequence coverage. The component table (Figure 2C) provides a comprehensive summary of all verified peptides and modifications identified in each sample along with their respective *m/z*, retention time, mass tolerance, charge states and number of primary fragment ions (b/y ions). The oxidative stress conditions used for NISTmAb, induced oxidation of both methionine and tryptophan. For routine CQA monitoring, a selected list of peptides from the component table was sent to a custom library. Similarly, the UNIFI processing method described here can be applied to data acquired on other Waters HRMS systems for peptide mapping and PTM confirmation.



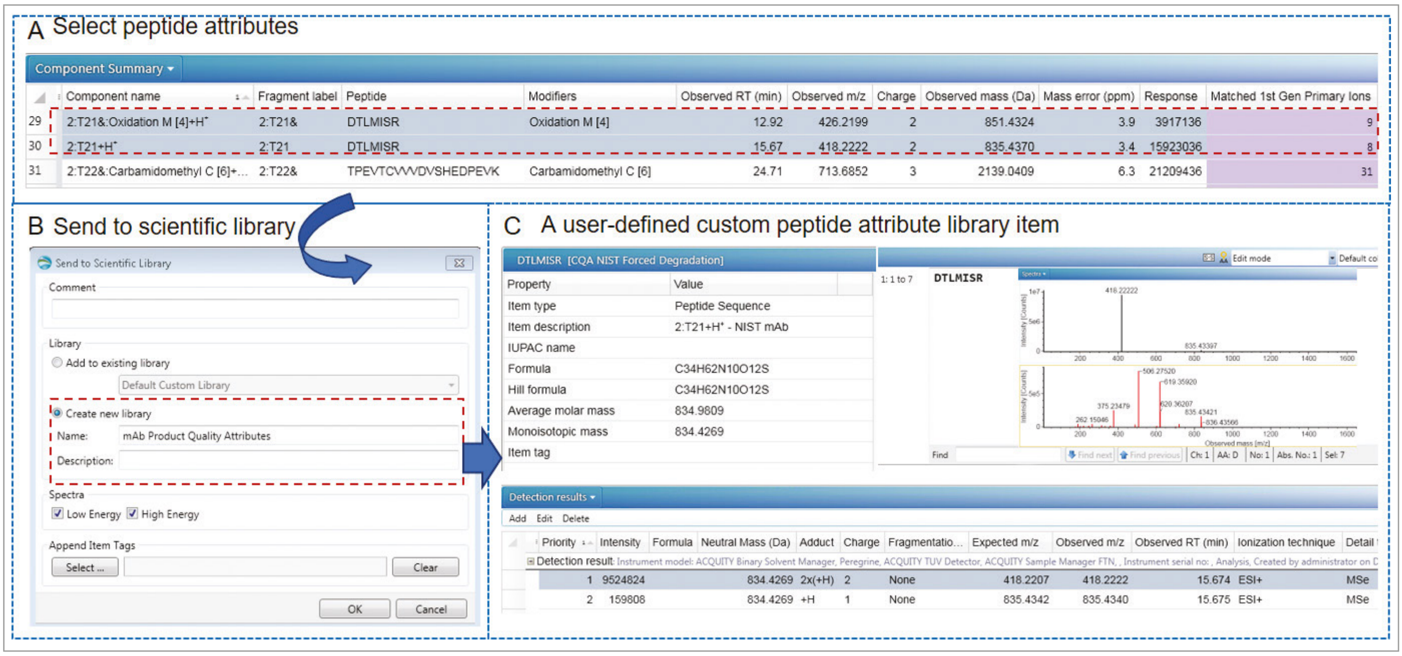
1: 1 to 50	DIQMTQSPST	LSASVGDRVT	ITCSASSRVG	YMHWYQQKPG	KAPKLLIYDT
1: 51 to 100	SKLASGVPSR	FSGSGSGTEF	TLTISSLQPD	DFATYYCFQG	SGYPFTFGGG
1: 101 to 150	TKVEIKRTVA	APSVFIFPPS	DEQLKSGTAS	VVCLLNNFYP	REAKVQWKVD
1: 151 to 200	NALQSGNSQE	SVTEQDSKDS	TYSLSSTLTL	SKADYEKHKV	YACEVTHQGL
1: 201 to 213	SSPVTKSFNR	GEC			
2: 1 to 50	QVTLRESGPA	LVKPTQTLTL	TCTFSGFSLS	TAGMSVGWIR	QPPGKALEWL
2: 51 to 100	ADIWWDDKKH	YNPSLKDRLT	ISKDTSKNQV	VLKVTNMDPA	DTATYYCARD
2: 101 to 150	MIFNFYFDVW	GQGTTVTVSS	ASTKGPSVFP	LAPSSKSTSG	GTAALGCLVK
2: 151 to 200	DYFPEPVTVS	WNSGALTSGV	HTFPAVLQSS	GLYSLSSVVT	VPSSSLGTQT
2: 201 to 250	YICNVNHKPS	NTKVDKRVEP	KSCDKTHTCP	PCPAPELLGG	PSVFLFPPKP
2: 251 to 300	K**DTLMISR**TP	EVTCVVVDVS	HEDPEVKFNW	YVDGVEVHNA	KTKPREEQYN
2: 301 to 350	STYRVVSVLT	VLHQDWLNGK	EYKCKVSNKA	LPAPIEKTIS	KAKGQPREPQ
2: 351 to 400	VYTLPPSREE	MTKNQVSLTC	LVKGFYPSDI	AVEWESNGQP	ENNYKTTPPV
2: 401 to 450	LDSDGSFFLY	SKLTVDKSRW	QQGNVFSCSV	MHEALHNHYT	QKSLSLSPGK

	Component name	Fragment label	Peptide	Modifiers	Observed RT (min)	Observed m/z	Charge	Observed mass (Da)	Mass error (ppm)	Response	Matched 1st Gen Primary Ions
29	2:T21&:Oxidation M [4]+H⁺	2:T21&	DTLMISR	Oxidation M [4]	12.92	426.2199	2	851.4324	3.9	3917136	9
30	2:T21+H⁺	2:T21	DTLMISR		15.67	418.2222	2	835.4370	3.4	15923036	8
31	2:T22&:Carbamidomethyl C [6]+…	2:T22&	TPEVTCVVVDVSHEDPEVK	Carbamidomethyl C [6]	24.71	713.6852	3	2139.0409	6.3	21209436	31
32	2:T2-3&:Carbamidomethyl C [1…	2:T2-3&	ESGPALVKPTQTLTLTCTFSGF…	Carbamidomethyl C [17]	38.32	1052.7989	4	4208.1739	5.2	294865	7
33	2:T23+H⁺	2:T23	FNWYVDGVEVHNAK		23.07	559.9411	3	1677.8086	3.9	23114436	20
34	2:T26&:Deamidation N [14]+H⁺	2:T26&	VVSVLTVLHQDWLNGK	Deamidation N [14]	34.81	603.6717	3	1809.0005	5.5	595790	8
35	2:T26&:Deamidation N [14]+H⁺	2:T26&	VVSVLTVLHQDWLNGK	Deamidation N [14]	34.48	603.6722	3	1809.0019	6.3	1309113	14
36	2:T26&:Oxidation x2 W [12]+H⁺	2:T26&	VVSVLTVLHQDWLNGK	Oxidation x2 W [12]	33.85	614.0080	3	1840.0095	7.2	249346	8

Figure 2. Review panel in UNIFI peptide mapping method. (A) Annotated high-energy spectra for unmodified and modified DTLMSIR peptide (HC:T21). Highlighted are the b and y ions (in blue and red) generated by collision energy ramping. (B) Protein sequence coverage is 92% (when the high confidence peptide fileting criteria uses ±10 ppm mass tolerance or less, and a minimum of three fragment ions per peptide). (C) Component table summarizing detection results for all peptides and their PTMs identified in the analysis, such as retention time, m/z, and MS intensity etc.

Step 2. Creating custom attribute libraries from peptide mapping results

Following peptide identification, a custom library was generated from the peptide mapping data in Step 1. This includes selecting peptide attributes of interest directly from the component table (Figure 3A) and creating a new custom library using “send to library” option in UNIFI (Figure 3B). Each peptide entry consists of several key parameters in addition to peptide sequence, chemical composition and neutral mass such as: retention time, *m/z*, MS intensity, MS and fragmentation spectra, and charge state information (Figure 3C) that can be selectively retrieved and used in targeted peptide monitoring methods. The case study stated here archived 28 potential peptide quality attributes associated with stressed NISTmAb samples in “mAb Product Quality Attributes” library using UNIFI scientific library feature. These custom libraries can be modified, saved, and shared among other UNIFI users within an organization.



A Select peptide attributes

Component Summary

	Component name	Fragment label	Peptide	Modifiers	Observed RT (min)	Observed m/z	Charge	Observed mass (Da)	Mass error (ppm)	Response	Matched 1st Gen Primary Ions
29	2:T21&:Oxidation M [4]+H⁺	2:T21&	DTLMISR	Oxidation M [4]	12.92	426.2199	2	851.4324	3.9	3917136	9
30	2:T21+H⁺	2:T21	DTLMISR		15.67	418.2222	2	835.4370	3.4	15923036	8
31	2:T22&:Carbamidomethyl C [6]+…	2:T22&	TPEVTCVVVDVSHEDPEVK	Carbamidomethyl C [6]	24.71	713.6852	3	2139.0409	6.3	21209436	31

B Send to scientific library

Send to Scientific Library

Comment

Library

○ Add to existing library

Default Custom Library

● Create new library

Name: mAb Product Quality Attributes

Description:

Spectra

☑ Low Energy ☑ High Energy

Append Item Tags

C A user-defined custom peptide attribute library item

DTLMISR [CQA NIST Forced Degradation]

Property	Value
Item type	Peptide Sequence
Item description	2:T21+H⁺ - NIST mAb
IUPAC name	
Formula	C34H62N10O12S
Hill formula	C34H62N10O12S
Average molar mass	834.9809
Monoisotopic mass	834.4269
Item tag	

Detection results

Priority	Intensity	Formula	Neutral Mass (Da)	Adduct	Charge	Fragmentatio…	Expected m/z	Observed m/z	Observed RT (min)	Ionization technique	Detail
Detection result: Instrument model: ACQUITY Binary Solvent Manager, Peregrine, ACQUITY TUV Detector, ACQUITY Sample Manager FTN, , Instrument serial no: , Analysis, Created by administrator on D											
1	9524824		834.4269	2x(+H)	2	None	418.2207	418.2222	15.674	ESI+	MSe
2	159808		834.4269	+H	1	None	835.4342	835.4340	15.675	ESI+	MSe

Figure 3. PTMs identified in peptide mapping method can be archived in a custom library for targeted peptide monitoring. The target peptides can be sent directly to a library from the peptide mapping results table. The steps are as follows: (A) identify peptides of interest and (B) select “Send to Library” menu option to create a new library. The example shows creation of “mAb Product Quality Attributes” library. The library created contains (C) detection results, MS and fragment ions spectra. A library can also be manually built by entering the MW, charge, RT, and fragment ion information.

Step 3. Potential CQA monitoring with accurate mass screening method

The application note further illustrates the use of UNIFI Accurate Mass Screening data processing method for routine peptide monitoring. With generic processing parameters, intensity thresholding for peptide attributes limit check criteria and reporting; the screening method has made routine peptide monitoring streamlined for improved robustness and productivity. In this case study, the 28-target peptide attributes selected for monitoring were imported from the custom CQA library, as given in Step 2 (Figure 4A). Each library item provides peptides’ neutral mass and retention time information for accurate peptide detection. In addition, this targeted method is equipped with a relative abundance measurement tool to calculate relative %modification levels of CQAs using single or multiple charge combined ion responses (Figure 4B). Customizable limit settings for %modifications were applied when visualizing the levels of oxidized peptides for easy identification of samples surpassing the standard oxidation levels. The example in Figure 4C demonstrates HC:T21 DTMLISR native and oxidized peptides after limit check using an arbitrary number for the threshold to demonstrate feature capability. Both oxidative stress conditions shown here trigged the limits for modified HC:T21 peptides and were flagged in yellow and red reflecting the preset warning (at 10%) and error limits (at 50%) respectively. The information gathered in each attribute monitoring step can be documented through the automated reporting feature and used in process development of therapeutic proteins.

	Component name	Label	Expected neutral mass (Da)	Expected RT (m...	Internal standard?	Description
1	DIQMTQSPSTLSASVGDR	DIQMTQSPSTLSASVGDR	1891.8946	22.55	☐	1:T1+H$^+$ - NIST mAb
2	DIQMTQSPSTLSASVGDR Oxidation M	DIQMTQSPSTLSASVGDR	1907.8895	18.74	☐	1:T1&:Oxidation M [4]+H$^+$ - NIST mAb
3	DMIFNFYFDVWGQGTTVTVSSASTK	DMIFNFYFDVWGQGTTVTVSSASTK	2800.3054	44.98	☐	2:T12+H$^+$ - NIST mAb
4	DMIFNFYFDVWGQGTTVTVSSASTK Oxidation M	DMIFNFYFDVWGQGTTVTVSSASTK	2816.3003	42.32	☐	2:T12&:Oxidation M [2]+H$^+$ - NIST mAb
5	DMIFNFYFDVWGQGTTVTVSSASTK Oxidation M_1	DMIFNFYFDVWGQGTTVTVSSASTK	2816.3003	44.99	☐	2:T12&:Oxidation M [2]+H$^+$ - NIST mAb
6	DTLMISR	DTLMISR	834.4269	15.67	☐	2:T21+H$^+$ - NIST mAb
7	DTLMISR Oxidation M	DTLMISR	850.4219	12.92	☐	2:T21&:Oxidation M [4]+H$^+$ - NIST mAb

Figure 4. (A) Targeted peptide library can be imported into another analysis method, such as the accurate mass screening processing method. Each entry contains peptide sequence, modifications, neutral mass, and retention time information for targeted monitoring. (B) The screening method uses XIC for relative quantitation. The example shows the BPI and XICs of modified and unmodified peptides. (C) The summary plots present the %relative abundance of each modified (top) and native (bottom) of HC:T21 peptide. Data from replicate injections are displayed here. The limits applied to HC:T21 oxidized peptide flagged the samples that exceed the set threshold.

Comparable performance in both MS-only and full scan with fragmentation modes

Most peptide monitoring assays use MS only acquisition to screen samples for a list of known target peptides to achieve robust quantification. However, the DIA acquisition with fragmentation spectra can contribute to identification and sequence verification of differentially expressed peaks in mAb samples. The ability to maintain consistent %modification measurements in both MS and MS with fragmentation methods is an important feature that demonstrates data integrity of the platform. In here, the data acquired for mAb control samples using both acquisition methods indicate comparable %relative modifications for the given peptide attributes. Therefore, both methods can be used for routine peptide monitoring (Figure 5) without compromising MS-based %modification levels.

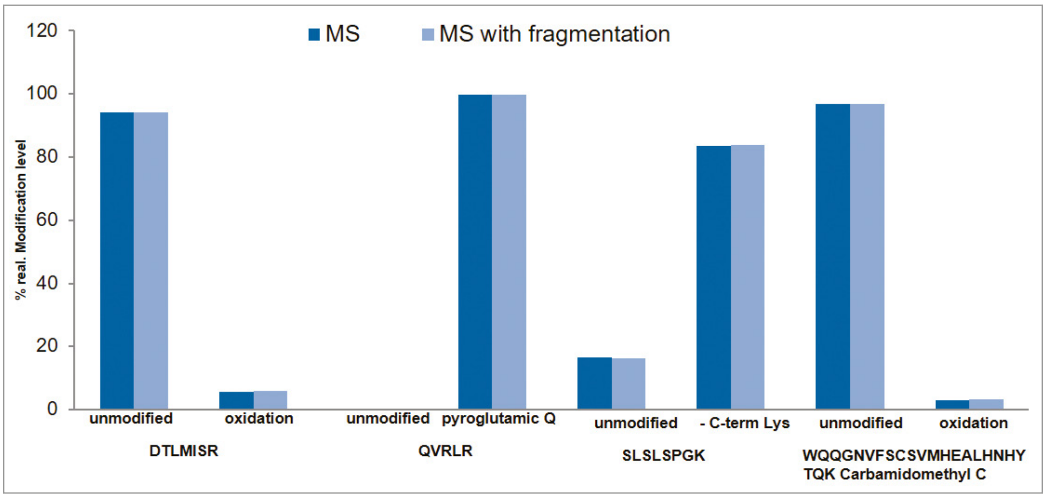


Figure 5. Relative abundance detected for a selected peptide in both MS-only and MS with fragmentation modes. The data shows comparable results in both acquisition modes.

CONCLUSIONS

The BioAccord System controlled by compliant-ready UNIFI Software is purposefully designed with integrated methods for routine therapeutic protein analysis. It can perform routine peptide characterization and accurate mass screening for CQA monitoring. The seamless integration of the two peptide analyses through the custom library feature is an effective way of transferring knowledge from characterization to monitoring that can be deployed across different analytical laboratory settings.

Further, the SmartMS System with intuitive informatics control (with promoted tune parameters, automated instrument health status monitoring, and calibration) provides a robust MS system suitable for users with any skill level.[5]

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5. Ranbaduge, N.; Shion, H.; Yu, Y.Q., Routine Peptide Mapping Analysis Using the BioAccord System. 720006466EN. Application Note. Waters Corporation. January 2019.

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[TECHNOLOGY BRIEF]

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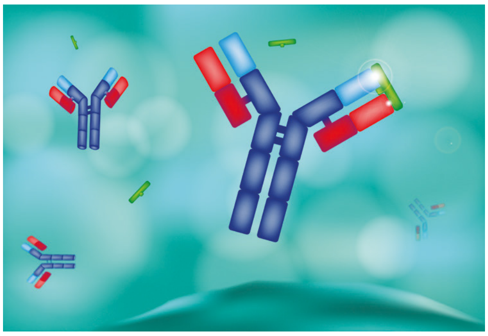
A Platform Method for the Molecular Mass Analysis of the Light Chains and Heavy Chains of Monoclonal Antibodies using the BioAccord System

Henry Shion, Ying Qing Yu, and Weibin Chen
Waters Corporation, Milford, MA, USA



A platform solution for rapid mass analysis of monoclonal antibodies light chain and heavy chain subunits using on-line denaturing SEC-MS.

GOAL

To demonstrate fast and routine mass analysis of reduced monoclonal antibodies based on a denaturing SEC-MS method using the Waters™ BioAccord™ System.

BACKGROUND

Therapeutic monoclonal antibodies (mAbs) still account for the largest percentage of biotherapeutics under development in the biopharmaceutical industry. Mass analysis of mAbs at the intact and subunit level is routinely conducted throughout the product development life cycle, serving to confirm sequence integrity, monitor product variations, and establish product identity. A simple and generic platform method for the mass measurement of mAbs with diverse physicochemical properties is highly desirable for improving the efficiency of analyses in the biopharmaceutical industry, particularly in the discovery and early development stages, where higher throughput screening of diverse mAb constructs is most common.

Size exclusion chromatography (SEC) is an isocratic protein separation mode, with selectivity predominantly based on the average stokes radius of the protein which correlates with the log molecular weight (MW) of the protein. Compared to reversed-phase (RP) based chromatography methods, SEC is inherently more generic as analyte recovery is not dependent on higher column temperature, and product peaks typically elute at predictable positions independent of the molecule's isoelectric point (pI) and hydrophobicity. Taking advantage of size-based separation capabilities, we developed a denaturing SEC method, employing mobile phases containing 30%

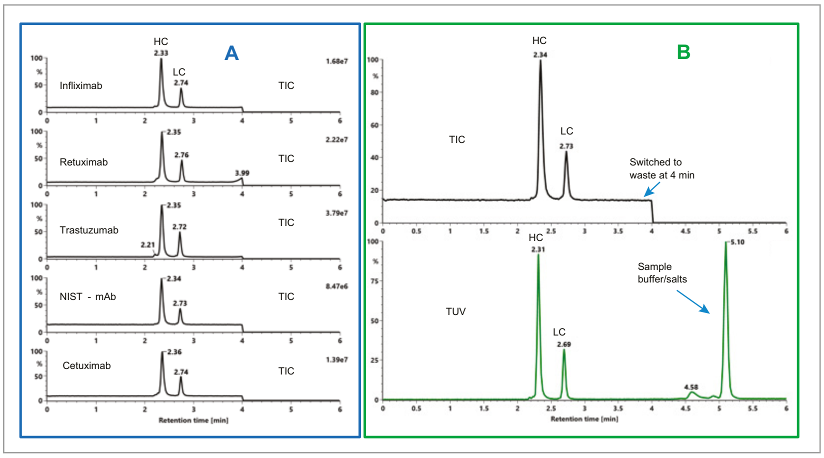
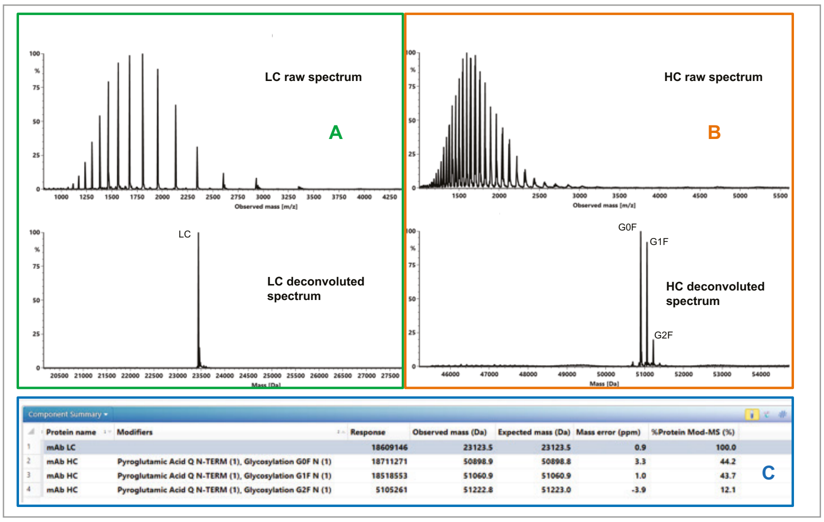


Figure 1. A. Total Ion Chromatograms (TIC) of multiple reduced mAbs subunits (heavy chains and light chains) from denatured SEC-MS analysis. B. TIC and TUV (280 nm) chromatograms of reduced NIST mAb subunits.



	Protein name	Modifiers	Response	Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	%Protein Mod-MS (%)
1	mAb LC		18609146	23123.5	23123.5	0.9	100.0
2	mAb HC	Pyroglutamic Acid Q N-TERM (1), Glycosylation G0F N (1)	18711271	50898.9	50898.8	3.3	44.2
3	mAb HC	Pyroglutamic Acid Q N-TERM (1), Glycosylation G1F N (1)	18518553	51060.9	51060.9	1.0	43.7
4	mAb HC	Pyroglutamic Acid Q N-TERM (1), Glycosylation G2F N (1)	5105261	51222.8	51223.0	-3.9	12.1

Figure 2. NIST mAb LC (A) and HC (B) summed raw spectra and deconvoluted spectra are shown from the denatured SEC-MS analysis. Good mass accuracies are observed for the automatically identified major LC and HC peaks as displayed in the components summary table (C).

acetonitrile, 0.1% trifluoroacetic acid (TFA), and 0.1% formic acid (FA) that achieved separation of reduced antibody light chain (LC) and heavy chain (HC) subunits. We transferred the SEC separation to the BioAccord System to obtain both UV and mass data for the reduced fragments. This method offers a robust workflow for mAb analysis, one highly tolerant of many buffers and formulants, and amenable to deployment as a platform analysis method.

THE SOLUTION

The employment of an MS-friendly mobile phase (30% acetonitrile, 0.1% TFA, and 0.1% FA) facilitates the direct coupling of SEC to inline UV and ESI-MS detection. The SEC separation on an ACQUITY™ UPLC™ BEH SEC 200Å Column (1.7 µm, 4.6 mm x 150 mm, p/n 186005225) was achieved with an ACQUITY UPLC I-Class PLUS System at a flow rate of 0.40 mL/min. The robust BEH hybrid particle technology ensures chemical and physical stability of the column under these aggressive denaturing SEC conditions. The flow passed through an ACQUITY UPLC TUV Detector (280 nm) inline with the ACQUITY RDa™ MS Detector. The separation achieved using sub-2-µm UPLC BEH SEC particle in the low-dispersion ACQUITY UPLC I-Class PLUS System enabled 6-minute SEC runs that completely resolved glycosylated heavy chain (HC) from the later eluting light chain (LC).

Figure 1A shows the Total Ion Chromatograms (TIC) of 5 reduced mAbs subunits (heavy chains and light chains) from denatured SEC-MS analysis. All the light chain and heavy chain peaks are baseline separated and eluted with the same retention times within the 6 minutes total runtime method. Figure 1B compares the TIC and TUV (280 nm) chromtagrams of the reduced NIST mAb subunits, which shows the late eluted salt species were switched to the waste to minimize potential contamination to the ACQUITY RDa MS Detector.

Figure 2 A and B shows the NIST mAb LC (A) and HC (B) summed raw spectra and deconvoluted spectra respectively. Good mass accuracies are observed from the automatically identified major LC and HC peaks as displayed in the components summary table in Figure 2C.

SUMMARY

A denaturing SEC/UV/MS method has been developed that enables the inline combination of size exclusion chromatography with optical and MS detection, for quantitative and qualitative mAb analysis at the subunit level. This well-established and robust separation technique could prove useful for scientists needing a higher throughput analytical solution to meet the demand to analyze a diverse array of mAbs without customized method development. UPLC-based sub-2-µm SEC separations enabled rapid antibody light and heavy chain separations under molecule and MS-friendly conditions. This separation based on size is complementary to RP chromatography, and when coupled with MS, offers a powerful, routine and platform solution for antibody analysis.

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[APPLICATION NOTE]

Increasing Productivity and Confidence for N-linked Glycan Analysis of Biosimilars Using the BioAccord System

Ximo Zhang, Corey Reed, Henry Shion, William Alley, Robert Birdsall, and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A compliance ready all-encompassing LC-FLR-MS solution for comprehensive N-linked glycan analysis
- Automated workflow from sample preparation to data reporting for improved productivity in biosimilar development
- Robust and specific platform solution that improves the confidence of released glycan analysis

WATERS SOLUTIONS

BioAccord™ System for Glycans

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

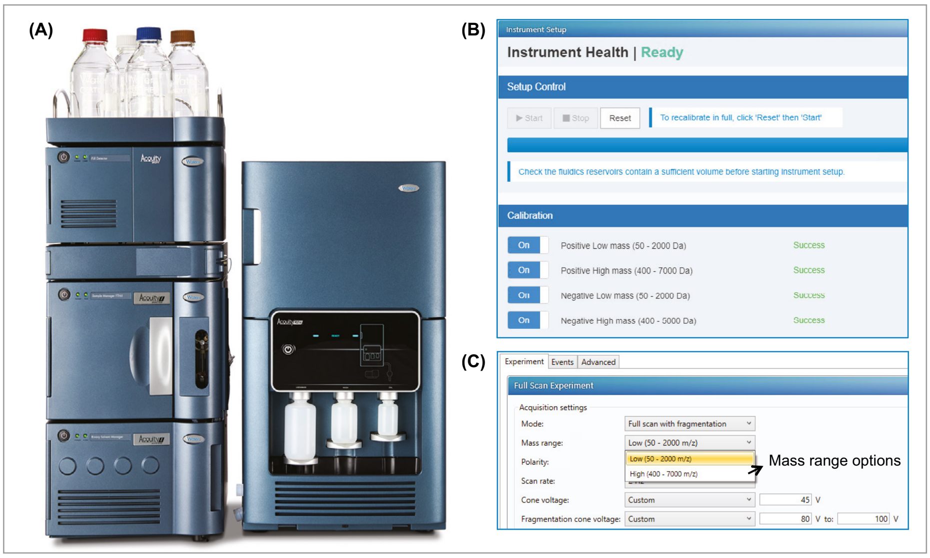
KEYWORDS

Released glycans, *Rapi*Fluor-MS, BioAccord System, RDa™ Detector, biosimilar

INTRODUCTION

Monoclonal antibody (mAb) based therapeutics have been well established in the effective treatment of various diseases due to their high efficacy and specificity. With the upcoming patent expiration of several commercially available mAb-based drugs in the next few years, growing efforts are being devoted to developing biosimilars as less expensive alternatives of innovator mAbs. To reduce the need for and size of expensive clinical trials and need to expedite the drug commercialization process, manufacturers must demonstrate high similarity in analytical properties of the biosimilar and its reference product through comprehensive analyses.[1] Due to the impact on drug efficacy and safety, glycosylation is one of the critical quality attributes of mAb based therapeutics that needs to be well characterized for similarity assessment and quality assurance of biosimilars.[2] With the high complexity of glycosylation profile of mAbs, orthogonal technologies such as mass spectrometry (MS) are commonly used to complement conventional LC-fluorescence (FLR) based methods for increased specificity. However, high resolution MS often requires experienced scientists for instrument operation, data processing and interpretation, which can be time and resource consuming. To this end, scalable technologies and methods that can add confidence and are easy to deploy for comprehensive glycan analysis in biosimilar development are highly desirable.

The SmartMS™-enabled BioAccord System is an easy-to-use LC-MS platform solution that was purposefully designed for comprehensive analysis of biotherapeutic drug products with built-in analytical workflows for specific analysis such as N-Glycan identification and profiling. Within the BioAccord System, highly robust chromatographic separation and accurate mass information can be obtained using the ACQUITY™ UPLC™ I-Class PLUS System and the ACQUITY RDa Detector controlled by UNIFI™ Scientific Information Software (Figure 1A). The ACQUITY RDa Detector is a compact bench top time-of-flight mass detector with built-in self-calibration function and straightforward instrument method setup (Figure 1B and 1C), which substantially reduces the complexity of operating MS instruments. Together, high quality LC-FLR and LC-MS data can be acquired and transformed into meaningful results for released glycan analysis using an all-encompassing workflow, reducing the cost and time for biosimilar development without compromising product quality.



Instrument Setup

Instrument Health | Ready

Setup Control

To recalibrate in full, click 'Reset' then 'Start'

Check the fluidics reservoirs contain a sufficient volume before starting instrument setup.

Calibration

On	Positive Low mass (50 - 2000 Da)	Success
On	Positive High mass (400 - 7000 Da)	Success
On	Negative Low mass (50 - 2000 Da)	Success
On	Negative High mass (400 - 5000 Da)	Success

Full Scan Experiment

Acquisition settings

Mode: Full scan with fragmentation

Mass range: Low (50 - 2000 m/z)

Low (50 - 2000 m/z)

High (400 - 7000 m/z)

Cone voltage: Custom 45 V

Fragmentation cone voltage: Custom 80 V to: 100 V

Figure 1. The BioAccord System. (A) Integrated instrument configuration for streamlined released glycan analysis. (B) ACQUITY RDa Detector setup page showing SmartMS™-enabled auto-calibration function for efficient instrument operation. (C) Instrument method page for MS data acquisition. For released glycan analysis, the low mass range option (50–2000 m/z) was selected.

The objective of this work is to demonstrate that the BioAccord System can increase confidence and productivity in released glycan analysis for the development of biosimilars. As an example of assessing the similarity of glycosylation, released glycans from innovator and biosimilar infliximab were analyzed using the BioAccord System.

EXPERIMENTAL

Chemical and reagents

Innovator and biosimilar infliximab samples were donated by external collaborators. LC-MS grade water and acetonitrile were purchased from Honeywell and used as received. Concentrated ammonium formate (p/n 186007081) was used as the additive to prepare mobile phase.

Sample preparation

Two infliximab samples from innovator (Remicade™) and one sample from Biosimilar (Inflectra™) were diluted with water to a final concentration of 1.5 µg/µL. N-glycans from infliximab were released from 15 µg of diluted mAb samples and labeled using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176004082)[3] via an Andrew Alliance automated sample preparation platform.[4] An amount of 2.5 pmol released glycan sample was injected for each analysis.

System setting

LC conditions

LC system:	ACQUITY UPLC I-Class PLUS
Detectors:	ACQUITY FLR Detector, λex=265 nm, λem=425 nm ACQUITY RDa MS Detector
LC column:	ACQUITY Glycan BEH Amide 1.7 µm, 130 Å, 2.1 × 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample vial:	12 × 32 mm glass vial Total Recovery (p/n 600000750cv)
Mobile phase A:	Water with 50 mM Ammonium formate, pH = 4.4
Mobile phase B:	Acetonitrile

Gradient table:

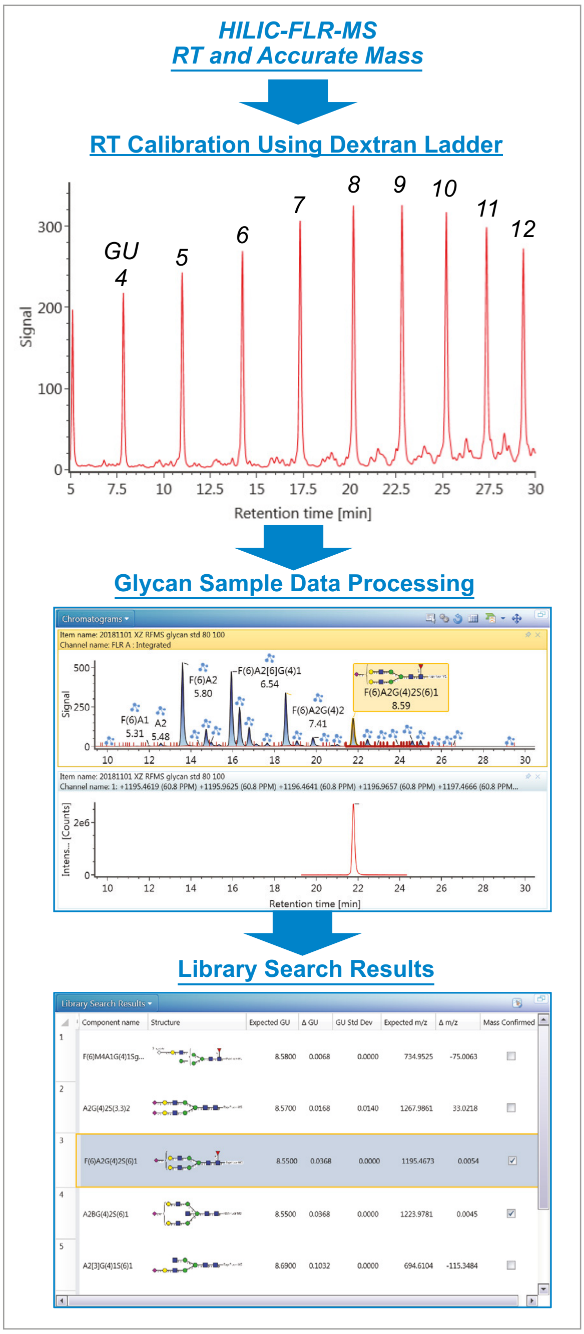
Time (min)	Flow rate (mL/min)	%A	%B
Initial	0.400	25.0	75.0
35.00	0.400	46.0	54.0
36.50	0.200	80.0	0.0
39.50	0.200	80.0	0.0
43.10	0.200	25.0	75.0
47.60	0.400	25.0	75.0
55.00	0.400	25.0	75.0

ACQUITY RDa Detector settings

Mass range:	50–2000 *m/z*	Desolvation temp.:	300 °C
Mode:	ESI+	Capillary voltage:	1.5 kV
Collection mode:	Continuum	Lock mass:	Leu-enkephalin at 50 fmol/µL in 50/50 water/acetonitrile with 0.1% formic acid
Sample rate:	2 Hz		
Cone voltage:	45 V for full scan 80–100 V for fragmentation	Informatics:	Glycan Application Solution within UNIFI 1.9.4

RESULTS AND DISCUSSION

As an integrated solution, the established glycan analysis workflow supported by the BioAccord System can be used to streamline the identification and comparison of glycosylation on biosimilars.[5] To demonstrate this workflow, *Rapi*Fluor-MS labeled N-linked glycans released from innovator and biosimilar infliximab were analyzed via HILIC separation followed by inline FLR and MS detection using the BioAccord System. With the self-calibration and self-tuning function of the RDa mass detector, high quality MS data were obtained in an efficient manner. The “Glycan FLR with MS confirmation” workflow within UNIFI was used for data interpretation, allowing automated data processing using the retention time and accurate mass information of separated glycans.[6] As shown in Figure 2, retention times of glycans were calibrated against a dextran ladder standard and converted to Glucose Units (GU) values, and then used along with accurate mass information to conduct a Glycan Scientific Library search within the UNIFI software for peak identification. With the use of GU values for library search, high confidence of peak assignment is ensured by minimizing the potential variation from retention times across analyses.



Item name: 20181101 XZ RFMS glycan std 80 100
Channel name: FLR A : Integrated

Item name: 20181101 XZ RFMS glycan std 80 100
Channel name: 1: +1195.4619 (60.8 PPM) +1195.9625 (60.8 PPM) +1196.4641 (60.8 PPM) +1196.9657 (60.8 PPM) +1197.4666 (60.8 PPM...

	Component name	Structure	Expected GU	Δ GU	GU Std Dev	Expected m/z	Δ m/z	Mass Confirmed
1	F(6)M4A1G(4)1Sg...		8.5800	0.0068	0.0000	734.9525	-75.0063	☐
2	A2G(4)2S(3,3)2		8.5700	0.0168	0.0140	1267.9861	33.0218	☐
3	F(6)A2G(4)2S(6)1		8.5500	0.0368	0.0000	1195.4673	0.0054	☑
4	A2BG(4)2S(6)1		8.5500	0.0368	0.0000	1223.9781	0.0045	☑
5	A2[3]G(4)1S(6)1		8.6900	0.1032	0.0000	694.6104	-115.3484	☐

Figure 2. The integrated “Glycan FLR with MS Confirmation” workflow for automated released glycan analysis within the UNIFI Informatics platform.

Figure 3 shows the processed FLR trace of innovator infliximab with each peak automatically assigned and annotated with its glycan name and associated structural information. As a result of the automated data processing, a total of 26 N-linked glycans were identified for both innovator infliximab samples and 27 for the biosimilar infliximab within a 10-ppm mass tolerance, demonstrating the high sensitivity and accuracy afforded by the ACQUITY RDa Detector. For the glycans identified, the similarities of glycan profiles between the innovator and biosimilar mAbs can be automatically assessed within the workflow.

During the development of biosimilar products, critical glycan structures often rank as high-risk quality attributes and need equivalence testing (Tier 1) for analytical similarity assessment according to the Three-tier approach recommended by FDA.[7] To this end, a straightforward approach to quantitatively compare glycan profiles is of particular importance to facilitate the biosimilar mAb development. Within the BioAccord System workflow, a direct comparison can be made in both qualitative and quantitative mode. Figure 4A shows the overlaid FLR chromatograms for the two innovator infliximab samples (black and red trace) and one biosimilar sample (blue trace), suggesting high similarity of glycan profiles between the two innovator mAb samples. Meanwhile, differences in relative abundance were observed for multiple sialylated glycan species between innovator and biosimilar mAb, as shown in the zoom-in chromatogram in Figure 4A (the inset figure). To confirm the differences, a Summary Plot can be accessed from the clickable workflow steps (Figure 4B) to quantitatively compare the FLR response or relative abundance of selected glycan species. As shown on the right side of Figure 4B, the Summary Plot for %Amount confirmed a consistent relative abundance of FA2G1Sg1 (F= Fucose, A2= biantennary, G=Galactose, Sg= NeuGc) in the two samples of the innovator mAb, and an elevated abundance in the biosimilar mAb. Collectively, the above results demonstrated the BioAccord System's ability to efficiently transform LC-MS data into meaningful results for biosimilarity assessment.

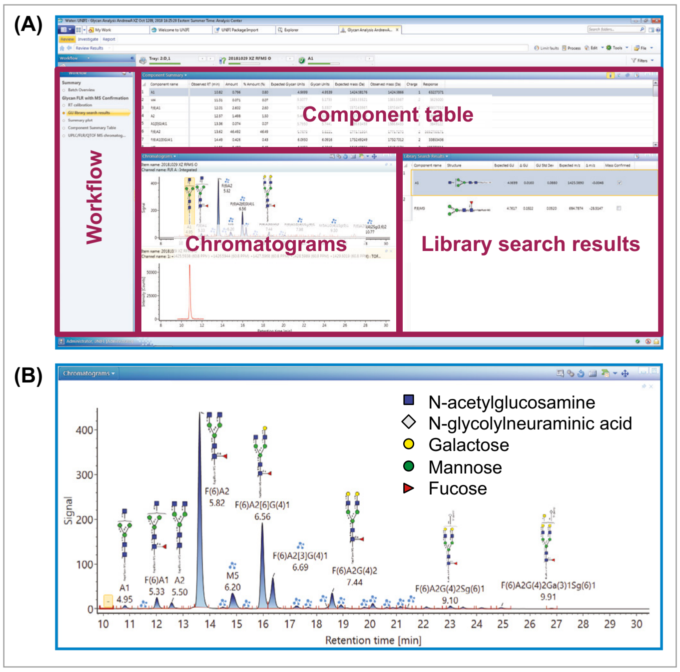


Figure 3. Review of the processed results. (A) The Review tab showing the clickable workflow steps, processed chromatograms, and library search results for identified peaks. (B) Processed FLR trance from the Review tab showing identified peaks annotated with glycan name, GU value, and associated structure information. To avoid over crowded structure display, detailed glycan structures can be viewed by simply mousing over the IBM connection icon. User has the option to choose between CFG or Oxford structure nomenclature to display (CFG is used for figures in this Application Note).

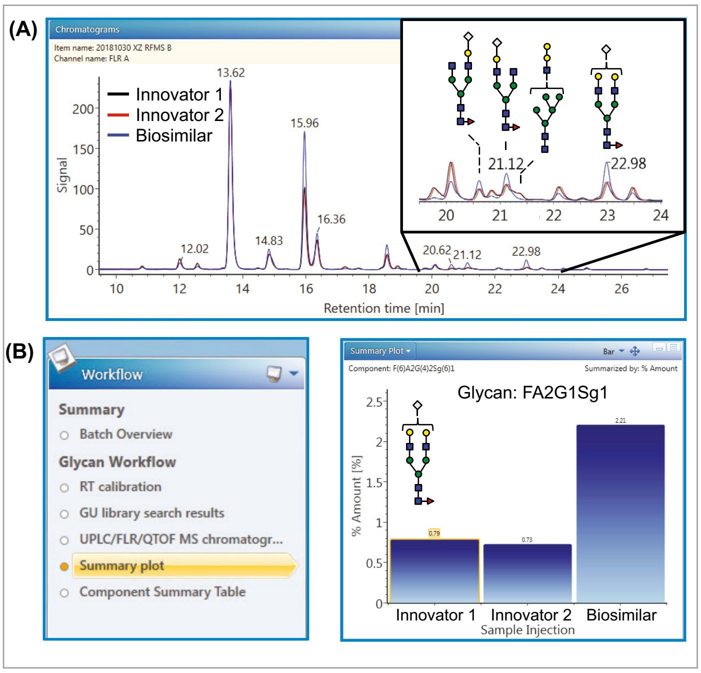


Figure 4. Comparison of glycan profiles between innovator infliximab samples and biosimilar sample. (A) Overlaid FLR chromatograms. Zoom-in chromatogram shows the region of low abundant glycan species. (B) An example of the Summary Plot as part of the clickable workflow steps showing the elevated abundance of a glycan, FA2G1Sg1 (Sg stands for n-glycolylneuraminic acid), in the biosimilar mAb.

When performing released glycan analysis of biosimilar mAbs, it is quite common to come across glycans with challenging MS spectra for correct assignment, such as isobaric glycans, which might lead to inconsistency in glycan analysis results. High collisional energy fragmentation is the common MS based method to help differentiating isobaric glycans with similar structures. The ACQUITY RDa Detector can acquire low collisional energy and high collisional energy MS fragmentation spectra in an alternative scan mode without pre-selecting the precursor ions, which provides additional diagnostic fragment ions to improve the confidence in structure assignment. (Note: The low energy MS channel is used for MW confirmation using the Scientific Glycan GU library by default). Figure 5 shows an example of how MS fragmentation data can be used to aid in the identification of a critical pair of glycans. As shown in Figure 5A, glycan FA2G2 eluted very close to its immunogenic isomer, FA2G1Ga1 (G1Ga1 = Gal-alpha (1,3)-Gal) resulting in highly similar GU values. The simultaneously acquired MS fragmentation data offers an opportunity to structurally differentiate the isobaric glycans. As shown in Figure 5B, high quality MS full scan and fragmentation data were obtained for both peaks, where a diagnostic ion (*m/z* 528) for α-1,3 Galactose was observed as the most dominant fragment ion in the MS fragmentation spectra of the shoulder peak confirming its identity as FA2G1Ga1.[3] To this end, the BioAccord System offers the flexibility to interrogate results to improve the robustness and confidence for released glycan analysis in biosimilar development.

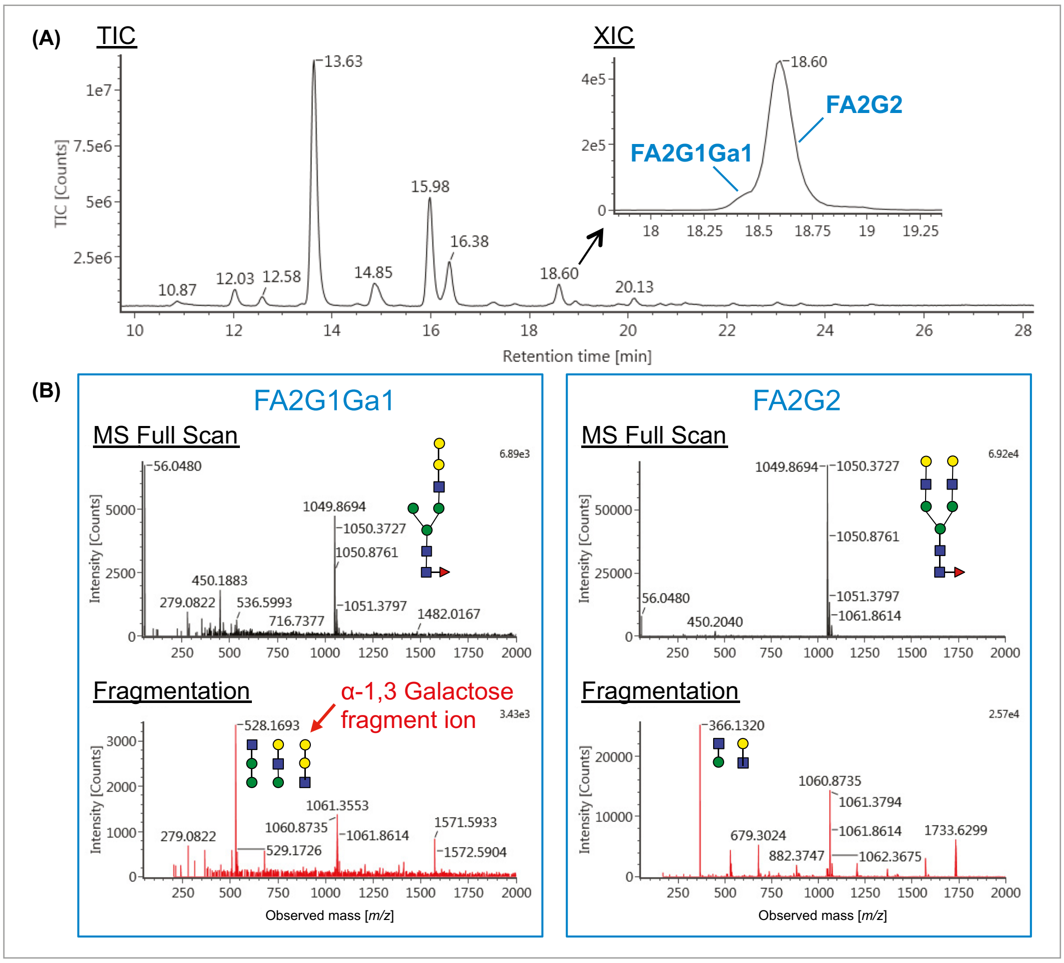
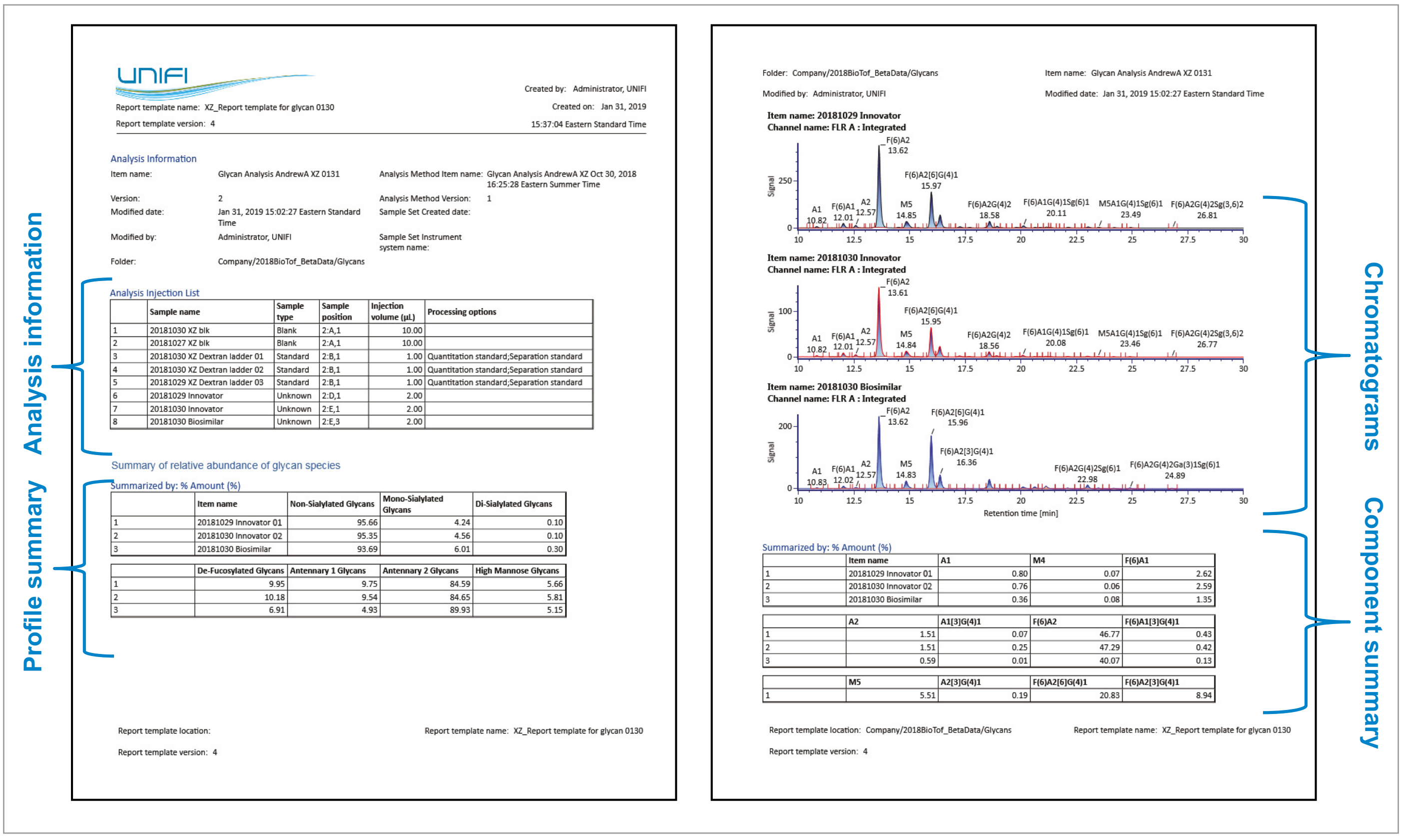


Figure 5. Additional details under Investigate tab for manual data interrogation. (A) MS (TIC) chromatogram of released glycans from innovator infliximab. Zoom-in chromatogram shows the XIC of the two isobaric glycans, FA2G1Ga1 and FA2G2. (B) MS full scan and fragmentation data of FA2G1Ga1 and FA2G2, showing a diagnostic ion (m/z 528) of α-1,3 Galactose.

To further accelerate the glycan analysis, UNIFI Software can automatically generate analysis reports to simplify the review, sharing, and filing of data. To demonstrate the reporting function of UNIFI Software, a report was created using a customizable template to summarize the glycan analysis results. As shown in Figure 6, the report was formatted to include Analysis Information, Chromatograms, and Summary Tables, showing the direct comparison for relative abundance of identified N-glycans across samples. The comparison based on different groups of glycans was also reported. To meet requirements in different laboratories, the report can be customized to include other desired information for simplified review of analysis results.



Report template name: XZ_Report template for glycan 0130
Report template version: 4

Created by: Administrator, UNIFI
Created on: Jan 31, 2019
15:37:04 Eastern Standard Time

Analysis Information

Item name: Glycan Analysis AndrewA XZ 0131
Version: 2
Modified date: Jan 31, 2019 15:02:27 Eastern Standard Time
Modified by: Administrator, UNIFI
Folder: Company/2018BioTof_BetaData/Glycans
Analysis Method Item name: Glycan Analysis AndrewA XZ Oct 30, 2018 16:25:28 Eastern Summer Time
Analysis Method Version: 1
Sample Set Created date:
Sample Set Instrument system name:

Analysis Injection List

	Sample name	Sample type	Sample position	Injection volume (µL)	Processing options
1	20181030 XZ blk	Blank	2:A,1	10.00	
2	20181027 XZ blk	Blank	2:A,1	10.00	
3	20181030 XZ Dextran ladder 01	Standard	2:B,1	1.00	Quantitation standard;Separation standard
4	20181030 XZ Dextran ladder 02	Standard	2:B,1	1.00	Quantitation standard;Separation standard
5	20181029 XZ Dextran ladder 03	Standard	2:B,1	1.00	Quantitation standard;Separation standard
6	20181029 Innovator	Unknown	2:D,1	2.00	
7	20181030 Innovator	Unknown	2:E,1	2.00	
8	20181030 Biosimilar	Unknown	2:E,3	2.00	

Summary of relative abundance of glycan species

Summarized by: % Amount (%)

	Item name	Non-Sialylated Glycans	Mono-Sialylated Glycans	Di-Sialylated Glycans
1	20181029 Innovator 01	95.66	4.24	0.10
2	20181030 Innovator 02	95.35	4.56	0.10
3	20181030 Biosimilar	93.69	6.01	0.30

	De-Fucosylated Glycans	Antennary 1 Glycans	Antennary 2 Glycans	High Mannose Glycans
1	9.95	9.75	84.59	5.66
2	10.18	9.54	84.65	5.81
3	6.91	4.93	89.93	5.15

Report template location:
Report template name: XZ_Report template for glycan 0130
Report template version: 4

Folder: Company/2018BioTof_BetaData/Glycans
Item name: Glycan Analysis AndrewA XZ 0131
Modified by: Administrator, UNIFI
Modified date: Jan 31, 2019 15:02:27 Eastern Standard Time

Item name: 20181029 Innovator
Channel name: FLR A : Integrated

Item name: 20181030 Innovator
Channel name: FLR A : Integrated

Item name: 20181030 Biosimilar
Channel name: FLR A : Integrated

Summarized by: % Amount (%)

	Item name	A1	M4	F(6)A1
1	20181029 Innovator 01	0.80	0.07	2.62
2	20181030 Innovator 02	0.76	0.06	2.59
3	20181030 Biosimilar	0.36	0.08	1.35

	A2	A1[3]G(4)1	F(6)A2	F(6)A1[3]G(4)1
1	1.51	0.07	46.77	0.43
2	1.51	0.25	47.29	0.42
3	0.59	0.01	40.07	0.13

	M5	A2[3]G(4)1	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1
1	5.51	0.19	20.83	8.94

Report template location: Company/2018BioTof_BetaData/Glycans
Report template name: XZ_Report template for glycan 0130
Report template version: 4

Figure 6. Report of analysis results (selected pages). The customizable report shows Sample List, Chromatograms, and Summary Table for easily reviewing the differences across samples.

CONCLUSIONS

Through this work, an efficient workflow has been demonstrated for comprehensive analysis of fluorescent labeled released glycans using the BioAccord System. Within the integrated workflow, highly robust separation and accurate mass measurement of N-linked glycans were obtained and transformed automatically to analysis results, allowing for fast determination of differences in identity and abundance of glycans. In summary, the BioAccord System simplifies the workflow of biosimilarity assessment and can be used to improve the productivity and confidence of released glycan analysis in biosimilar mAb development.

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Exploiting Rapi Fluor-MS Labeling to Monitor Diverse N-Glycan Structures via Fluorescence and Mass Detection (PN 720005353EN)

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