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APPLICATION SOLUTIONS FOR BIOPHARMACEUTICALS

A Focus on Protein Therapeutics

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Unlimited characterization. Routinely.

The growing biopharmaceutical pipeline

Major advances in protein characterization, biochemistry, and molecular biology continue to revolutionize the diagnosis and treatment of disease, as well as increase our understanding of fundamental life processes. Recombinant proteins and monoclonal antibodies represent the largest portion of biopharmaceuticals in development. A variety of challenging techniques are required to fully characterize biopharmaceutical products. Due to their size and complexity, they are inherently more complex to analyze than small molecule drugs. Laboratories need to ensure they can meet the significant analytical challenges for this class of therapeutics.

Increasing regulatory requirements

As the biopharmaceutical pipeline grows, regulators have increased their knowledge and understanding of the importance of analytical techniques. Robust characterization capabilities are also emerging as a critical requirement as approval pathways for biosimilars are defined. This increased focus has inevitably led to more stringent requirements for product approval and process changes. On the other hand, regulators have emphasized the potential for reduction in clinical trial costs by increased product knowledge through state-of-the-art analytics.

Significant business challenges

You need to accelerate development of your product candidates. Processes for existing products must be optimized to improve yield and decrease cycle time, thereby reducing costs and increasing asset utilization. Competitive products are threatening to take market share.

Keep research moving

Waters has decades of experience in supporting the analytical technology needs of pharmaceutical and life sciences organizations, from drug discovery and development to the development and formulation of biologics. We know what it takes to move efficiently through the research and development pipeline – from routine biochemical measurements to advanced conformational studies.

Waters solutions

Waters offers application-focused system solutions for protein characterization, from intact protein mass analysis, peptide mapping, and glycan analysis to higher order structure and amino acid analysis. Waters will help your laboratory meet these increasing analytical demands with solutions that deliver the accuracy, sensitivity, stability, reproducibility, and versatility required to ensure you can characterize and analyze biopharmaceuticals more efficiently.

With end-to-end solutions including chromatography, mass spectrometry, column and sample prep chemistries, data management software, and standards and reagents, our technologies continue to advance your laboratory's capabilities in biopharmaceutical analysis and characterization. We are committed to developing new solutions that will help you meet new and emerging business opportunities, as well as their accompanying scientific and regulatory challenges.

Look to Waters as a partner who delivers scientific expertise with flexible, superior service and a commitment to your success.

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INTACT PROTEIN MASS ANALYSIS

Intact protein mass analysis is a holistic technique yielding a set of deconvoluted masses with associated intensities that are typically used to confirm protein identity and profile product-related variants. Typically applied alongside orthogonal approaches, such as peptide mapping and released glycan analysis, intact mass analysis can be used for the initial characterization of a biotherapeutic, for subsequent monitoring of product quality and consistency throughout primary development, and for establishing comparability during subsequent post-approval process improvement studies.

FROM CONFIRMATION TO COMPARABILITY

The experimental goals of intact mass analysis vary from simple mass confirmation to executing full comparability exercises requiring more rigorous quantification of each detected variant. Several experimental elements are common to all intact mass analyses:

- The need to efficiently remove buffers, salts, and other sample components that would interfere with mass analysis or reduce the quality of the resulting data.
- The need to present narrow concentrated protein peaks to a mass detector for optimum sensitivity and component resolution.
- The desire to efficiently utilize the analysis system to achieve greater sample throughput or reduced sample analysis times.
- The desire to reduce the burdens of routine and repetitive data analysis.

COMPREHENSIVELY DESIGNED FOR PERFORMANCE

Waters delivers a comprehensive system solution for intact protein mass analysis with performance that leverages our extensive expertise in protein chromatography, mass spectrometry, and informatics.

- UPLC® Technology provides superior resolution of variants and greater sensitivity for more in-depth protein characterization, while offering robust, integrated system operation.
- Intact mass analysis can be combined with a versatile array of detection options, from UV/Vis detection to high-resolution mass spectrometry that delivers structural information on biomolecules – all optimized for UPLC.
- Analysis of intact protein LC/MS data can be productivity-limiting for many laboratories. Waters has developed powerful bioinformatics tools to automate raw data processing, annotate intact protein LC/MS data sets, and report results.

PROTEIN SEPARATION TECHNOLOGY (PrST) COLUMNS

Higher resolution separations are often needed for biotherapeutic analysis to address higher sample complexity or the need to resolve closely-related product variants. The core ethylene-bridged hybrid (BEH) particle used in PrST Technology Columns performs exceptionally well for high-resolution separations of immunoglobulins and other recombinant biotherapeutics.

Waters provides a full suite of application-specific columns for ion exchange, size exclusion, reversed phase, affinity, and hydrophobic-interaction chromatography, as well as sample preparation approaches such as on-line protein desalting. Every batch of PrST Columns is QC tested with a diverse protein mixture to assure researchers obtain consistent batch-to-batch column performance. For a full listing of PrST chromatography columns and products, go to **www.waters.com/proteins** or for the Waters bioseparations products catalog, **www.waters.com/biosep**.

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Comprehensive and Routine Characterization of Proteins and Peptides by LC/MS and LC/MS^E

Asish Chakraborty, St John Skilton, Weibin Chen, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The comprehensive characterization of biopolymers as drug candidates is a requirement for safety and regulatory agencies. Challenges include the high molecular weight of biopolymers and the heterogeneous nature of protein drugs, which require extensive characterization to achieve regulatory approval. Comparability during manufacturing changes must be demonstrated, or to provide intellectual property (IP) protection against biosimilars.

This application note describes how different analyses can be performed to provide comprehensive information more quickly to meet these business objectives. All of these analyses can be done by non-specialists on the same platform.

The combination of liquid chromatography and electrospray mass spectrometry (LC/MS) provides enabling technology for well-characterized and comparable biotherapeutics. LC/MS analysis of proteins and peptides provides high levels of detail to aid characterization. However, the ability to routinely generate and interpret LC/MS data in a timely manner has been challenging in the past because involvement of an expert was required. Additionally, a major hindrance has been, until now, the lack of automated tools to complete the analysis. In order to increase laboratory productivity, high-performance mass spectrometers need to be made more accessible.

The Waters Xevo™ QTof MS System is a benchtop instrument designed to provide organizations with easy access to the most sensitive, high-performance, accurate mass MS, MS^E, and MS/MS analysis available. The system incorporates Waters' design philosophy of Engineered Simplicity™ and features tools for automated calibration and system monitoring, allowing organizations to obtain the very best MS performance in routine analyses.

This application note focuses on intact protein measurement and peptide mapping for biotherapeutics. We demonstrate a system solution that integrates ACQUITY UPLC® separations, application-specific column chemistries, Xevo QTof MS mass detection, and industry-leading biopharmaceutical informatics to deliver improvements in laboratory productivity. Tasks that took two weeks to complete, such as peptide mapping, can now be accomplished in a day.

There are good reasons to determine the intact mass of a monoclonal antibody (mAb): in addition to providing an accurate mass of the protein, intact mass analysis provides an overall view of the heterogeneity of the protein, showing relative amounts of the various forms. Minimal sample preparation and chromatographic separation is required, so intact mass analysis provides results in minutes. Furthermore, generic methods can be used for rapid analysis of many different sample types.

In an organization where there is a large increase in the number of biopharmaceutical candidates advancing through the pipeline, such an approach provides results rapidly without having to request additional headcount.

For example, if there is a need to quickly confirm that the correct protein has been made, samples may be submitted to the analytical characterization group by biochemists or biologists who are not experts in mass spectrometry. An accurate molecular weight of the protein would ensure that the overall mass matches the expected mass. Without this confirmation, the wrong protein may be tested in several expensive bioassays, increasing time and costs for the organization.

METHODS

Sample preparation

Intact monoclonal antibody (IgG1)

A humanized IgG1 was received as a buffered solution (21.0 mg/mL). The solution was diluted to 0.5 mg/mL with 50 mM ammonium bicarbonate in preparation for intact mass analysis.

Reduced antibody

The antibody sample was reduced with DTT at 37 °C for 20 min using a published method.[1] The solution was diluted to 0.10 mg/mL (pH 3.0) with 2% (v/v) formic acid aqueous solution.

Protein digest (for peptide mapping)

RapiGest™ SF (0.05% in final solution) was added to the monoclonal antibody (mAb) stock solution, and the sample was heated at 60 °C for 30 minutes. The protein was then reduced with 10 mM DTT at 60 °C for 30 min, and alkylated with 13 mM of IAA in the dark for 45 minutes. Trypsin digestion was performed at 37 °C overnight (trypsin/protein ratio was 1:50). The digest was diluted to 0.015 mg/mL with 0.1% formic acid.

UPLC conditions

LC system: Waters ACQUITY UPLC® System

Columns:
- For intact IgG protein: MassPREP™ Micro desalting column 2.1 x 5 mm, 20 µm, 1000Å
- For separation of IgG heavy/light chains: ACQUITY UPLC C_4 BEH300 1.7 µm, 2.1 x 50 mm
- For peptide mapping: ACQUITY UPLC C_{18} BEH300 1.7 µm, 2.1 x 150 mm

Column temp.:
- Intact IgG protein: 80 °C
- Reduced monoclonal antibody: 80 °C
- Peptide mapping: 60 °C

Mobile phase A: 0.1% Formic acid (Water)
Mobile phase B: 0.1% Formic acid (ACN)
Flow rates: 0.2 mL/min

Gradients:
- Intact monoclonal antibody: 10-90% B in 1.5 min.
- Reduced monoclonal antibody: 25-35 %B over 15 min.
- Peptide mapping: 2%B-40%B in 90 min.

Mass spectrometry conditions

MS system: Waters Xevo QTof MS
Ionization mode: ESI positive
Capillary voltage: 3.0 kV
Cone voltage: 25 V (peptide) / 45 V (protein)
Desolvation temp.: 350 °C
Source temp.: 150 °C
Desolvation gas: 800 L/Hr
Acquisition range: m/z: 50 to 1990 (peptide) / 600 to 4500 (protein)

Informatics/data processing

BiopharmaLynx™ Application Manager, v. 1.2, for MassLynx Software

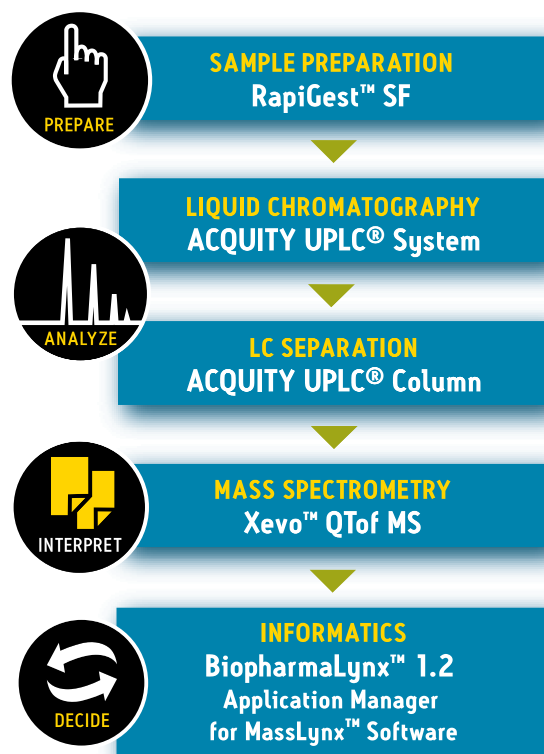

Figure 1. The general Waters LC/MS workflow logically steps from sample preparation to data analysis, with integrated instrumentation and software enabling the process to occur seamlessly.

RESULTS AND DISCUSSION

Analysis of humanized IgG was used as an example to demonstrate the general LC/MS workflow as well as Xevo QTof MS's performance. UPLC® Technology provides significant advantages for all of these analyses by avoiding the need for flow splitting and by providing increased sensitivity for MS detection

The therapeutic was analyzed in three ways:

1. As an intact antibody
2. In reduced form to show the masses of the light chain and heavy chain after minimal chromatographic separation
3. Enzymatic digestion followed by LC/MS with the annotation of each peptidic element of the antibody

Analysis 1: Intact mass analysis

Information on heterogeneity is provided by intact mass measurement. Incomplete characterization of a candidate protein could lead to a delay in achieving regulatory approval, costing the company millions. In the worst case, if a protein is approved and later found to be incompletely characterized because of the presence of an undesired form, the product may have to be removed from the market, costing the company millions of dollars and hurting its reputation.

For choosing the optimum cell line, determining the protein intact mass analysis is a powerful, high-throughput method used to show changes in the protein's heterogeneity with different cell lines, helping to reduce development and manufacturing costs. In QC functions, an intact mass analysis would give general confirmatory information.

Figure 2 shows mass spectra of an intact IgG from a traditional QTof mass spectrometer and the Xevo QTof MS, respectively. Both spectra show a charge envelope with a distribution of multiply-charged ion peaks. Although great similarity between the two spectra can be found, the Xevo QTof MS data show a six-fold sensitivity increase in comparison to traditional QTofs.

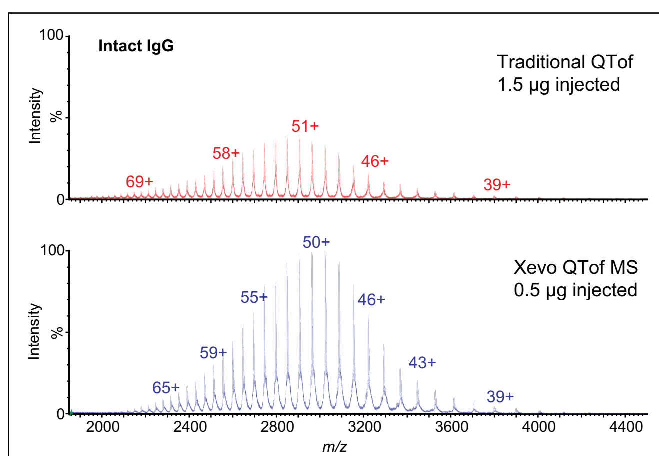

Figure 2. Improved sensitivity of Xevo QTof for intact mass analysis. For the intact IgG analysis, the Xevo QTof MS showed a six-fold improvement in sensitivity in comparison to tradition quadrupole time-of-flight MS.

In a manual analysis, a scientist typically transfers this data to a software package to deconvolute the multiply-charged states to a zero charge molecular mass or distribution for the intact protein. With BiopharmaLynx 1.2 Software, spectral deconvolution is performed automatically on the acquired data. The user is provided with an interactive browser page that displays a comparison of a control sample and an analyte so that differences are easily seen (Figure 3). The use of such software removes the need for intermediate manual transfers and avoids human bias. A large number of samples can be rapidly compared to a control and reports can be generated automatically for external users.

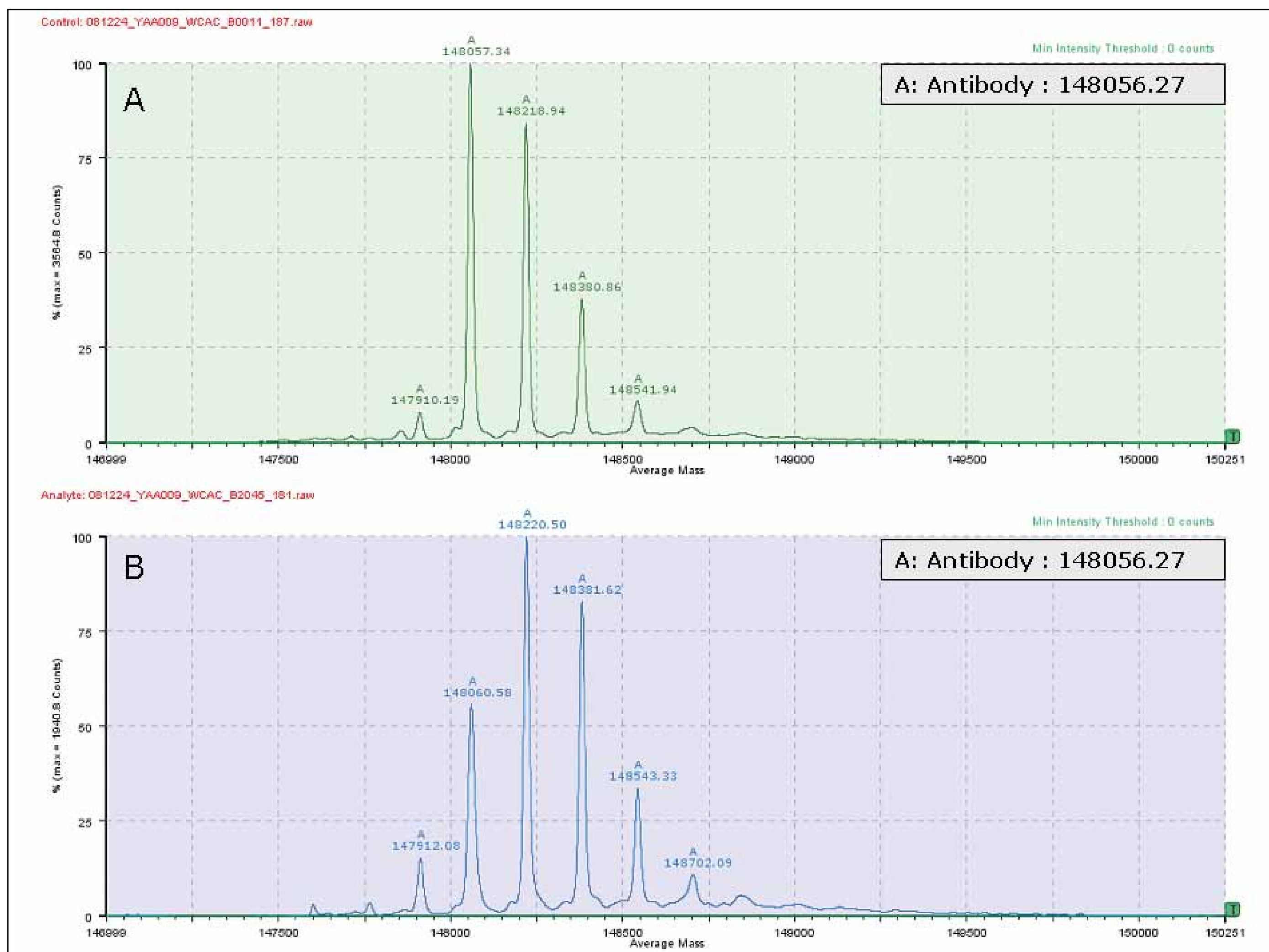

Figure 3. Deconvoluted mass spectra of a humanized monoclonal antibody from BiopharmaLynx 1.2. Panel A and Panel B display the batch comparison of the same antibody to demonstrate the glycoform variation caused by the production process.

Analysis 2: Analysis of heavy and light chains from a reduced monoclonal antibody

Chromatographic separation of the heavy and light chains adds further detail to the characteristics of an mAb, confirms that the glycoprotein profiles of candidate expression clones match the expectation, or checks whether there are unusual distributions of glycoforms. When specific glycoforms are of interest, the separation of light and heavy chains helps to examine glycoforms more closely.

The separation of light chain and heavy chain also allows the relative component quantitation to be achieved, and modifications specific to the light or heavy chains can be characterized. Mass changes associated with specific residues (such as succinimide formation) can be detected by analysis of partially reduced monoclonal antibodies (or of peptides from enzymatic digestion as discussed below) with chromatographic separation.

Figure 4 displays separate spectra for the heavy and light chains from a reduced monoclonal antibody. The corresponding deconvoluted spectra for the light chain and heavy chain are also shown. The chromatogram shown in inset demonstrates that high resolution separation by an ACQUITY UPLC BEH C_4 Column enables the differentiation of minor isoforms of either heavy chain or light chains, providing enhanced assessment on the heterogeneity of the sample.

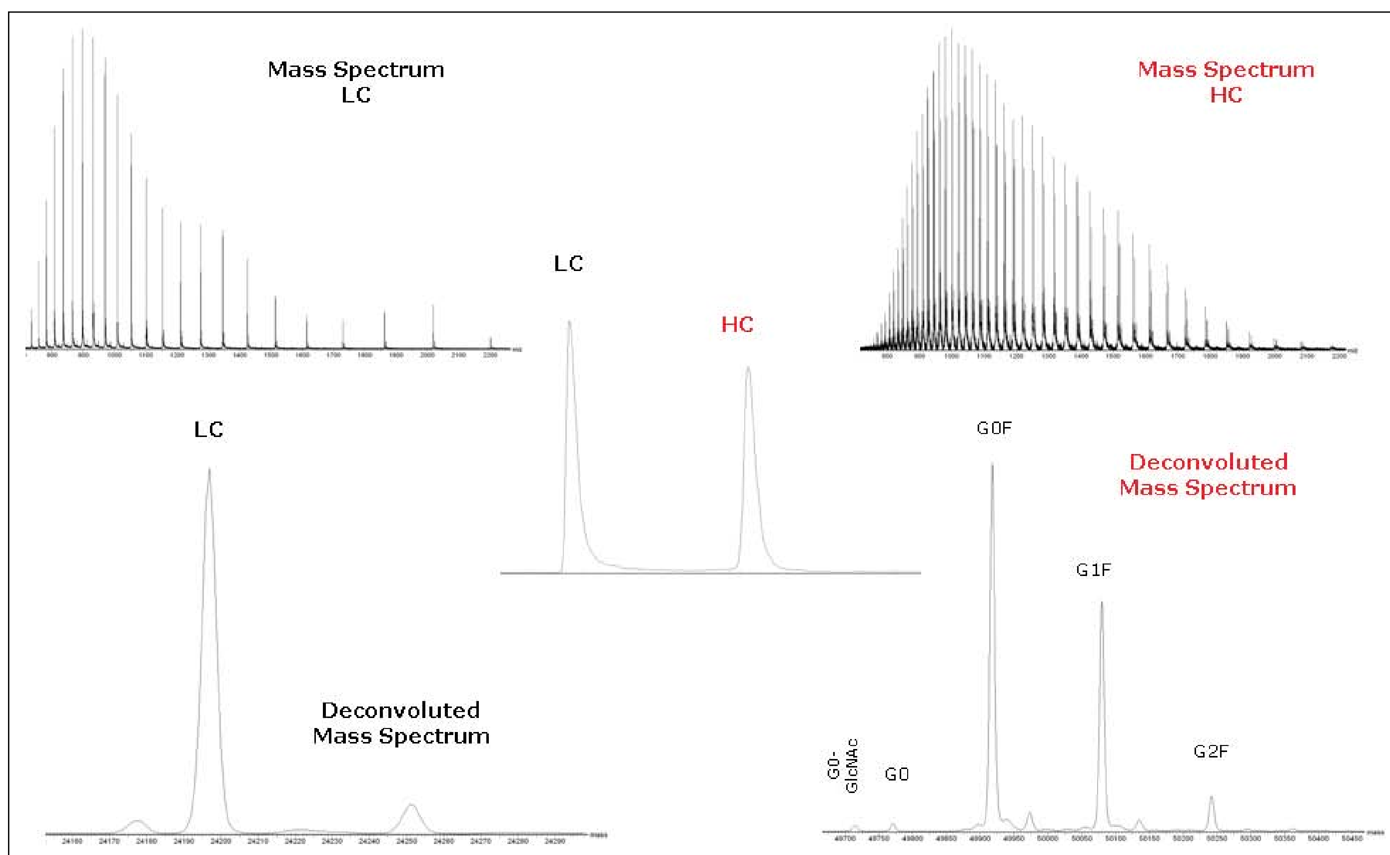

Figure 4. LC/MS analysis of a reduced monoclonal antibody. Complete resolution of the light chain and heavy chain were achieved using a UPLC BEH C_4 column.

Analysis 3: Peptide mapping analysis

In intact mass analysis, the overall heterogeneity of the protein is obtained. In order to characterize modifications that lead to small or no mass changes in the intact protein mass (e.g., deamidation or structural isomers of glycans), peptide mapping with MS detection provides a valuable technique for detailed and comprehensive modification coverage in a simple manner.

In LC/MS this can be done with ease using only one enzyme to digest the protein and obtain sequence coverage above 95%. The ability of a peptide map to highlight small changes in the primary structure of a protein makes it valuable for establishing the identity, purity, and composition of a protein. For example, in shelf life studies the ability to detect modifications is vital. In the optimization of *in vitro* folding processes the arrangement of disulfide bridges is crucial.

LC/MS analysis has greatly expanded the capability to develop and interpret data from peptide mapping experiments, compared to traditional LC-UV/VIS detection. However, single-stage MS analysis may not be capable of resolving peptides that are isobaric, nor can suspected peptide modifications be identified and localized from MS data alone. MS/MS data are therefore used to generate fragment ion mass spectra. In the past, MS/MS data were acquired with selected peptides using a traditional data-dependent (or directed) MS/MS acquisition (DDA). We present here an approach that is a significant improvement on DDA by using MS^E analysis to confirm sequence information.

MS^E provides a comprehensive MS/MS picture without any prior knowledge of the sample and is applicable across all charge states. (See references 2-7 for details of MS^E.) This means that the peptide map can be performed in an unbiased, systematic manner and provide consistent results without the need for an expert. The methodology also means that the same dataset could potentially be re-analyzed at a later stage for additional information without having to re-run the samples – saving significant costs, time, and capital investment.

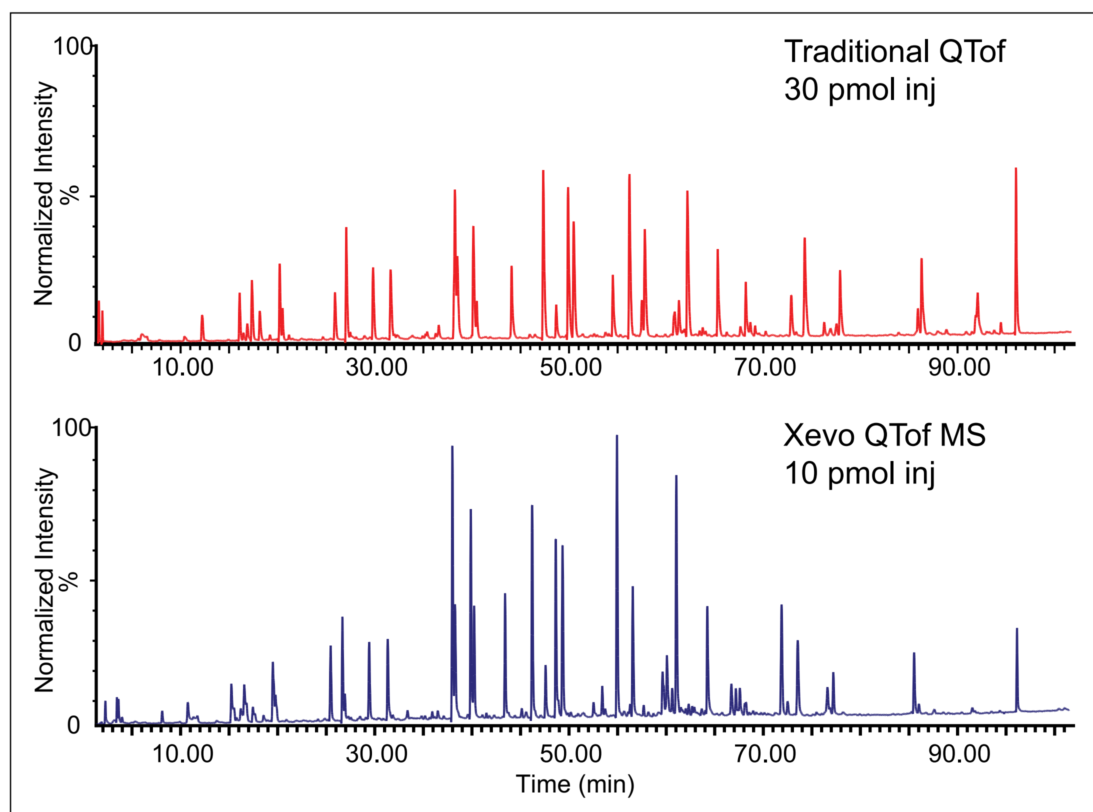

Figure 5. Improved sensitivity of Xevo QTof MS for peptide mapping experiment.

In the BiopharmaLynx browser, the user is able to interactively annotate data that might previously have been overlooked. Modifications are annotated automatically and disulfide bridges can be shown on the peptide map. Increased sensitivity provided by the Xevo QTof MS enhances the information available with no compromise in productivity.

In Figure 5, an Enolase digest was analyzed by UPLC/MS^E. The comparison between a traditional QTof and the new Xevo QTof MS highlights the improvements in sensitivity for this workhorse instrument.

In BiopharmaLynx 1.2, additional information on peptide sequence is also provided. This confirms the sequence of the peptides from the protein. Figure 6 shows a screenshot from the tabular browser display presented to the user. In the right-most column shown, the y- and b-ions are listed to indicate exactly which backbone fragments were automatically identified from the MS/MS spectra. The user is also able to select alternative sequences where they are presented or reject them if the user prefers a different interpretation.

Protein	Peptide	Fragment Number	Modifiers	Control b/y Found	Control b/y List
humanized mab	DIQMTQSPSSLSASV	1:T001		28	1/b2;1/b3;1/b4;1/b5;1/b6;1/b7;1/b9;1/b10;1/b11;1/b12;1/b13;1/b14;1/b15
humanized mab	DIQMTQSPSSLSASV	1:T001*	decom of carbamid	9	1/b2;1/b7*;1/b10*;1/y3;1/y4;1/y5;1/y6;1/y7;1/y8
humanized mab	DIQMTQSPSSLSASV	1:T001-002*	CarbamidomethylC(1)		
humanized mab	VTITCR	1:T002*	CarbamidomethylC	4	1/b2;1/y1;1/y2*;1/y3*
humanized mab	VTITCRASQDVNTAV	1:T002-003*	CarbamidomethylC(1)		
humanized mab	ASQDVNTAVAWYQC	1:T003		22	1/b2;1/b3;1/b4;1/b5;1/b18;1/y1;1/y2;1/y3;1/y4;1/y5;1/y6;1/y7;1/y8;1/y9;
humanized mab	ASQDVNTAVAWYQC	1:T003*	Deamidation N(1)	14	1/b2;1/b4;1/b18*;1/y2;1/y3;1/y4;1/y6;1/y7;1/y8;1/y9;1/y13*;1/y14*;1/y15
humanized mab	ASQDVNTAVAWYQC	1:T003-004			
humanized mab	APK	1:T004			
humanized mab	APKLLIYSASFLYSGV	1:T004-005			
humanized mab	LLIYSASFLYSGVPSF	1:T005		14	1/b2;1/b3;1/b9;1/b13;1/y3;1/y5;1/y6;1/y7;1/y8;1/y9;1/y10;1/y11;1/y12;1.
humanized mab	LLIYSASFLYSGVPSF	1:T005*	carbamidomethy Y	0	
humanized mab	LLIYSASFLYSGVPSF	1:T005-006			
humanized mab	FSGSR	1:T006		4	1/b5;1/y1;1/y3;1/y4
humanized mab	FSGSRSGTDFTLTISS	1:T006-007*	CarbamidomethylC(1)		
humanized mab	SGSR	1:T006/y4		2	1/y1;1/y3
humanized mab	SGTDFTLTISSLQPED	1:T007*	CarbamidomethylC	24	1/b4;1/b5;1/b6;1/b7;1/b8;1/b9;1/b10;1/b11;1/b12;1/b13;1/b28*;1/b37*;1/
humanized mab	SGTDFTLTISSLQPED	1:T007-008*	CarbamidomethylC(1)		
humanized mab	VEIK	1:T008		4	1/b2;1/y1;1/y2;1/y3
humanized mab	VEIKR	1:T008-009			
humanized mab	R	1:T009			
humanized mab	RTVAAPSVFIFPPSD	1:T009-010			
humanized mab	TVAAPSVFIFPPSDE	1:T010		16	1/b2;1/b3;1/b4;1/b6;1/b8;1/b9;1/b10;1/y7;1/y8;1/y9;1/y10;1/y11;1/y12;1.
humanized mab	TVAAPSVFIFPPSDE	1:T010-011*	CarbamidomethylC(1)		
humanized mab	TVAAPSVFIF	1:T010/b10		6	1/b2;1/b3;1/b4;1/b6;1/b8;1/b9
humanized mab	PPSDEQLK	1:T010/y8		1	1/y7
humanized mab	SGTASVVCLLNNFYP	1:T011*	CarbamidomethylC	25	1/b2;1/b3;1/b4;1/b5;1/b6;1/b7;1/b8*;1/b9*;1/b10*;1/b11*;1/b12*;1/b13*;1
humanized mab	SGTASVVCLLNNFYP	1:T011*	Deamidation N(1),C	10	1/b14*;1/y2;1/y3;1/y4;1/y5;1/y6*;1/y7*;1/y8*;1/y9*;1/y10*
humanized mab	SGTASVVCLLNNFYP	1:T011*/b16*	CarbamidomethylC	13	1/b2;1/b3;1/b4;1/b5;1/b6;1/b7;1/b8*;1/b9*;1/b10*;1/b11*;1/b12*;1/b13*;1

Figure 6. Results of BiopharmaLynx-processed Xevo QTof MS data, showing MS and MS/MS (MS^E) data. Fragment ions (b/y ions) are identified and listed in the Peak Match Data Table.

Figure 7 shows the sequence information superimposed on a spectrum in the BiopharmaLynx browser. The y-series amino acid sequence information is shown in red underneath the b-series sequence in blue.

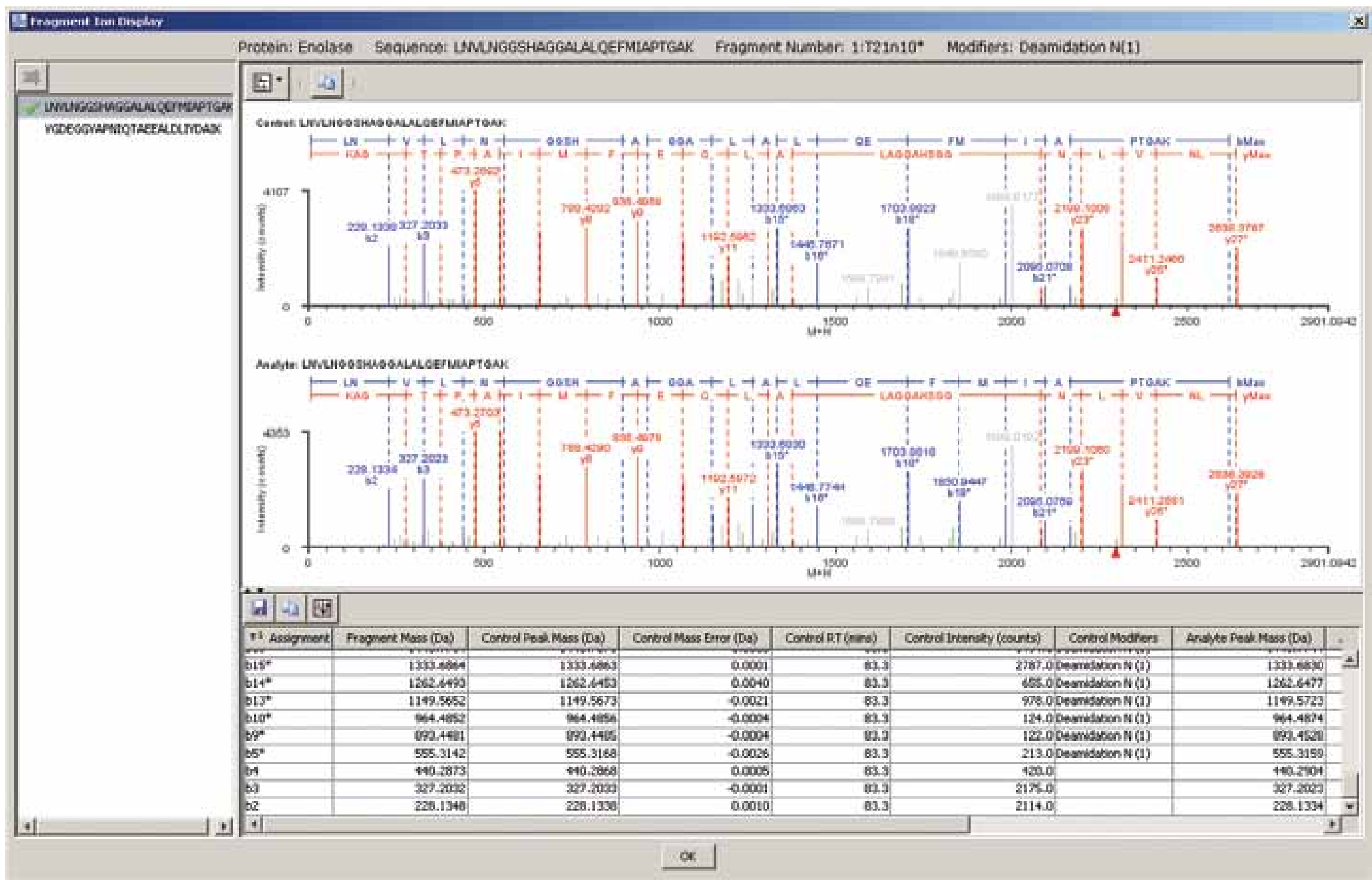

Figure 7. The Fragment Ion display from BiopharmaLynx 1.2 showing sequenced peptides on an MS/MS spectrum for a control (top) and an analyte (bottom). Additional information is available in the tabular display showing modifications and locations of these modifications.

CONCLUSION

- This application note has demonstrated high-performance, routine characterization of intact proteins and peptide maps, saving days of time for providing a complete analysis.
- This LC/MS system solution featured:
  - Optimized LC resolution, sensitivity, and speed with the ACQUITY UPLC System
  - Robustness and reproducibility with ACQUITY UPLC Columns and 1.7 µm particles
  - High-sensitivity, accurate mass MS^E with the Xevo QTof Mass Spectrometer
  - Targeted, streamlined, and sophisticated conversion of data into usable information with BiopharmaLynx informatics
- We presented an improved system that provides more comprehensive and faster characterization of:
  - Intact proteins
  - Peptide maps
- Automated peptide sequence confirmation is now possible with MS^E and BiopharmaLynx.
- The ACQUITY UPLC/Xevo QTof MS system combines into a comprehensive analytical workflow solution for the biopharmaceutical laboratory, intelligently integrating instrumentation with software to provide improvements in productivity and efficiency: from instrument performance, to data generation, to interpretation and decision-making.

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

[APPLICATION NOTE]

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Characterization of an IgG1 Monoclonal Antibody and Related Sub-structures by LC/ESI-TOF MS

Asish B. Chakraborty, Scott J. Berger, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Monoclonal antibodies (mAb) comprise a significant proportion of biotechnology-derived molecules used for diagnostic and therapeutic applications. The inherent heterogeneity of such products has dictated the need for thorough analytical characterization methodologies so that safe, effective and reproducible products can be produced. LC/MS has become a powerful tool as part of the standard analytical package used to characterize these important biomolecules.

IgG1s comprise a subclass of the antibodies developed as therapeutic and diagnostic agents. Specificity of interaction is produced by hypervariable regions of sequence present within a constant sequence backbone structure. Thus, while antibodies can have vastly differing binding selectivity, the overall structure is highly conserved between antibodies of the same class, and standard analytical methods can often be used as a starting point towards developing an optimized analytical strategy for an individual molecule.

Like many biotechnology-derived products, IgG1s have a complex heterogeneous structure, where multiple glycoforms and sub-stoichiometric modifications produce a wide population of structures (variants) for a single antibody. The overall molecule (MW ~150 kD) comprises two identical heavy chains (HC, Figure 1, dark blue) linked together through two disulfide bonds, and two light chains (LC, Figure 1, light blue), each linked to a heavy chain by a single disulfide bridge.

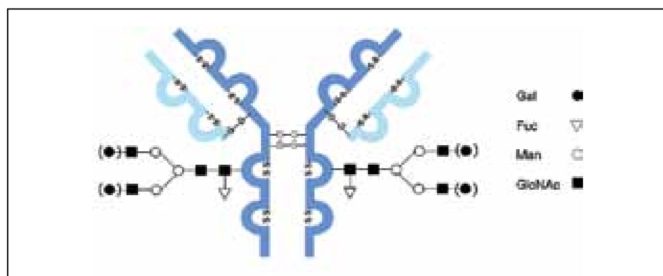


Figure 1. Structure of a monoclonal IgG1 antibody used in these studies. This particular IgG1 was found to contain N-linked biantennary carbohydrates linked each of the heavy chains. The major product variants that were observed correspond to glycan heterogeneity (0-4 galactose residues per antibody).

The typical IgG1 contains an additional 12 intra-chain disulfide bonds for a total of 16 disulfide bonds in the intact molecule[1]. Major product variants observed with IgG1s include the presence of a lysine on one or both C-termini of the heavy chains, pyroglutamic acid modification of free N-termini containing glutamine and heterogeneous heavy chain glycosylation (e.g., in Figure 1 where an N-linked biantennary core glycan is shown with up to four terminal galactose residues). A variety of less specific modifications (e.g., deamidation, oxidation, etc.) can also appear at multiple sites on an antibody and levels of these modifications have been found to be altered by production and storage conditions.

The increased interest in recombinant monoclonal antibodies as therapeutic biomolecules has presented a challenge for the development of analytical methodologies required to permit their full characterization. High resolution mass spectrometry of the intact antibody and its variants provides a powerful approach for assessing batch-to-batch variation and for the study of antibody structural stability over time.

Additional insight has also been achieved by targeted processing of the intact antibody to generate smaller and less complicated antibody substructures. This has been achieved by chemical reduction of disulfides to produce free heavy and light chains, enzymatic or chemical deglycosylation and targeted cleavage of the antibody hinge region to generate characteristic antibody fragments. As with the intact antibody, the primary structure of these substructures will vary from antibody to antibody, but they will be sufficiently similar to permit analysis using a generic LC/MS methodology.

Most antibodies are stored in a matrix of biological buffers and non-volatile salts and stabilizers. Thus, one of the most significant challenges encountered during mass analysis of intact antibodies is processing of the sample to remove these agents, which often form non-covalent adducts that reduce MS response and further complicate the resulting mass spectral data.

In this study, we have developed two rapid, sensitive and efficient generic desalting/cleanup LC/electrospray time-of-flight (ESI-TOF) MS methods that can be used for the characterization of an intact antibody and its variants, deglycosylated forms of that antibody, resolution of constituent heavy and light chain structures and analysis of the common antibody fragments that are generated following papain cleavage.

MATERIALS AND METHODS

Materials

Protein A affinity purified mouse monoclonal antibody (IgG1,κ) was obtained from VICAM, a Waters Business (Milford, MA). Papain was purchased from Boehringer Mannheim (Indianapolis, IN). Dithiothreitol (DTT) and cysteine-HCl were obtained from Pierce (Rockford, IL). Sodium phosphate and EDTA were purchased from Sigma (St. Louis, MO). RapiGest™ SF and MassPREP™ MALDI Matrix, DHB were obtained from Waters Corporation (Milford, MA). Peptide N-Glycosidase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA).

Preparation of intact IgG1

Intact IgG1 stock (11.3 µg/µL, 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3) was diluted with 50 mM ammonium bicarbonate to achieve 1.0 µg/µL IgG1. Lesser concentrations (0.1-0.5 µg/µL) were obtained by serial dilution with ammonium bicarbonate. LC/MS analyses were performed on 10 µL of diluted IgG1 samples.

Preparation of reduced IgG1 (to form heavy and light chains)

Reduction of disulfides in the IgG1 (0.5 µg/µL) was accomplished using 20 mM DTT at 80 °C for 15 min. The reduced sample was injected onto the column for LC/MS analysis (10 µL) without further processing or storage.

Preparation of deglycosylated intact IgG1

PNGase F digestion of IgG1 was performed in IgG1 stock solution buffer (0.1 M sodium bicarbonate buffer, 0.5 M NaCl, pH 8.3). RapiGest SF (0.05% final) was added to the stock IgG1 solution (11.3 µg/µL) and heated at 60 °C for 30 min. The resulting solution was cooled to room temperature, and deglycosylation was initiated by the addition of PNGase F (11 U/µg IgG1), and incubated at 37 °C for 18 h. Following deglycosylation, the sample was diluted with 5% acetonitrile in 0.1% formic acid to 0.1 µg/µL, then treated with neat hydrochloric acid (2% v/v) for 1 h at 37 °C. LC/MS analysis was performed on a 10 µL of 0.1 µg/µL processed sample.

Preparation of deglycosylated and reduced intact IgG1

PNGase F digestion of IgG1 in the presence of DTT was performed in IgG1 stock solution buffer (0.1 M sodium bicarbonate buffer, 0.5 M NaCl, pH 8.3. RapiGest SF (0.05% final) was added to IgG1 solution (11.3 µg/µL) and heated at 60 °C for 5 mins. DTT was then added at a concentration of 20 mM and heated at 60 °C for 25 min. The resulting solution was cooled to room temperature, and deglycosylation was initiated by the addition of PNGase F (11 U/µg IgG1), and incubated at 37 °C for 18 h. Following deglycosylation, the sample was diluted with 5% acetonitrile in 0.1% formic acid to 0.5 µg/µL, then treated with neat hydrochloric acid (2% v/v) for 1 h at 37 °C. LC/MS analysis was performed on a 10 µL of 0.5 µg/µL processed sample.

Papain digestion (no cysteine)

Stock IgG1 was buffer exchanged against cysteine-free papain digestion buffer (1 mM EDTA, 50 mM sodium phosphate buffer, pH 6.3) by centrifugal ultrafiltration (VIVASPIN, 5000 MWCO, 11,000 x g, 5 °C). Papain was activated by adding one part papain suspension (10 mg/mL) to nine parts freshly prepared activation buffer (1 mM EDTA, 10 mM cysteine, 50 mM sodium phosphate buffer, pH 7.0), and incubating for 15 min at 37 °C. The excess cysteine was removed by buffer exchange (against 6 vol. cysteine-free digestion buffer) using centrifugal ultra-filtration.

The activated papain was then diluted in cysteine-free digestion buffer (1 µg/µL), added to the IgG1 solution at an enzyme:antibody ratio of 1% (w/w), and incubated at 37 °C for 2 h. The papain digest was diluted with 5% acetonitrile in 0.1% formic acid to obtain 0.5 µg/µL, and used for LC/MS analysis (10 µL).

Papain digestion (addition of cysteine)

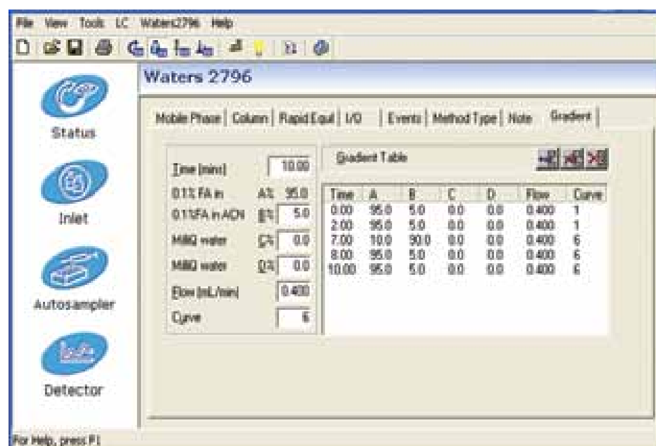
Stock IgG1 was buffer exchanged against papain digestion buffer plus cysteine (10 mM cysteine, 1 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0) by centrifugal ultra-filtration (VIVASPIN, 5000 MWCO, 11,000 x g, 5 °C).

Papain was activated by adding one part papain suspension (10 mg/mL) to nine parts freshly prepared activation buffer (1 mM EDTA, 10 mM cysteine, 50 mM sodium phosphate buffer, pH 7.0), and incubating for 15 min at 37 °C. The excess cysteine was removed by buffer exchange (against 6 vol. cysteine-free digestion buffer) using centrifugal ultra-filtration.

Papain digestion was carried out in digestion buffer plus cysteine at 37 °C overnight at an enzyme:antibody ratio of 1% w/w. The papain digest was diluted with 5% acetonitrile in 0.1% formic acid to obtain 0.5 µg/µL, and used for LC/MS analysis (10 µL).

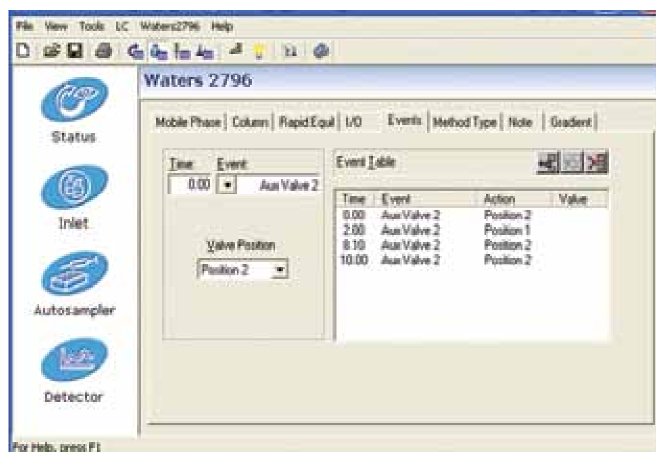
HPLC or UPLC separations of intact and deglycosylated intact monoclonal antibody

A Waters® Alliance® HPLC 2796 Bioseparations System or ACQUITY UPLC® System was directly coupled to a Waters orthagonal acceleration time-of-flight (Tof) mass spectrometer for all experiments. Reversed phase separations of intact antibody and PNGase F treated antibody were performed on a MassPREP Desalting Cartridge (2.1 x 10 mm) using a 5 min gradient (5 to 90% B) at a flow rate 0.4 mL/min and a column temperature of 30 °C (see Tables 1 and 2). Mobile phase A was 0.1% formic acid in water, while mobile phase B contained 0.1% formic acid in acetonitrile. The column effluent was diverted from the MS source to waste using a 10-port valve for the first 2 min of the run to prevent salt contamination of the ESI source. Following each separation, the column was re-equilibrated with 5% B for 2 min.



Time	A	B	C	D	Flow	Curve
0.00	95.0	5.0	0.0	0.0	0.400	1
2.00	95.0	5.0	0.0	0.0	0.400	1
7.00	10.0	90.0	0.0	0.0	0.400	6
8.00	95.0	5.0	0.0	0.0	0.400	6
10.00	95.0	5.0	0.0	0.0	0.400	6

Table 1. Gradient profile used for the intact and deglycosylated IgG1 monoclonal antibody.

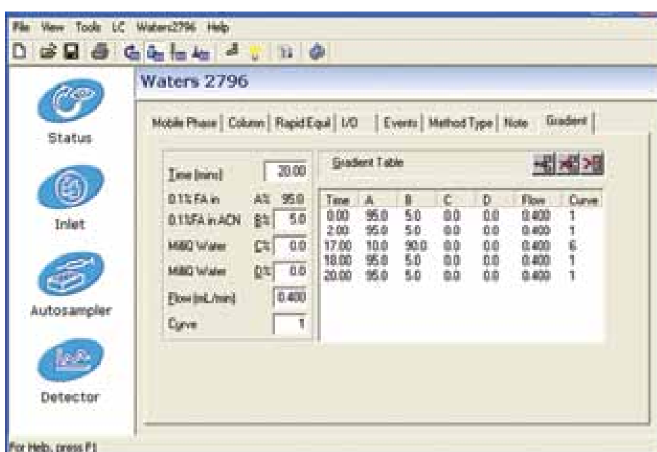


Time	Event	Action	Value
0.00	Aux Valve 2	Position 2	
2.00	Aux Valve 2	Position 1	
8.10	Aux Valve 2	Position 2	
10.00	Aux Valve 2	Position 2	

Table 2. Salt divert valve events used for the intact and deglycosylated IgG1 monoclonal antibody.

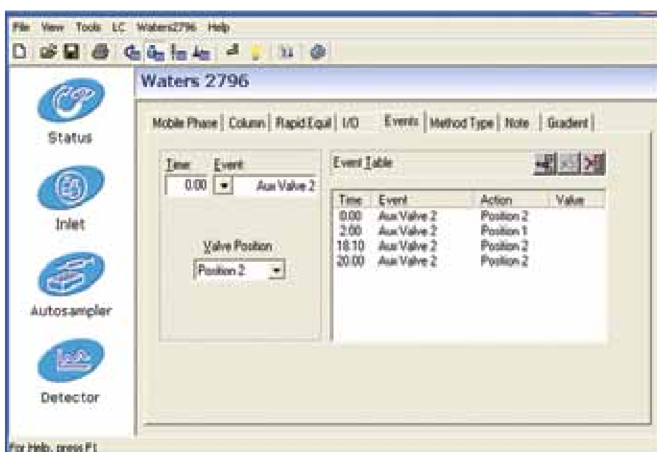
HPLC separations of reduced IgG1 (LC/HC), deglycosylated reduced IgG1 (LC/HC), and papain digested IgG1 fragments

Reversed phase separations of reduced IgG1 (light and heavy chain fragments), deglycosylated LC and HC and papain digest fragments were performed on a MassPREP Desalting Cartridge (2.1 x 10 mm) using a 15 min gradient (5 to 90% B) unless otherwise stated. All other conditions for the separation were identical to those used for the intact antibody analysis. Tables 3 and 4 display the gradient table and salt divert valve events used for these experiments.



Time	A	B	C	D	Flow	Curve
0.00	95.0	5.0	0.0	0.0	0.400	1
2.00	95.0	5.0	0.0	0.0	0.400	1
17.00	10.0	90.0	0.0	0.0	0.400	6
18.00	95.0	5.0	0.0	0.0	0.400	1
20.00	95.0	5.0	0.0	0.0	0.400	1

Table 3. Gradient profile used for reduced deglycosylated IgG1 (LC/HC) and papain digest fragments.



Time	Event	Action	Value
0.00	Aux Valve 2	Position 2	
2.00	Aux Valve 2	Position 1	
18.10	Aux Valve 2	Position 2	
20.00	Aux Valve 2	Position 2	

Table 4. Salt divert valve events used for reduced deglycosylated IgG1 (LC/HC) and papain digest fragments.

Purification of released glycans

Glycans released from the intact IgG1 by PNGase F treatment were enriched using the 96-well MassPREP HILIC µElution plate according to the Waters Care and Use Instructions and a published paper by Yu and colleagues[2]. Briefly, the method used to extract glycans was as follows:

- A µElution plate well was washed with 200 µL of Milli-Q water (Millipore, Billerica, MA), and equilibrated with 200 µL of 85% acetonitrile
- The deglycosylated IgG1 (9 µg/µL) that was acid-treated to break down RapiGest was diluted with acetonitrile to obtain the equivalent of 0.3 µg/µL IgG1 in 85% organic solvent
- 200 µL of diluted sample was loaded on the plate by gravity
- Deglycosylated IgG1 and salts were washed off with 200 µL of 85% acetonitrile
- Glycans were eluted with 10 mM ammonium citrate in 25% acetonitrite (50 µL, twice), and the collected sample was dried under vacuum (Labconco, Kansas City, MO)

Analysis by electrospray ionization mass spectrometry

Protein mass information was obtained using the Waters ESI-TOF mass spectrometer operated in the positive ion W-mode. The electrospray (ESI) source was operated under the following conditions:

Source temp.: 150 °C
Desolvation temp.: 350 °C
Desolvation gas: 800 L/h
Cone voltage: 40 V
Capillary voltage: 3.2 kV
Ion guide 1: 100 V

The mass spectrometer was calibrated using an external multi-point calibration based on singly-charged CsI ions (2 mg/mL CsI dissolved in 50% isopropanol). Mass spectra were acquired in the m/z range of 600 to 5000. A 1 Hz scan rate with 0.1 sec interscan delay was used to acquire data over the entire analysis.

Analysis by MALDI mass spectrometry

Purified glycans were reconstituted in 30 µL of 25% acetonitrile. Sample (0.5 µL) and an equal volume of matrix (20 mg/mL DHB in 80% acetonitrile) were co-spotted on a stainless steel MALDI plate and allowed to dry at ambient temperature. Ethanol (0.5 µL) was overlaid on the dried well to form a more homogeneous crystalline sample.

Glycan masses, and tandem (MS/MS) spectra from the purified glycans, were determined using a Waters MALDI quadrupole time-of-flight mass spectrometer (MALDI Q-Tof). It was equipped with a N_2 laser (337 nm) source operated at 10 Hz laser firing rate for data acquisition. Calibration was accomplished using polyethylene glycol plus sodium iodide in 50% acetonitrile with CHCA matrix (MassPREP Calibration Mix-MALDI Reflectron) in the mass range of m/z 50 to 3000. Mass spectra were acquired in the positive ion mode with V-optics. Argon was used as the collision gas and the collision cell pressure was maintained at 5.30 10^{-3} mBar. Collision energy in MS mode was 10V and 500 laser shots were combined to generate an MS spectrum; glycan fragmentation spectra were acquired using collision energy of 125 V and combining 1,200 laser shots.

Data processing

Summed electrospray mass spectra were generated by combining all spectra over chromatographic peaks as indicated. The resulting summed spectra (m/z regions as indicated in subsequent figures) were deconvoluted by the Waters MaxEnt1 algorithm to produce neutral mass information. MaxEnt1 processing parameters included an output bin size of 1 Da, a Gaussian damage model with a peak width of 1 Da (intact and deglycosylated IgG1) or 0.7 Da (reduced IgG1, reduced and deglycosylated IgG1, and papain fragments), minimum intensity ratio of 33% (right and left) and processing to model convergence.

RESULTS AND DISCUSSION

It is essential that nonvolatile buffers, salts and stabilizing agents are removed prior to MS analysis, as they reduce MS response and produce noncovalent adducts that complicate interpretation of antibody mass spectra. Here we have utilized a robust large-pore polymeric reversed phase trap column (MassPREP On-Line Desalting Cartridge) for sample cleanup and desalting prior to online mass analysis.

Although the key function of the column was to remove interfering substances for LC/MS analysis, sufficient separation capacity exists within the trap column to achieve partial resolution between heavy and light chains, deglycosylated heavy and light chains and the major fragments of papain digestion. Two LC/MS methods (differing primarily by length and slope of reversed phase gradient) are provided within this document to accomplish the desalting or desalting/separation functions described below.

Analysis of an intact IgG1

A fast and efficient LC/ESI-MS method was used to identify multiple structural variants of a model IgG1 molecule, with a total injection-to-injection cycle time of 10 min. The corresponding gradient table and salt divert valve event times are shown in Tables 1 and 2, respectively.

Figure 2 depicts the fluidic configuration used for LC/MS analysis, including a post-column salt diversion valve, and the direct flow of eluent into the mass spectrometer. Column effluent was diverted from the mass spectrometer to waste using a post-column 10-port two-position valve (in position 1) for the first 2 min, to prevent salt contamination of the ESI source. Eluent flow was restored to the mass spectrometer (valve position 2) and the IgG1 was eluted using a 5 min gradient of 5 to 90% acetonitrile in 0.1% FA, followed by a 2 min column regeneration period with 5% acetonitrile in 0.1% FA.

Figure 2. LC/ESI-TOF MS configuration. A Waters Alliance HPLC 2796 Bioseparations System was directly coupled to a Waters LCT Premier ESI-TOF MS for antibody mass analysis. A modern configuration would use the Waters ACQUITY UPLC H-Class Bio System with a Waters Xevo® G2 Tof Mass Spectrometer.

Protein separation and desalting were accomplished using a 2.1 x 10 mm polymeric RP cartridge column. During the LC/MS analysis, the column effluent was diverted to waste for the first two minutes each run, using a post-column 10-port two-position valve, to prevent salt contamination of the ESI source.

Carryover and binding capacity of the trap column were briefly investigated by loading increasing amounts (1, 5, 10 µg) of intact antibody with intervening blank runs. The overlaid (y-axis linked) total ion current chromatograms (TICs) for this experiment and the associated summed mass spectra are shown as Figures 3 and 4, respectively. The results show no detectable carryover in the blank following a 1 µg injection, and less than 4% carryover at the 5 µg and 10 µg loadings (Figure 4). An increase in MS response (max counts of the summed spectra) of ~3 fold was seen in the 5 µg loading compared to the 1 µg loading, whereas the 10 µg loading resulted in only a ~4 fold signal increase relative to the 1 µg loading (Figure 4).

Thus, minimal (but measurable) carryover is evidenced when approaching the maximum loading capacity (~10 µg) of the column. Loads on the order of 1 µg, intervening blank runs, or post-run sawtooth (rapid up-down) gradient cycles would be utilized with this particular antibody to obtain the best quantitative data.

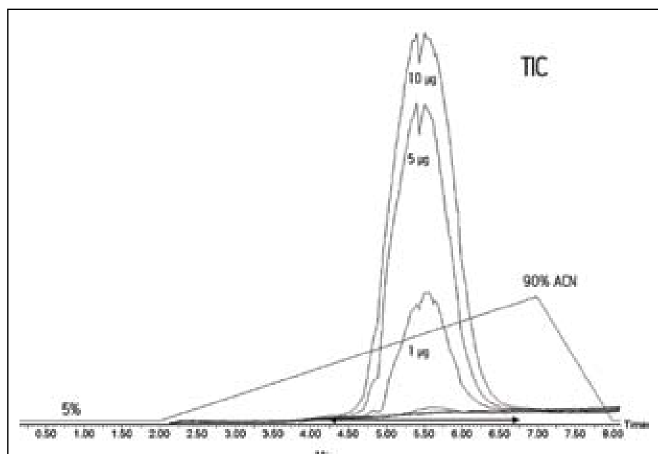


Figure 3. Total ion current chromatograms (TIC) from LC/ESI-MS analyses of an intact IgG1 loaded at varying amounts (1 to 10 µg). Blank LC/MS runs conducted between each of the samples were used to demonstrate the low amount of sample carryover for this method.

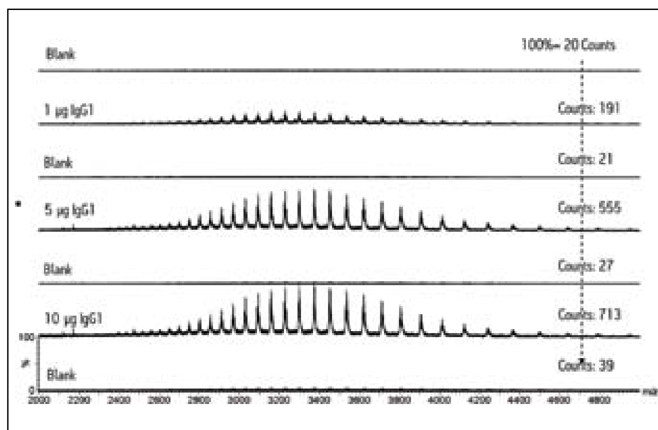


Figure 4. Combined ESI-TOF mass spectra of an intact IgG1 demonstrating the relative carryover at varying amounts (1 to 10 µg) from the intervening blank runs. There was no carryover detected with the 1 µg loading, and less than 5% carryover associated with the 5 and 10 µg loadings.

Monoclonal antibodies demonstrate significant structural heterogeneity (variants) due to complex patterns of post-translational modifications. The most common set of protein variants differ with respect to processing of terminal amino acids (N-terminal pyroglutamic acid formation, C-terminal Lysine processing of heavy chains), and heterogeneity in the glycoform structures present on each of the heavy chains. Other variants (e.g. those containing deamidation and oxidation) arise due to environmental conditions of growth, storage and processing.

The isotopic distribution of an intact antibody is sufficiently wide (~25 Da at half height for the neutral molecule) that lower mass modifications may not be spectrally resolved from the unmodified antibody, but patterns of glycosylation (140 to 200 Da) and lysine processing (128 Da) can be easily discerned. The presence of low mass modifications can be observed as partially resolved peaks, or as a mass shift for a peak that will be in direct proportion to the relative levels of the species. The analysis of antibody substructures (e.g. light and heavy chains, or Fab and Fc fragments) or peptide mapping analysis is typically used to directly confirm the presence of these low mass modifications.

The summed ESI-TOF mass spectrum for our intact IgG1 (5 μg load) revealed a symmetrical charge state envelope over 2400 to 5000 m/z (Figure 5). The +44 charge state region, at the center of the envelope, has been enlarged (Figure 5, inset) to show greater detail, and reveals at least six major antibody variants, even before MaxEnt1 processing. The maximum entropy approach uses all MS information to discern the most likely deconvoluted spectrum to have given rise to the observed m/z spectrum. Because of this reliance on the "global view," the effective resolution achievable following MaxEnt1 deconvolution is greater than revealed from examination of any given charge state.

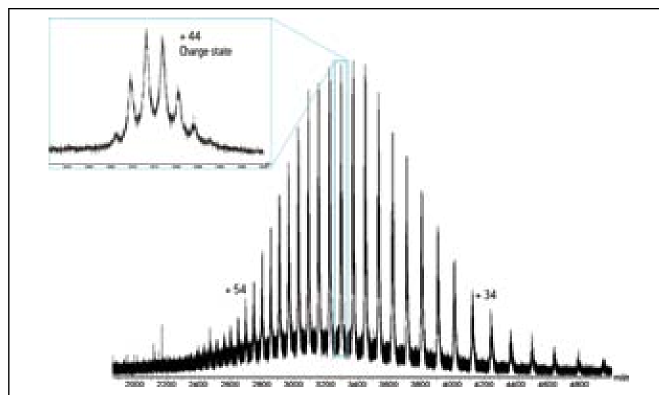


Figure 5. The summed ESI-MS mass spectrum over the TIC peak shown for the 5 μg IgG1 load. A charge envelope of 2400 to 4800 m/z was observed for the intact IgG1, centered on +44 charge state of the protein.

The MaxEnt1 deconvoluted spectrum (Figure 6) for the intact IgG clearly revealed heterogeneity in the carbohydrate moieties attached to the two heavy chains. Five peaks demonstrate a characteristic sequential mass difference of ~162 Da consistent with extension of the two core glycan structures by up to four hexose (galactose) residues. An additional peak pair (148,214 Da and 148,068 Da) demonstrates a mass difference of 146 Da corresponding to incomplete occupancy of a fucosylation site on the core glycans.

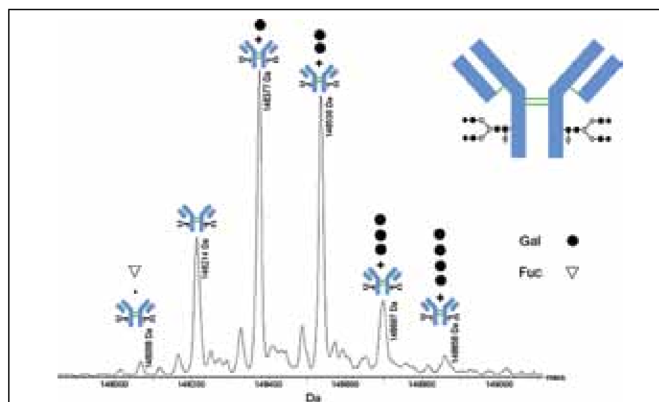


Figure 6. MaxEnt1 deconvoluted mass spectrum of the intact IgG1. Major variants observed were due to carbohydrate heterogeneity. The deconvoluted mass spectrum shows the intact IgG1 with up to four terminal galactose residues (•), and slightly incomplete core glycan fucosylation (Δ). No C-terminal lysine processing variants were observed on this antibody.

MaxEnt1 produces a result that is quantitatively conserved with the raw spectral data, and for chemically similar analytes (as we expect IgG1 variants to be), the MaxEnt1 result can be used for relative quantitation for each of the identified species. There were no additional variants observed that would reveal incomplete lysine processing on one or both heavy chains.

Overall, the data demonstrates that a single IgG1 monoclonal antibody exists as a complex population of variants, and that mass spectrometry on the intact antibody is capable of characterizing much of this heterogeneity.

Analysis of a reduced IgG1

Reduction of the intact IgG1 with dithiothreitol (DTT) breaks intra-chain disulfide bonds, and inter-chain disulfide bonds that connect the light chains to the heavy chains and two heavy chains to one another. The resulting liberation of light and heavy chains provides the opportunity for more detailed structural studies on the individual subunits.

Finding proper conditions for this reduction can be somewhat challenging as the various IgG subtypes have differing susceptibility to reduction of the inter-chain bonds, and the intra-chain disulfides often require even more stringent conditions to achieve complete reduction. In some LC/MS studies, we have observed additional species and TIC peaks arising due to incomplete reduction of an antibody. For our IgG1 antibody, complete reduction of both intra-chain and inter-chain disulfide bonds was achieved using elevated temperature and DTT concentration (20 mM, 15 min, and 80 °C) than may be typical (10 mM, 10 min, and 60 °C). The minimum effective treatment should be used, as heat and other environmental stresses have the potential to produce undesirable structural changes in proteins.

Separation of the heavy and light chains was obtained by applying a longer gradient (5 to 50% acetonitrile in 0.1% FA over 8 min) for an 11 min injection-injection cycle time. The first 1 min of a chromatographic run was diverted to waste for sample desalting, and the last 3 min of the run was utilized for column washing and regeneration. Gradient entries and salt divert valve timing for these methods are shown in Table 5.

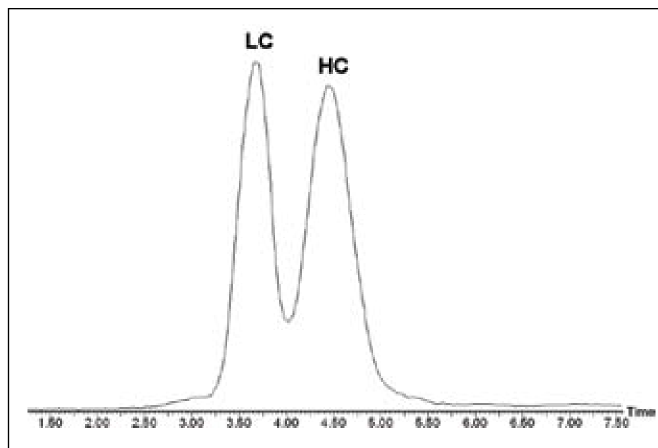


Figure 7. TIC chromatogram from ACQUITY UPLC/MS analysis of the reduced IgG1 antibody. Separation was achieved using an 8 min linear gradient (5 to 50%) of acetonitrile. The column temperature was maintained at 80 °C throughout the run.

A post-column salt diversion valve (top-left corner of the LCT Premier) was used to divert buffers and non-volatile salts to waste during sample loading and column washing steps.

The TIC of the reduced IgG1 (heavy and light chain fragments) is displayed in Figure 7. In this chromatogram, the light chain is an earlier eluting peak, while the heavy chain elutes later. Figure 8 shows the summed mass spectrum (inset) and deconvoluted spectrum of the light chain, which reveals a single major peak at 24,199 Da. Minor peaks (sodium adduct and loss of water) are also visible in the deconvoluted spectrum.

Time (min)	%B	Flow (mL/min)	Curve	
0.00	5	0.2	Initial	Load/Wash -Divert Flow-
1.0	5	0.2	6	
1.01	10	0.2	6	Gradient
8.11	50	0.2	6	
8.5	90	0.5	6	Column Washing and Regeneration
8.6	5	0.5	6	
9.1	90	0.5	6	
9.6	5	0.5	6	
9.7	90	0.5	6	
9.8	5	0.5	6	
11	5	0.5	6	

Table 5. Gradient profile and salt divert timing used for a reduced IgG1 (LC/HC).

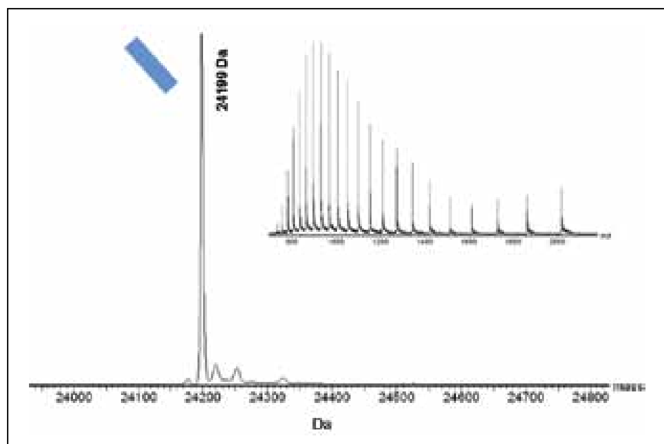


Figure 8. Combined mass spectrum (inset) and deconvoluted mass spectrum of the light chain derived from the IgG1. The complete reduction of intrachain and interchain disulfide bonds was achieved using the condition referenced in the methods section. The light chain mass was determined as 24,199 Da.

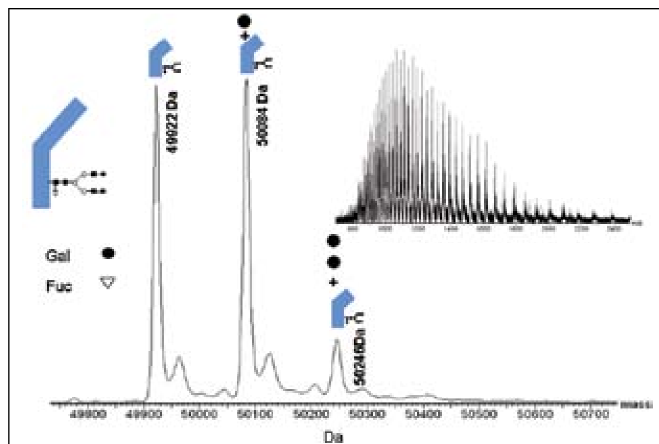


Figure 9. Combined mass spectrum (inset) and deconvoluted mass spectrum of the glycosylated heavy chain derived from the IgG1. Three major peaks differing by ~162 Da, corresponded to the heavy chain with no (Mass 49,922 Da), one (Mass 50,084 Da), and two (Mass 50,246 Da) terminal galactose residues.

Figure 9 depicts the summed mass spectrum (inset) and deconvoluted mass spectrum of the glycosylated heavy chain. Three major peaks with a mass differential of ~162 Da correspond to the heavy chain containing the core glycan, and variants where the core glycan is extended by one or two galactose residues. This is in agreement with our previous findings for the intact IgG1 antibody, where up to four additional galactose residues were observed.

In basic antibody math, the mass of the intact IgG1 can be calculated from the combined masses of two light chains, two heavy chains, and restoration of the 16 disulfide bonds (-32 Da). Based on our mass observations of the light chain and heavy chain (containing only the core glycan), we would predict an IgG1 mass of 148,210 Da. This prediction is only 4 Da (or 30 ppm) lighter than the observed intact IgG1 mass of 148,214 Da (LC/MS of intact IgG1 analysis). This result was obtained using a 1 Da precision to MaxEnt1 deconvolution, which potentially accounts for much of that mass difference.

This result confirms that LC/ESI-TOF MS can produce a mass measurement for the intact IgG1 of similar precision to that determined for the much smaller constituent heavy and light chains. The main advantage of working with the reduced antibody becomes the ability to directly detect lower mass modifications such as oxidation (+16 Da) that are obscured under the isotopic envelope of the intact antibody.

Analysis of a deglycosylated IgG1

PNGase F is an glycosidase that releases the oligosaccharides attached to an asparagine residue (N-glycosylation sites) within a glycoprotein by cleaving the beta-aspartylglycosylamine linkage. In this process, the attachment site Asn is converted to Asp via a glycosidase-mediated hydrolysis reaction.[3] Consequently, the observed mass for a deglycosylated protein or peptide shifts upwards by one Da from the predicted value. Factoring in this small difference, the mass difference between the deglycosylated antibody and intact antibody is the combined mass of the two heavy chain glycans.

There are several types of core glycan structures that differ by composition and extent and location of branching points, but a thorough discussion is beyond the scope of this document. There are, however, several common core glycan structures for which the masses have been calculated,[4, 5] and are commonly deduced from mass analysis of an antibody with and without enzymatic deglycosylation.

The deglycosylated IgG1 was analyzed under identical LC/MS conditions to the intact antibody. As with the intact antibody, LC/MS of the PNGase F deglycosylated IgG1 reveals a single TIC peak (Figure 10), and there was no obvious shift in retention time between the intact and deglycosylated IgG1.

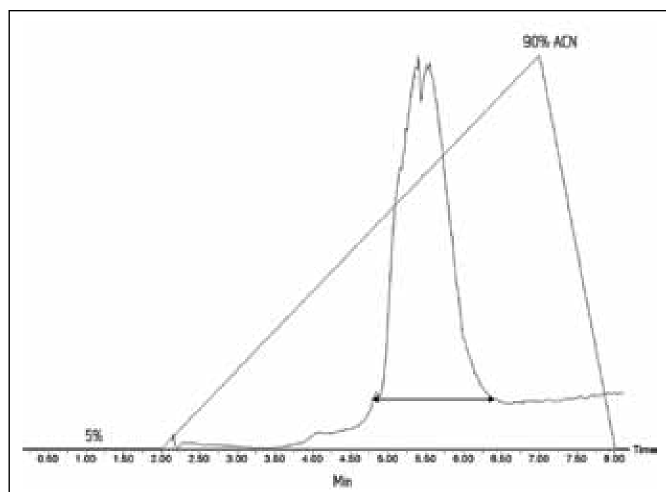


Figure 10. TIC chromatogram from LC/ESI-MS analysis of deglycosylated IgG1 produced by PNGase F digestion (37 °C, 18 h).

The combined ESI mass spectrum of the deglycosylated IgG1 (Figure 11, inset) resulted in a deconvoluted mass spectrum (Figure 11) containing one major component (deglycosylated IgG1 with a mass of 145,328 Da), and one minor component (Unidentified, mass of 147,508 Da). The mass difference between the intact IgG1 (containing 2 core glycans) of 148,214 and the deglycosylated IgG1 145,328 Da is 2,886 Da.

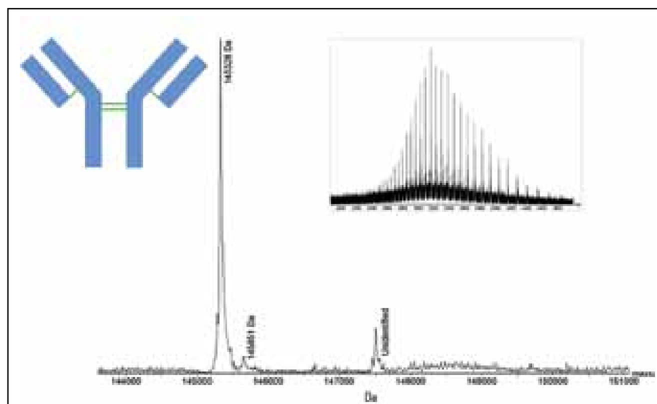


Figure 11. Combined ESI-TOF mass spectrum (inset) and deconvoluted mass spectrum of the deglycosylated IgG1 reveals a single major component with a mass of 145,328 Da.

Applying the correction factor of two Da for the N → D product of the enzymatic reaction produces a "real" mass difference of 2,888 Da or 1,444 Da per glycan chain. This mass is consistent with that arising from a common core fucosylated biantennary glycan structure (1,446 Da), as shown in Figure 1. This core structure has N-acetyl-glucosamine residues at the four non-reducing ends of the glycan branches, which are the common sites for extension by single galactose residues, as was observed to varying extent with the intact antibody.

Analysis of a reduced and deglycosylated IgG1

LC/MS data for the reduced and deglycosylated IgG1 was acquired under the same conditions as for the reduced IgG1, with partial resolution achieved between heavy and light chains using the extended gradient (Figure 12). The mass of the earlier eluting light chain (Figure 13) was determined to be 24,199 Da, in exact agreement with the mass determined for the reduced IgG without PNGase F processing.

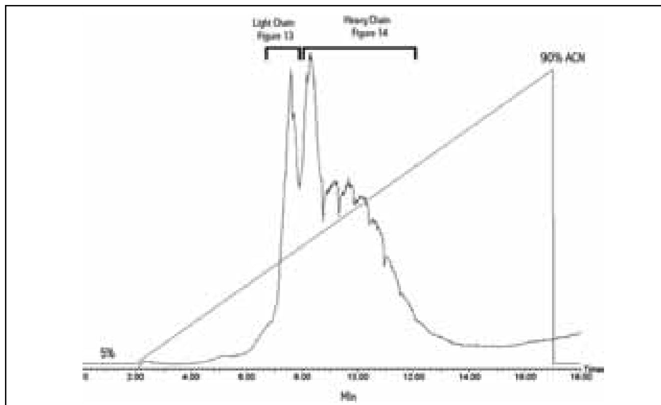


Figure 12. LC/MS TIC chromatogram of deglycosylated LC and HC generated from PNGase F deglycosylated IgG1 in the presence of 20 mM DTT. Partial separation of LC and HC was achieved using a 15 min (5 to 90%) gradient of acetonitrile.

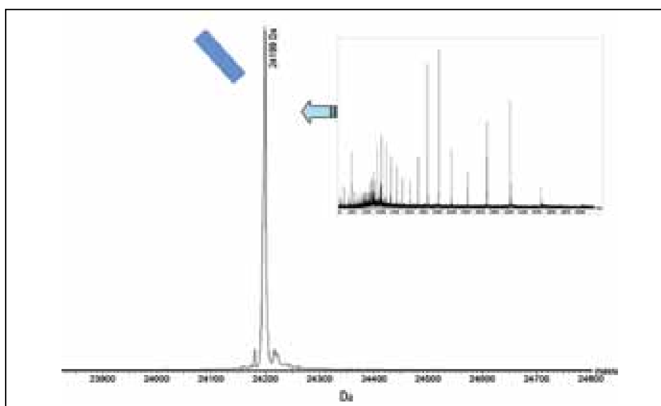


Figure 13. Combined (inset) and deconvoluted mass spectrum of the light chain (24,199 Da) derived from reduced deglycosylated IgG1. As expected, this mass corresponds to the mass of the light chain from the unprocessed antibody.

As would be expected, the later eluting heavy chain (Figure 14) is simplified to a single major species (48,476 Da) following deglycosylation. The mass difference between glycosylated (49,922 Da) and deglycosylated heavy chain was 1,447 Da, also accounting for the +1 Da N → D transformation of the glycosylation site. This measurement is once again consistent with an N-linked biantennary fucosylated carbohydrate core structure (1,446 Da) commonly found in the Fc region of IgG1 heavy chain.

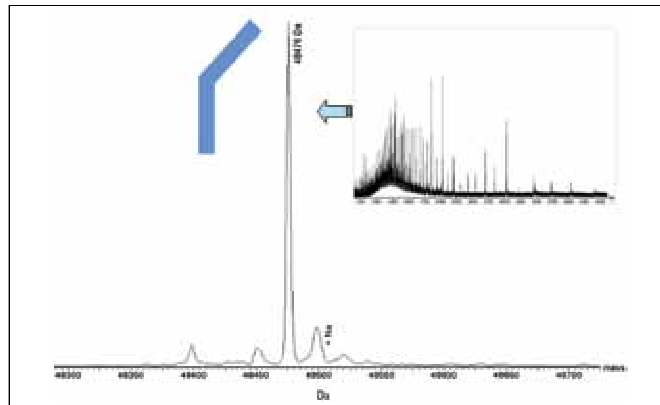


Figure 14. Combined (inset) and deconvoluted mass spectrum of the heavy chain (48,476 Da) derived from reduced deglycosylated IgG1. This mass corresponds to the mass of the heavy chain lacking the core glycan structure and related galactosylated variants.

PNGase F releases N-linked oligosaccharides as glycosylamines which are not retained by typical reversed phase columns used in these studies. The carbohydrates can be collected in the diverted flowthrough desalting fraction, and subjected to targeted methods for glycan sequencing to confirm the assigned glycan structures.

Analysis of an IgG1 subjected to papain digestion

Fab fragments and other truncated antibody structures offer many potential advantages over intact antibodies as immunochemical tools, diagnostic agents and therapeutic molecules. They demonstrate improved pharmacokinetics, lower incidences of patient immunogenicity and eliminate non-specific binding interactions associated with the glycosylated Fc portion of the IgG.[4, 5]

Papain is a nonspecific, thiol-endopeptidase that preferentially cleaves antibodies in the hinge region of the molecule. In an IgG1, the hinge region is proximal to the two disulfides that bridge the heavy chains. Depending on the location of papain cleavage within this region (above or below the disulfide bonds), processing can generate bivalent F(ab')2 fragments (~100 kDa) or univalent Fab fragments (~ 50 kDa), as well as their corresponding Fc fragments (~ 25 kDa). These papain fragment structures are illustrated in Figure 15.

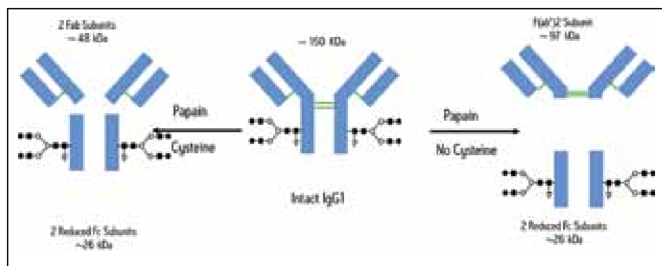


Figure 15. Papain processing of an IgG1 in the presence and absence of cysteine.

Interpreting these processing events by LC/MS can be complicated, as differential cleavage susceptibility of various IgG sub-classes and antibody source species creates some variability in processing results.[6, 7] In addition, the broad enzyme substrate specificity of papain may dictate the need for tight controls on reaction conditions (pH, time, enzyme concentration) to achieve consistently processed antibody fragments.

It has been reported[6] that the presence or absence of cysteine as well as solution pH can determine whether a Fab or F(ab')2 fragment is generated by papain. In that study, the production of F(ab')2 and Fc fragments was achieved by preactivating papain with cysteine, removal of excess cysteine following activation and subsequent digestion at pH 6.3. The TIC (Figure 16) of IgG1 digested under similar conditions reveals two peaks corresponding to these expected digestion products. This LC/MS analysis was accomplished using the more resolving LC/MS method that was previously used to analyze the reduced IgG1 sample.

The spectrum of the earlier eluting Fc fragment (Figure 17) demonstrates a wide biphasic charge state envelope (1200 to 4000 m/z), that reveals three major components (25,726 Da, 25,888 Da, and 26,050 Da) upon deconvolution with mass differentials of 162 Da; this corresponds to the Fc fragment with one or two galactose residues. The spectrum of the later eluting F(ab')2 fragment (Figure 18) reveals a single major component with a mass of 96,795 Da. Evidence for formation of any Fab fragments was not observed under cysteine free/pH 6.3 digestion conditions.

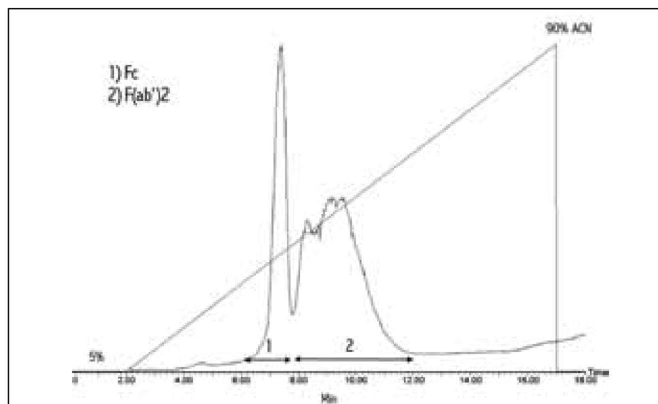


Figure 16. LC/MS TIC from the analysis of IgG1 papain digestion without cysteine in the digestion solution. Partial separation of the Fc (Peak 1) and F(ab')2 (Peak 2) fragments was achieved by using a 15 min linear gradient (5 to 90%) of acetonitrile.

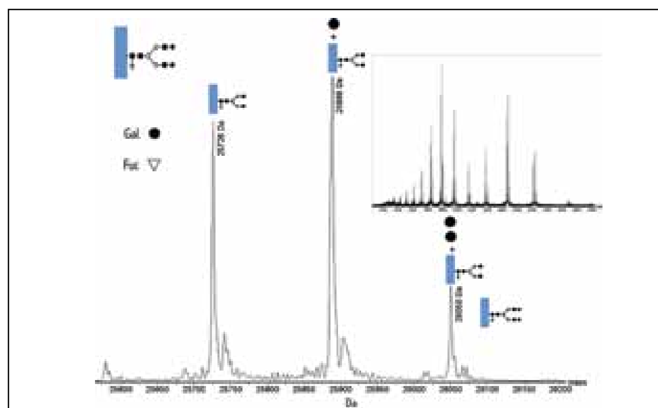


Figure 17. Combined mass spectrum (inset) of Fc fragment (peak 1) revealed a series of components upon MaxEnt1 deconvolution. Three components with a mass differences of 162 Da were observed that represented the Fc and core carbohydrate with no (25,726 Da), one (25,888 Da), or two (26,050 Da) terminal galactose residues.

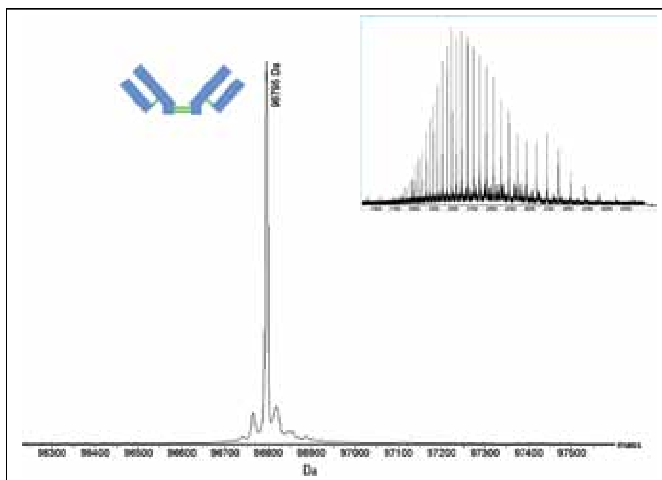


Figure 18. Combined (inset) and deconvoluted mass spectrum generated from the F(ab')2 fragment (peak 2) of the IgG1. A single major component with a mass of 96,795 Da was observed.

As with the other analyses, the masses observed correlate with the observed mass of the intact IgG1. In this case, the mass of the F(ab')2 plus two Fc (core glycan only) equals 148,247 Da, or 33 Da greater than the 148,214 Da observed for the intact IgG1. This is accounted for by the mass of two water molecules (18 Da each) added during hydrolytic cleavage of the two peptide bonds by papain.

The overnight digestion of IgG1 with papain (pH 7) in the presence of cysteine resulted in processing of the IgG1 to Fab and Fc fragments. Intermediates of this processing were observed when 2 h digestion was employed (not shown), but overnight papain digestion showed two partially resolved TIC peaks (Figure 19) that correspond to the expected products. The deconvoluted mass spectrum of the earlier eluting Fc fragment (Figure 20) again showed three components (25,726 Da, 25,888 Da, and 26,050 Da) corresponding to the Fc fragment with one or two galactose addition variants.

The combined mass spectrum (Figure 21, inset) generated from the later eluting peak revealed one major component with a mass of 47,752 Da that is attributed to the processed Fab fragment (Figure 20). Complete processing to the Fab was achieved, and no evidence of the F(ab')2 fragment was observed under these optimized conditions.

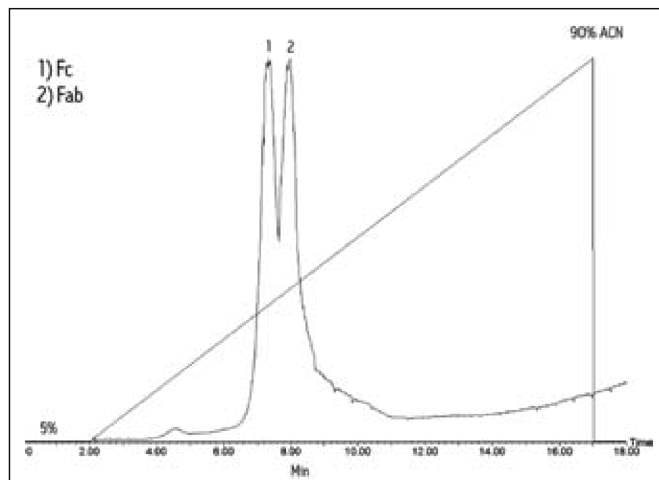


Figure 19. LC/MS TIC from the analysis of an IgG1 that was digested with papain at pH 7 in the presence of cysteine shows partial separation of the Fc (Peak 1) and Fab (Peak 2) fragments using a 15 min linear gradient (5 to 90%) of acetonitrile.

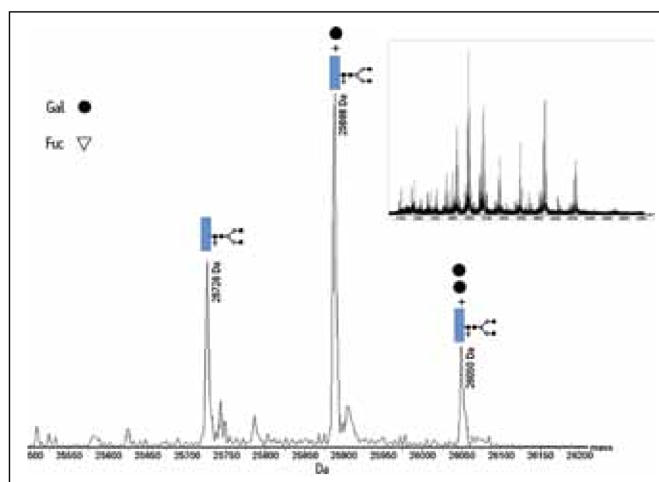


Figure 20. The combined mass spectrum (inset) of the Fc fragment generated by papain digestion in the presence of cysteine shows three components varying intensity differing by 162 Da. These correspond to the Fab fragment containing a core glycan with no (25,726 Da), one (25,888 Da), or two (26,050 Da) terminal galactose residues. This Fc fragment directly corresponds to the Fc generated using the cysteine free papain digestion conditions.

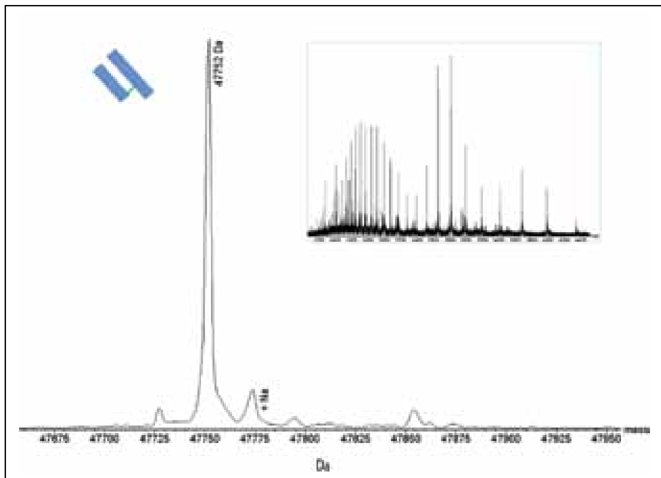


Figure 21. Combined (inset) and deconvoluted mass spectrum corresponding to the Fab fragment (peak 2) generated by papain digestion in the presence of cysteine. Complete processing of the F(ab')2 fragment to the Fab fragment (47,752 Da) was achieved under the optimized conditions.

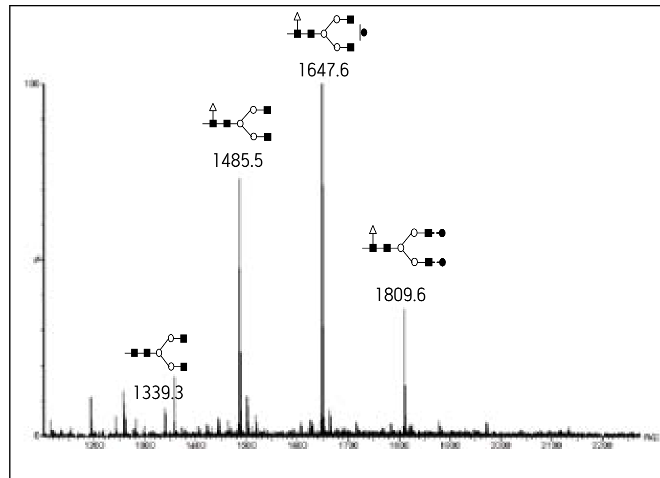


Figure 22. MALDI MS spectrum of purified glycans obtained from the intact antibody. Observed masses were consistent with the monosodiated adducts of the biantennary fucosylated glycan structures shown.

Confirmation of glycan structure

Data from the deglycosylation experiments on the intact and reduced antibody revealed PNGase F induced mass differences consistent with N-glycosylation with a fucosylated biantenerary core glycan. This result was validated by mass and tandem mass analysis of glycans released and purified from the intact antibody using MALDI. The MS spectrum of the purified glycans (Figure 22) revealed a pattern mirroring the glycan distribution observed with the heavy chain (Core glycan + 1 Galactose > Core glycan > Core glycan + 2 Galactose), all with masses consistent with the monosodium adducts of the expected glycan structures. The MS/MS spectrum of the sodiated core glycan (Precursor ion @ 1485.5 m/z) in Figure 23 provides additional high coverage confirmation of the predicted glycan structure.

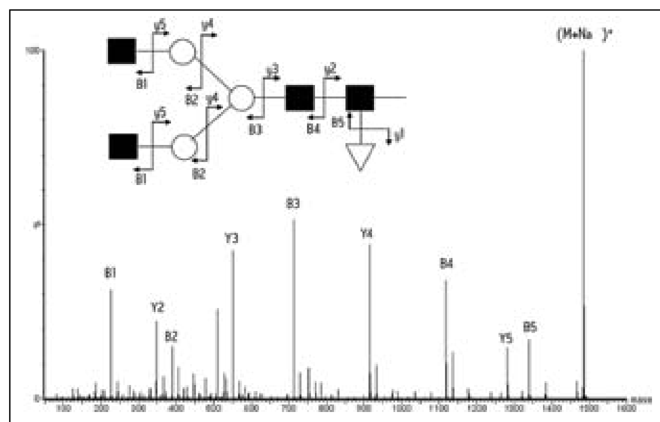


Figure 23. MALDI MS/MS of the sodiated non-glucosylated core glycan (Figure 22, Precursor ion 1485.5 m/z). High sequence coverage was obtained as detailed on the associated glycan structure.

CONCLUSION

In this work, we have demonstrated that antibodies and a broad subset of common antibody sub-structures can be efficiently analyzed by LC/MS using a common instrument configuration and two generic separation methods.

The MassPrep Desalting Cartridge proved effective for LC/MS analysis of an IgG1 antibody and for partial resolution of the reduced (or digested) substructures of this antibody.

ESI-TOF MS analysis is capable of generating precise mass measurements (<30 ppm) over the wide range of antibody structures and substructures studied.

Using MALDI for MS and MS/MS analysis of released glycans can confirm the tentative assignments generated by comparing an intact antibody or substructure with the deglycosylated version.

The use of multiple antibody analysis conditions can generate a wealth of complementary – and entirely self-consistent – LC/MS results.

Acknowlegements

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Rapid Profiling of Monoclonal Intact Antibody by LC/ESI-TOF MS

Asish B. Chakraborty, Scott J. Berger, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The pipeline of therapeutic antibodies is growing, as the pharmaceutical industry increasingly focuses on development of biotechnology-derived drugs and diagnostic agents. This dynamic has put pressure on bioanalytical groups to produce more generic methodologies for antibody characterization, with higher throughput and faster sample turnaround times.

While antibody selectivity varies appreciably, overall antibody structures are highly conserved. Standard analytical methods can often be employed as the basis for optimizing analysis of individual molecules and their variants. High resolution mass spectrometry provides a powerful holistic approach for profiling batch-to-batch variations, and studying the structural changes associated with drug production, formulation, and storage.

Most antibodies are stored in a matrix of nonvolatile buffers, salts, and stabilizers, and their removal (desalting) is a common challenge encountered for antibody mass analysis.

In this study, we have devised a methodology using UPLC®/MS technology for rapid desalting and efficient mass profiling of an intact antibody and its associated variants.

Figure 1. The ACQUITY UPLC System with LCT Premier XE Mass Spectrometer.

EXPERIMENTAL

UPLC conditions

LC system: Waters ACQUITY UPLC® System
Column: Waters MassPREP™ Micro Desalting Column (2.1 x 5 mm)
Column temp.: 80 °C

MS conditions

MS system: Waters LCT Premier™ ESI-TOF MS
Ionization mode: ESI positive, V mode
Capillary voltage: 3200 V
Cone voltage: 40 V
Desolvation temp.: 350 °C
Source temp.: 150 °C
Desolvation gas: 800 L/Hr
Ion guide 1: 100 V
Acquisition range: 600 to 5000 m/z

System configuration

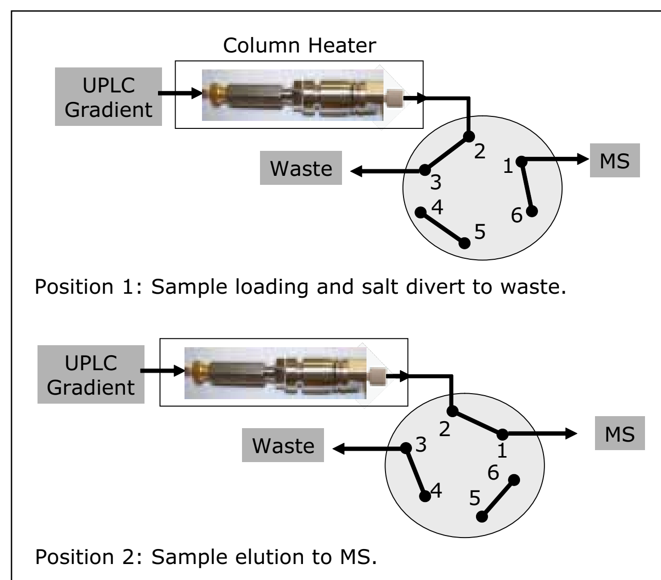

Figure 2. Fluidic configuration for LC/MS analysis. A post-column salt diversion valve (top-left corner of the LCT Premier XE) was utilized to divert buffers and nonvolatile salts to waste during the sample loading step.

Time (min)	%B	Flow (mL/min)	Curve	
0.00	5	0.5	Initial	Load/Wash -Divert Flow-
0.50	5	0.5	6	
0.51	5	0.2	6	Gradient
2.00	90	0.2	6	
2.10	5	0.5	6	Column Washing and Regeneration
2.70	90	0.5	6	
2.80	5	0.5	6	
3.40	90	0.5	6	
3.50	5	0.5	6	
4.00	5	0.5	6	

A = 0.1% formic acid (water) B = 0.1% formic acid (ACN)

Table 1. Gradient profile used for antibody analysis.

Preparation of intact IgG1

A monoclonal murine IgG1 (11.3 µg/µL, 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3) was diluted with 50 mM NH_4HCO_3 to 0.1 µg/µL for analysis. Following microcentrifugation, LC/MS analyses were performed on 5 µL of the diluted sample.

RESULTS

A fast (4 min cycle time) and efficient LC/ESI-MS method was used to profile multiple structural variants of an IgG. To minimize cycle time and maximize system performance, higher flow rates were used for loading, desalting, and column regeneration. A system controlled post-column valve was used for waste diversion of sample buffers and salts.

Additional sawtooth (rapid) gradient cycles were applied to regenerate the column to pre-injection conditions as part of each analysis (Figure 3). This avoided the potential need to separate difficult samples with inter-run blank injections.

Overlaid TICs (y-axis linked) for this experiment and the associated summed mass spectra are shown as Figures 3 and 4, respectively. The results reveal no detectable carryover following a 0.5 µg injection of the antibody.

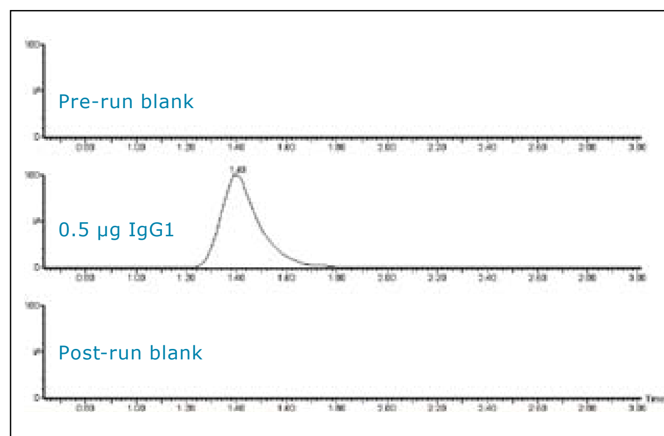

Figure 3. Total ion chromatograms (TICs) resulting from LC/MS analyses of an intact antibody.

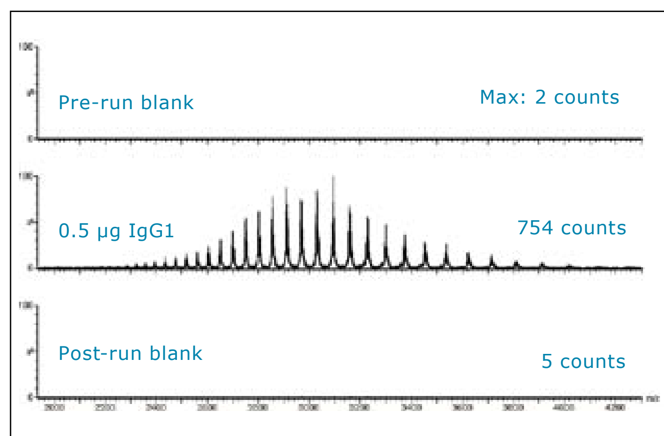

Figure 4. Combined ESI-TOF mass spectra (1.2 to 1.7 min, Figure 3) demonstrating system regeneration post-injection.

The summed ESI-TOF mass spectrum for the antibody revealed a charge state envelope over 2300 to 4000 m/z (Figure 4). The +51 charge state region has been enlarged (Figure 5, inset) to show greater detail, indicating at least six significant antibody variants.

The MaxEnt1 deconvoluted spectrum (Figure 5) clearly revealed carbohydrate-related heterogeneity. Five peaks demonstrate characteristic sequential mass differences of ~162 Da consistent with extension of the two core glycan structures by up to four hexose (galactose) residues. The earliest peak pair shows a mass difference of 146 Da corresponding to incomplete occupancy of a fucosylation site on the core glycans.

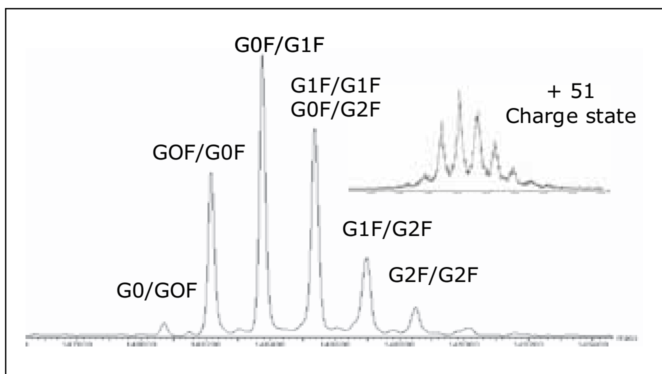

Figure 5. MaxEnt1 deconvoluted mass spectrum of the intact IgG1. Major variants observed were due to carbohydrate heterogeneity.

The MaxEnt1 algorithm produces a result that is quantitatively conserved with the raw spectral data, and is often used for profiling and monitoring this glycoform pattern. The analysis also confirmed that other potential variants (e.g., incomplete heavy chain lysine processing) were absent from this molecule.

CONCLUSION

A simple LC/MS configuration has been demonstrated to permit efficient desalting and rapid (4 min) LC/MS analysis of an intact murine IgG1 monoclonal antibody. This methodology yielded a simple holistic view of the molecule by providing intact mass information that can be used to confirm primary structure, and profiles of macro-heterogeneity (glycosylation, Lys processing) that can be used to assess consistency of IgG production.

Overall, the adoption of rapid characterization methodologies using UPLC/MS should permit analytical groups to make maximal use of their personnel and resources, and adapt to the demand of R&D organizations for greater sample capacity, and decreased analysis turnaround times.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
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Routine Profiling of Reduced Antibodies by LC/ESI Quadrupole MS

Asish B. Chakraborty, Scott J. Berger, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Biopharmaceutical companies are requiring their analysts to screen increasingly larger quantities of monoclonal antibody samples to support clone selection, stability, and product formulation studies. Much of this analysis involves routine confirmation of reduced antibody (light/heavy chain) masses and rapid profiling of their structural variants.

Analysis of reduced antibody subunits enables the detection and subunit localization of large mass difference variants (e.g., proteolytic clipping, glycosylation, lysine processing) and lower mass modifications such as oxidation and pyroglutamate formation. These smaller mass modifications are obscured under the isotopic envelope during intact antibody analysis.

Antibody light and heavy chains produce electrospray charge envelopes well within the ion transmission and detection capabilities of both high resolution time-of-flight (TOF) MS systems and lower resolution quadrupole-based mass detectors. For antibodies of typical complexity, heterogeneity of the reduced subunits can be profiled using both mass detection technologies.

While TOF instruments provide greater precision for mass determination and the additional resolution to discern more complex variant profiles, the utility of the Waters ACQUITY® SQD and its single quadrupole mass detection for routine screening and mass confirmation also provides tangible benefits. In fact, the system's features – such as low start-up costs, easy-to-use automated tuning and calibration, and walk-up open access operation – enable organizations to provide their analytical groups with mass analysis capabilities for routine antibody screening using UPLC®/MS.

Antibodies are typically stored in a nonvolatile matrix of buffers, salts, and stabilizers. Their removal (desalting) is one of the challenges encountered during routine mass analysis. In this study, we have combined a quadrupole-based LC/MS system with robust methodology for rapid sample desalting and efficient variant profiling of reduced monoclonal antibodies.

Figure 1. The ACQUITY SQD LC/MS System.

EXPERIMENTAL

UPLC conditions

LC system:	Waters ACQUITY® SQD
Column:	MassPREP™ Micro Desalting Column (2.1 x 5 mm)
Column temp.:	80 °C

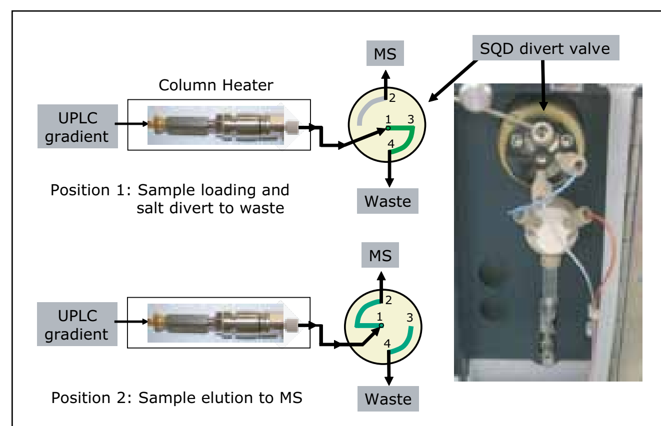

Figure 2. Fluidic configuration for LC/MS analysis. An integrated post-column valve used for salt diversion during sample loading is located behind the right-hand door of the SQ mass detector. This valve is also utilized to divert LC flow and direct the AutoTune calibrant during automated system startup.

Time (min)	%B	Flow (mL/min)	Curve	
0.00	5	0.2	Initial	Load/Wash -Divert Flow-
0.50	5	0.2	6	
0.51	10	0.2	6	Gradient
7.61	50	0.2	6	
8.00	90	0.5	6	
8.10	5	0.5	6	Column Washing and Regeneration
8.60	90	0.5	6	
8.70	5	0.5	6	
9.20	90	0.5	6	
9.30	5	0.5	6	
9.80	5	0.5	6	

A = 0.1% Formic Acid (Water)
B = 0.1% Formic Acid (ACN)

Table 1: Gradient profile used for reduced IgG1 analysis.

MS conditions

MS system: Waters SQ Detector
Ionization mode: ESI positive
Capillary voltage: 4500 V
Cone voltage: 30 V
Desolvation temp.: 450 °C
Source temp.: 150 °C
Desolvation gas: 800 L/Hr
LM 1/HM 1 resolution: 11.2/14.8
Acquisition range: 600 to 2000 m/z

Reduction of an IgG1 to heavy (HC) and light (LC) chain subunits

Reduction of disulfides in an IgG1 (0.5 µg/µL) was achieved using 20 mM DTT at 80 °C for 15 min. The reduced sample was acidified with formic acid (to 1%), microcentrifuged, and injected onto the column for LC/MS analysis (2.5 µL).

RESULTS AND DISCUSSION

A rapid LC/ESI-MS method (Table 1) was used for resolution and mass analysis of IgG1 heavy and light chains. For efficient sample desalting, a system-controlled post-column valve (Figure 2) was used for waste diversion of sample buffers and salts prior to initiating the 7.5 min analysis gradient. Additional sawtooth (rapid) gradient cycles were applied following the analysis gradient to regenerate the column back to pre-injection conditions (Figure 3). To minimize run cycle times and maximize system performance, higher flow rates were applied for column regeneration.

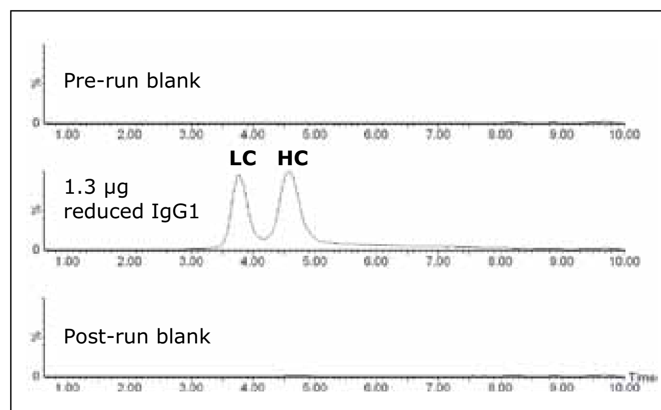

Figure 3. Total ion chromatograms (TICs) from LC/MS analysis of light and heavy chains from a reduced antibody.

The total ion chromatogram (TIC) from a reduced antibody LC/MS analysis is displayed in Figure 3. The 10-minute LC/MS run largely resolved the light chain from the later-eluting heterogeneously glycosylated heavy chain. Comparison of pre-run and post-run TIC traces demonstrates the efficient regeneration of the LC system following the reduced antibody analysis.

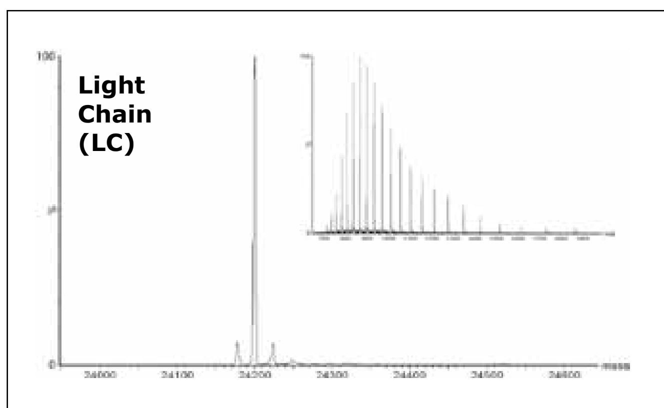

Figure 4. Combined ACQUITY SQD-ESI mass spectrum (inset, 3.5 to 4.0 min, Figure 3) and resulting MaxEnt1 deconvoluted mass spectrum of the antibody light chain.

Figure 4 displays the summed mass spectrum (inset) and MaxEnt1 deconvoluted spectrum of the light chain, which reveals a single major peak at 24,200 Da. Minor peaks (corresponding to the sodium adduct and loss of water) were also visible in the deconvoluted spectrum.

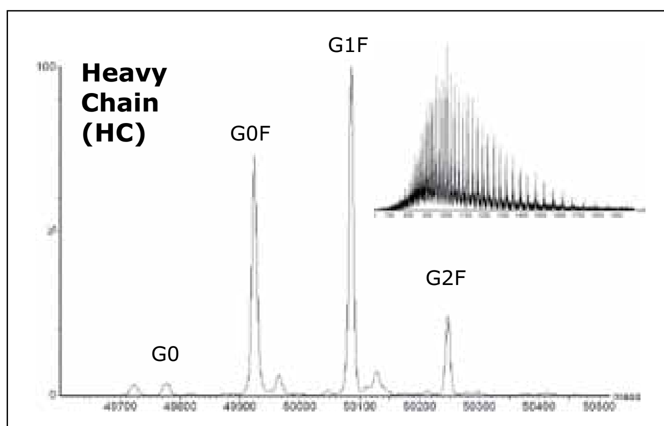

Figure 5: Combined mass spectrum (inset, 4.3 to 5.0 min, Figure 3) and resulting MaxEnt1 deconvoluted mass spectrum of the heavy chain.

Figure 5 depicts the summed mass spectrum (inset) and resulting MaxEnt1 deconvoluted mass spectrum of the glycosylated heavy chain. The major peaks correspond to the heavy chain containing the core fucosylated glycan (G0F 49,924 Da), a minor non-fucosylated form (G0), and core glycan variants extended by one or two terminal galactose residues (G1F, G2F). These results are fully consistent with studies of this antibody by LC/ESI-TOF MS shown in earlier application notes.

CONCLUSION

In this application note, we have demonstrated a quadrupole-based LC/MS methodology that is capable of rapidly resolving and profiling the light and heavy chain variants obtained from a reduced IgG1 monoclonal antibody.

Many bioanalytical groups are looking to balance high-end protein characterization capabilities with lower-cost solutions for routine confirmation of protein structures. The combination of TOF and quadrupole UPLC/MS platforms can help laboratories achieve both analytical goals without over-extending their financial or human resources.

The utility of quadrupole mass detection for reduced antibody mass profiling, combined with the throughput, robustness, and ease-of-use of an integrated LC/MS system, presents biopharmaceutical organizations with flexible capabilities to respond to their ever-expanding demands for routine antibody mass analysis.

References

1. Rapid Profiling of Monoclonal Intact Antibodies by LC/ESI-TOF MS. Waters Application Note; 2007: 720002393EN.

2. Rapid Screening of Reduced Monoclonal Antibodies by LC/ESI-TOF MS. Waters Application Note; 2007: 720002394EN.

3. Characterization of an IgG1 Monoclonal Antibody and Related Substructures by LC/ESI-TOF MS. Waters Application Note; 2007: 720002107EN.

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

[APPLICATION NOTE]

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Rapid Screening of Reduced Monoclonal Antibody by LC/ESI-TOF MS

Asish B. Chakraborty, Scott J. Berger, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Biopharmaceutical R&D groups are asking their analysts to screen increasingly larger numbers of monoclonal antibody samples to support clone selection, stability, and product formulation studies. While various antibodies can exhibit unique selectivity, their overall structures are conserved within an antibody class, and standard analytical methods can often be employed for their routine analysis.

While intact antibody LC/MS analysis is useful for profiling glycovariants and C-terminal Lys processing, even more information can be gleamed from analysis of the light and heavy chain subunits from the disulfide-reduced antibody. LC/MS analysis of reduced antibodies offers the ability to detect the lower mass modifications such as oxidation (+16 Da) and pyroglutamate formation (-17 Da) that are obscured under the isotopic envelope of an intact antibody. Additionally, larger mass modifications such as nonenzymatic glycations (+162 Da) can be more clearly revealed with analysis of individual subunits.

Most antibodies are stored in a nonvolatile matrix of buffers, salts, and stabilizers. Their removal (desalting) is one of the challenges encountered during routine mass analysis.

In this study, we have devised an LC/MS methodology, using UPLC®/MS technology, for rapid sample desalting and efficient characterization of the heavy and light chain structures from a reduced antibody.

Figure 1. The ACQUITY UPLC System with LCT Premier XE Mass Spectrometer.

EXPERIMENTAL

UPLC conditions

LC system:	Waters ACQUITY UPLC® System
Column:	Waters MassPREP™ Micro Desalting Column (2.1 x 5 mm)
Column temp.:	80 °C

MS conditions

MS System:	Waters LCT Premier™ XE ESI-TOF MS
Ionization mode:	ESI positive, V mode
Capillary voltage:	3200 V
Cone voltage:	40 V
Desolvation temp.:	350 °C
Source temp.:	150 °C
Desolvation gas:	800 L/Hr
Ion guide 1:	5 V
Acquisition range:	600 to 2500 m/z

System configuration

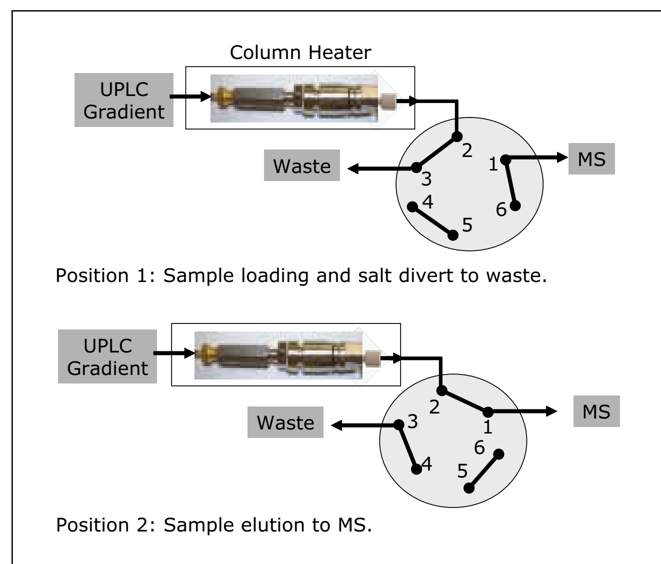

Figure 2. Fluidic configuration for LC/MS analysis. A post-column salt diversion valve (top-left corner of the LCT Premier XE) was utilized to divert buffers and nonvolatile salts to waste during the sample loading step.

Time (min)	%B	Flow (mL/min)	Curve	
0.00	5	0.2	Initial	Load/Wash -Divert Flow-
0.50	5	0.2	6	
0.51	10	0.2	6	Gradient
7.61	50	0.2	6	
8.00	90	0.5	6	
8.10	5	0.5	6	Column Washing and Regeneration
8.60	90	0.5	6	
8.70	5	0.5	6	
9.20	90	0.5	6	
9.30	5	0.5	6	
9.80	5	0.5	6	

A = 0.1% formic acid (water) B = 0.1% formic acid (ACN)

Table 1. Gradient profile used for reduced IgG1 analysis.

Preparation of reduced IgG1 (to form HC and LC)

Reduction of disulfides in an IgG1 (0.5 µg/µL) was achieved using 20 mM DTT at 80 °C for 15 min. The reduced sample was acidified with formic acid (to 1%), microcentrifuged, and injected onto the column for LC/MS analysis (2.5 µL).

RESULTS

A rapid LC/ESI-MS method was used to resolve and characterize IgG heavy and light chains. For efficient sample desalting, a system-controlled post-column valve was used for waste diversion of sample buffers and salts prior to initiating the analysis gradient.

Additional sawtooth (rapid) gradient cycles were applied following the analysis gradient to regenerate the column back to pre-injection conditions (Figure 3). This avoids the need to separate difficult samples with inter-run blank injections. To minimize run cycle time, and maximize system performance, higher flow rates were applied for column regeneration.

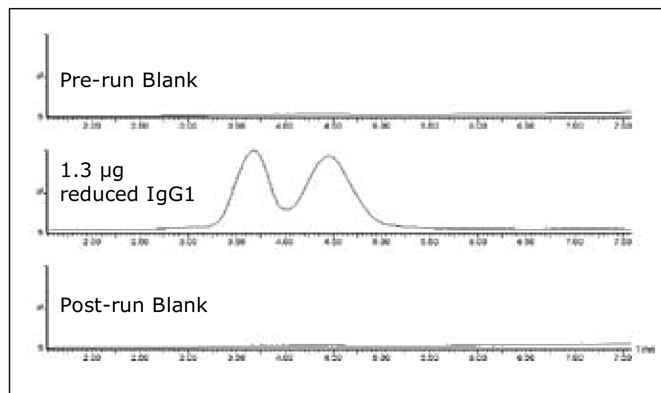

Figure 3. Total ion chromatograms (TICs) from LC/MS analyses of light and heavy chains from a reduced antibody.

The TIC of the reduced antibody analysis is displayed in Figure 3. The 10-minute LC/MS run largely resolved the earlier eluting light chain from the later eluting glycosylated heavy chains.

Figure 4 displays the summed mass spectrum (inset) and MaxEnt1 deconvoluted spectrum of the light chain, which reveals a single major peak at 24,199 Da. Minor peaks (sodium adduct and loss of water) are also visible in the deconvoluted spectrum.

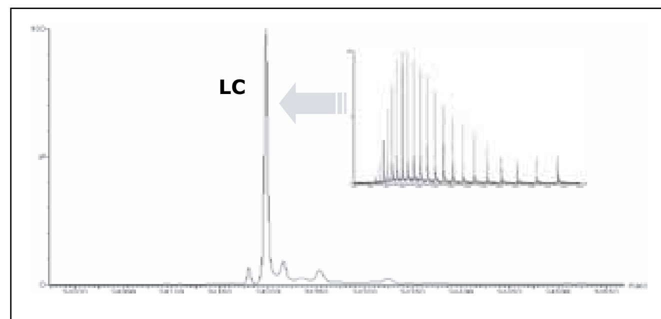

Figure 4. Combined ESI-TOF mass spectrum (inset, 2.3 to 3.9 min, Figure 3) and resulting MaxEnt1 deconvoluted mass spectrum of the light chain.

Figure 5 depicts the summed mass spectrum (inset) and resulting MaxEnt1 deconvoluted mass spectrum of the glycosylated heavy chain. Three major peaks with mass differentials of ~162 Da correspond to the heavy chain containing the core fucosylated glycan, and variants extended by one or two terminal galactose residues.

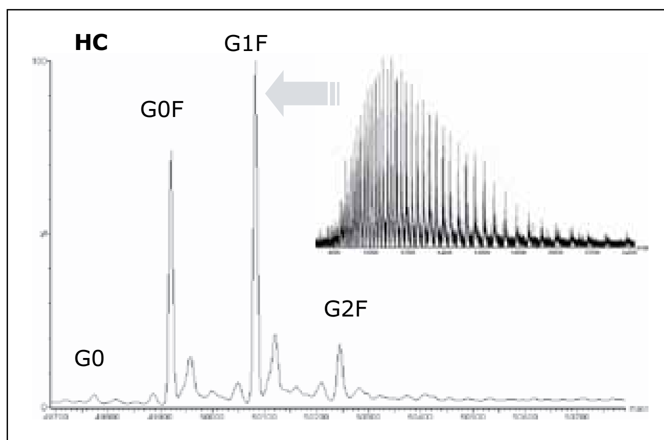

Figure 5. Combined mass spectrum (inset, 4.1 to 5.0 min, Figure 2) and resulting MaxEnt1 deconvoluted mass spectrum of the heavy chain. The major peaks differing by 162 Da corresponded to zero (49,922 Da), one, and two terminal galactose on the bound core N-glycan structure.

CONCLUSION

We have demonstrated an LC/MS methodology that is capable of rapidly resolving and efficiently characterizing light and heavy chains obtained from a reduced monoclonal antibody. The inclusion of additional sawtooth gradient cycles in the method eliminated the need for blank injections between samples, further increasing the utility of this approach for routine antibody screening.

Overall, sample throughput gains achieved with rapid characterization methods using UPLC/MS should provide bioanalytical groups the flexibility and capacity to adapt to the ever expanding demands of their parent organizations.

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

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Rapid Characterization of Variable Regions of Monoclonal Antibodies by Top-Down Sequencing using SYNAPT HDMS

Petra Olivova, Weibin Chen, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The Waters® SYNAPT™ High Definition Mass Spectrometry™ (HDMS™) System combines high-efficiency ion mobility with high-performance tandem mass spectrometry. This enables the analysis of samples differentiated by size, shape, and charge, as well as mass, to deliver increased specificity and sample definition.

In this study, we describe the use of the Synapt HDMS System to quickly obtain a partial amino acid sequence of a monoclonal antibody (MAb) via a top-down approach.

In this method, reduced MAb is introduced to the Synapt HDMS System via on-line desalting. Selected charge states of the intact light chain subunit are subjected to collision-induced dissociation (CID) prior to separation of the resulting fragment ions based on their gas phase mobilities.

Post-acquisition data processing produces simplified top-down fragmentation spectra containing fragment ions predominantly from one charge state, from which N-terminal amino acid sequence (up to 11 residues) of IgG can be readily deduced.

This structural information, coupled with molecule weight measurement of the intact MAb, can be used to support comparability studies and as a rapid ID test, reducing testing times and allowing for more efficient manufacturing processes.

EXPERIMENTAL

Partial reduction of the IgG1 followed a published procedure.[1] The IgG1 was reduced using DTT at 37 °C in the presence of Tris-HCl buffer (pH 8.0) containing EDTA. The sample was then diluted with formic acid and stored at 10 °C prior to analysis.

Reversed phase desalting of the reduced antibody was performed on a nanoACQUITY UPLC® System configured with a desalting column (MassPREP™ micro, Part No. 186004032). After injection (2.5 μg), the column was washed to remove the salts and other contaminants. Then the analyte was eluted (10 to 90% B in 15 min, flow rate of 20 μL/min, column temperature of 65 °C) and the column was re-equilibrated with initial conditions.

Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in ACN

MS conditions

MS system:	Waters SYNAPT HDMS System
Ionization mode:	ESI positive (V mode)
Capillary voltage:	3200 V
Cone voltage:	35 V
Desolvation temp.:	150 °C
Desolvation gas:	100 L/Hr
Source temp.:	100 °C
Acquisition range:	100 to 2500 m/z
Trap collision energies:	Ramp 25 to 45 V
Transfer collision energies:	4 V
Scan time:	1s (0.2s interscan)
IMS gas:	N2 gas
IMS gas pressure:	6.29e-1 mbar
Pulse height:	Fixed, 8.4 V

Protein ions produced during electrospray ionization are sampled by a ZSpray™ source and passed through a quadrupole, which was set to isolate light chain ions of a specific m/z. The selected ions are subjected to CID in the TRAP portion of the Triwave™ ion guiding device (Figure 1).[2] The TRAP T-Wave™ traps, accumulates, and fragments ions, in this case for up to 100 msec. The ions are then gated into the IMS T-Wave, where the high-efficiency ion mobility separation (IMS) +1, +2, and +3, and multiply-charged peptide fragments occurs.

RESULTS

The partially reduced IgG1 was initially analyzed using the instrument in time-of-flight (TOF) mode. The TOF spectrum of the light chain obtained by on-line LC/MS analysis is shown in Figure 2A.

In the following acquisition experiment, five different charge states [+17 (m/z 1424.1), +18 (m/z 1345.0), +19 (m/z 1274.3), +20 (m/z 1210.6), +21 (m/z 1153.0)] were selected for fragmentation in the TRAP T-Wave (Figure 2A). Data were acquired with the instrument in mobility TOF mode (IMS-TOF).

Figure 2B displays a two-dimensional DriftScope™ plot diagram obtained from the ion mobility separation of fragments from the light chain of partially reduced IgG1. This figure shows that there is systematic difference in the drift time for +1, +2 and multiply charged fragments at a given m/z. Species with different charges are clearly separated according to their ion mobility.

As a result, all the +1 ions can be located in one ion cloud. Likewise, the +2 are represented by the data in a separate region. Figure 2C shows the raw mass spectrum of the light chain fragments from the selected region of +1 ions. The spectrum contains consecutive mass peaks from the N-terminal region of light chain sequence (up to 11 residues).

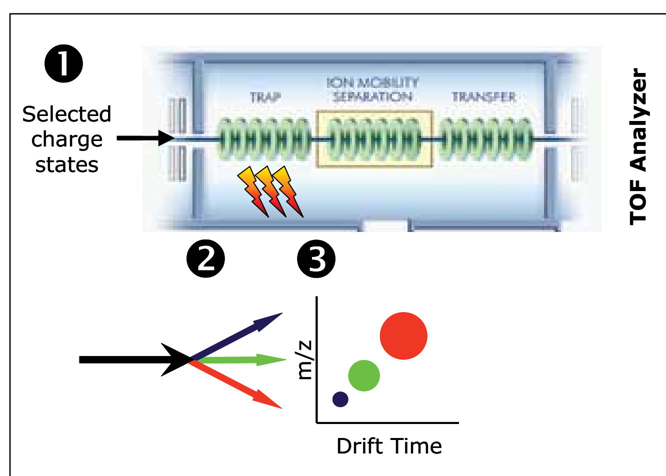

Figure 1. Schematic of the top-down experiment using the SYNAPT HDMS System, which incorporates Triwave technology.

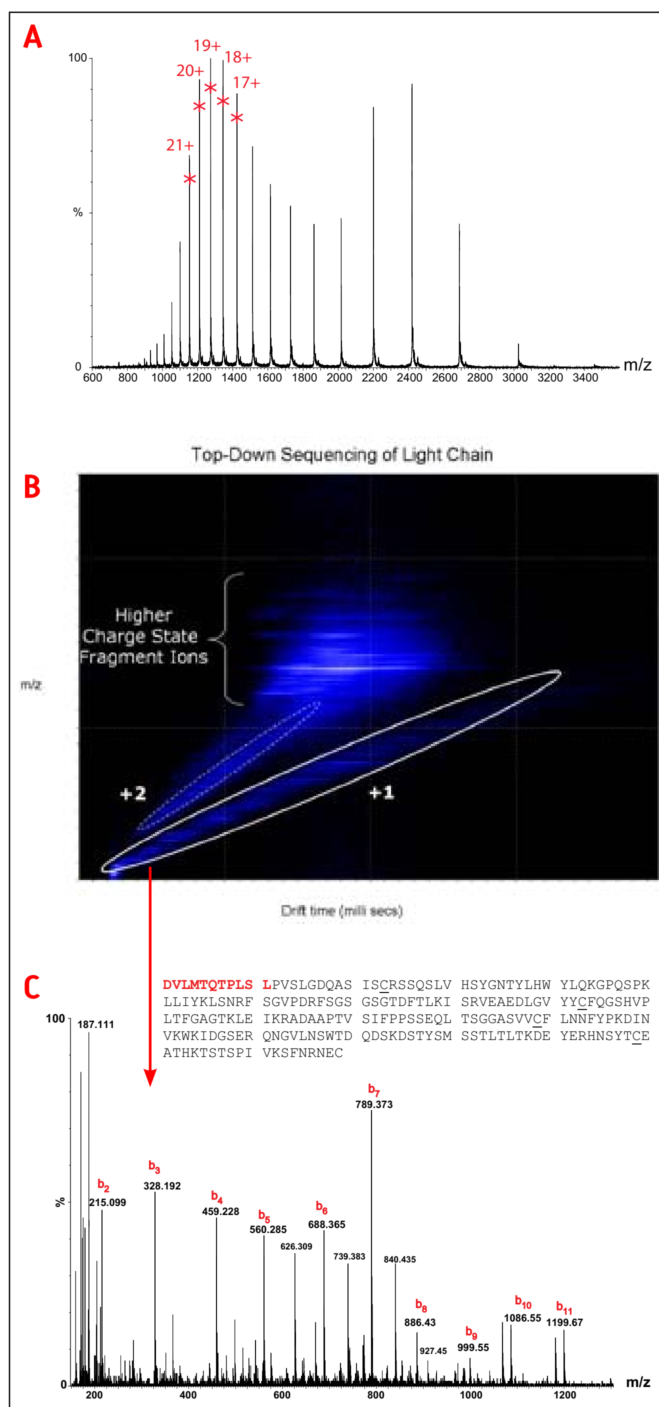

Figure 2. (A) The combined ESI-TOF raw spectrum of the light chain with labeled charge states selected for fragmentation. (B) Driftscope plot (HDMS data) showing the gas-phase separation of the fragment ions from fragmentations of the intact light chain. (C) The summed mass spectrum of all the singly charged ions generated from the intact light chain fragmentation process.

CONCLUSION

This work presents the top-down characterization of the variable region of a monoclonal antibody using the SYNAPT HDMS System. The method allows sequencing of the N-terminal region of the intact light chain in a simple, fast experiment that can be used for high-throughput screening of mutation variability during antibody production.

The method can also be used for a fast ID test, reducing QC testing time so product can be realized faster. The results demonstrate that the Synapt HDMS System is a superior tool to characterize MAb and other proteins.

References

1. Yu, L.; et al. Rapid Commun. Mass Spectrom. 2006, 20, 3674-3680.
2. Giles K; et al. Rapid Commun. Mass Spectrom. 2004, 18, 2401-2414.

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

[TECHNOLOGY BRIEF]

Enhancing the Characterization of Biomolecules with High Resolution LC/MS

GOAL

Technology advances for biomolecule characterization enable more in-depth information to be acquired for managing the pipeline, ensuring comparability and biosimilarity of biologics, and avoiding problems downstream. We demonstrate how the improved sensitivity available with the Xevo® G2-S QTof System, featuring StepWave™ ion transfer optics technology and QuanTof™ technologies, supports the characterization goals of biopharmaceutical organizations.

BACKGROUND

The efficient characterization of biotherapeutics is increasingly important to both regulatory agencies and to pharmaceutical companies to ensure the safety and efficacy of biotherapeutic products. Analytical solutions that are able to provide more detailed characterization about a potential biotherapeutic have always been highly desirable to the biopharmaceutical industry.

StepWave and QuanTof technologies for the high-performance Xevo family of mass spectrometers empower scientists with the analytical tools that are required to achieve more in-depth characterization of biomolecules. Data from the mass analysis of an intact monoclonal antibody, from its subunits, and from peptide mapping will demonstrate the superior MS sensitivity and resolution that can be achieved with the Xevo G2-S QTof System, while maintaining robust operation.

StepWave ion transfer optics deliver maximum sensitivity using a larger sampling orifice to capture significantly more ions compared to earlier generations of MS instruments. This unique off-axis T-Wave™ ion guide transfers ions from the ion source to the quadrupole MS analyzer with the highest possible efficiency while actively filtering out undesirable neutral contaminants. This keeps the critical components in the device untarnished, thus maintaining the robustness of the system. This novel design dramatically increases MS signals and minimizes background noise, thus significantly improving detection limits and the repeatability of quantitative assays.

Additionally, QuanTof Technology simultaneously delivers UPLC®-compatible mass resolution, matrix-tolerant dynamic range, quantitative performance, mass accuracy and speed of analysis.

The Xevo G2-S QTof dramatically increases sensitivity for large biomolecule characterization with novel off-axis ion transfer optics, StepWave Technology.

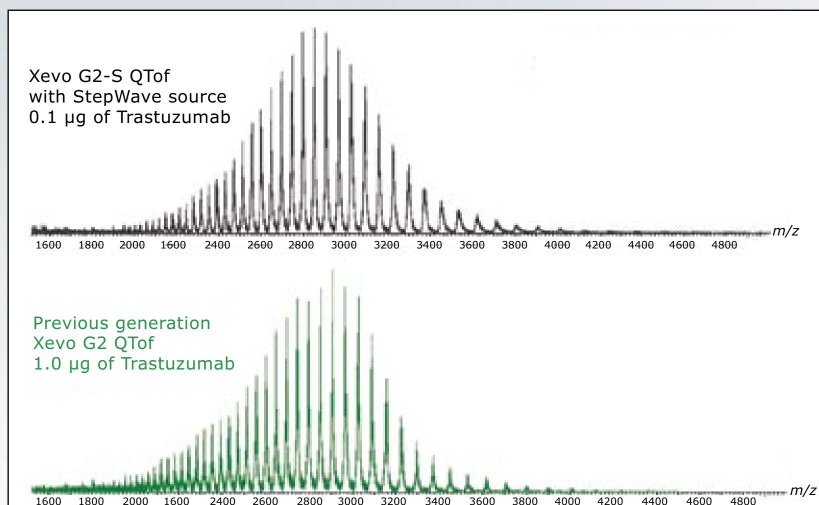

Figure 1. UPLC/MS analysis of intact monoclonal antibody. Summed mass spectra display that the intact mass data generated with the Xevo G2-S QTof with 0.1 µg on-column loading (10X dilution) is equivalent to that of the Xevo G2 QTof with 1.0 µg on-column loading (no dilution).

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THE SOLUTION

Trastuzumab, an IgG1 monoclonal antibody was used to compare the UPLC/MS performance of the new StepWave ion optics source incorporated in the Xevo G2-S QTof System against the previous generation (source) Xevo G2 QTof.

The mass analysis of intact trastuzumab data shown in Figure 1 were obtained during back-to-back experiments performed using two Xevo G2 QTof systems, one of which utilized StepWave ion transfer optics (top). The UPLC/MS analysis of 1.0 µg of intact trastuzumab was acquired in the resolution mode of the Xevo G2 QTof instrument, whereas the analysis of 0.1 µg of intact trastuzumab was acquired in the sensitivity mode of the Xevo G2-S QTof. The resolution was constant at 20,000 FWHM in both experiments.

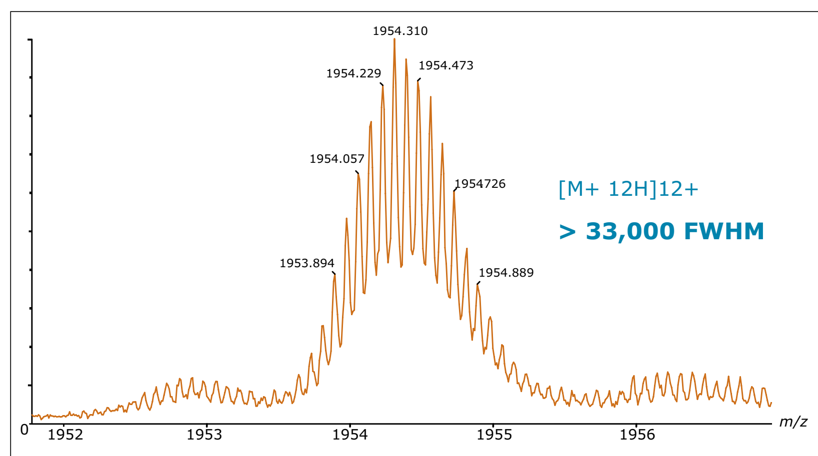
As can be seen in Figure 1, similar spectra were generated by the two instruments at an equivalent resolution, although 10-fold less material was loaded on column for the Xevo G2-S QTof, demonstrating its enhanced sensitivity for the mAb analysis. Increasing ion currents entering the Xevo G2-S QTof mass spectrometer enables high quality spectra to be acquired with less sample consumption.

In addition to the sensitivity increase, with QuanTof technology, the Xevo G2-S QTof is capable of achieving isotopic resolution for the examples shown here between 20 to 30 kDa and yielding low ppm mass accuracy results under routine LC/MS conditions.

Figure 2 shows the spectrum of the light chain of trastuzumab from the analysis of reduced antibody. Data collected in the resolution mode of Xevo G2-S QTof display the isotopic resolution of the light chain (25 kDa). The spectrum in Figure 2 is a zoomed-in region of the m/z scale of the mAb light chain, showing over 33,000 FWHM resolution of the 12+ charge state.

The clearly defined isotopic pattern of the 25 kDa trastuzumab light chain subunit enables isotopic models to be applied for the detection of lower mass modifications such as oxidation (+16 Da) and pyroglutamate formation (-17 Da) that are obscured under the isotopic envelope of an intact antibody.

For IgGs, such sensitive characterization with greater structural detail from the intact protein analysis may improve earlier decision making for clone selection, or assist in screening or variant analysis.



The effect of incorporating StepWave ion optics and QuanTof in the Xevo G2-S QTof was further demonstrated by peptide mapping experiments. Peptide mapping is an essential technique for the comprehensive characterization of biopharmaceutical products, including sequence confirmation, post-translational modifications (PTMs) and their site determination, and quantitation.

The tryptic digest of trastuzumab was analyzed using the UPLC/MS^E technique on both Xevo G2 QTof and Xevo G2-S QTof systems under the same instrument resolution. UPLC/MS^E is a simple, patented method of unbiased data acquisition that comprehensively analyzes all components in a single analysis. Both parent and associated fragment ions are generated in a single run with no prior knowledge of the sample.

Figure 3 shows the tryptic maps of trastuzumab acquired on both Xevo G2-S QTof (top) and Xevo G2 QTof (bottom) systems at 20,000 FWHM instrument resolution. The peptide map data from both instruments were then processed by BiopharmaLynx™ Application Manager for MassLynx™ Software.

The processed trastuzumab tryptic peptide map acquired on the Xevo G2-S QTof yielded 100% sequence coverage of the light chain and 98% of the heavy chain with even 7-fold less digest loaded on the column. Figure 4 shows the mass accuracy of a heavy chain peptide, T15, over 44 hours acquired on the Xevo G2-S QTof. The enhanced sensitivity and mass accuracy of the Xevo G2-S QTof, with automated data processing by BiopharmaLynx has enabled scientists to achieve more complete sequence coverage of light and heavy chains of mAb trastuzumab.

Figure 2. Zoomed mass spectrum of the reduced mAb, Trastuzumab, showing the isotopic distribution for the light chain (25 kDa).

SUMMARY

The performance of the Xevo G2-S QTof System for biomolecular characterization has been demonstrated using an intact antibody drug, trastuzumab (MW ~148 kDa), and its subunit light chains (~24 kDa) and tryptic digest.

The result shows that the Xevo G2-S QTof enables the efficient mass analysis of an intact antibody with enhanced sensitivity (10X) and mass accuracy.

The ability to provide isotopic resolution of proteins at the subunit level (with a size of 25 kDa) in a routine UPLC/MS analysis has also been demonstrated with the Xevo G2-S QTof System. The data suggests that this compact benchtop instrument possesses the capability to achieve finer structural details that is normally offered by advanced MS instrumentation.

The enhanced sensitivity and mass accuracy gained by StepWave and QuanTof technologies, along with automated data processing by BiopharmaLynx, enables scientists to achieve complete sequence coverage of their biomolecules with less sample consumption, and to confidently identify and quantify the PTMs with greater mass accuracy.

The superior sensitivity, resolution, and mass accuracy of Xevo G2-S QTof empowers organizations with the analytical solutions that are able to provide more detailed characterization of their biotherapeutics.

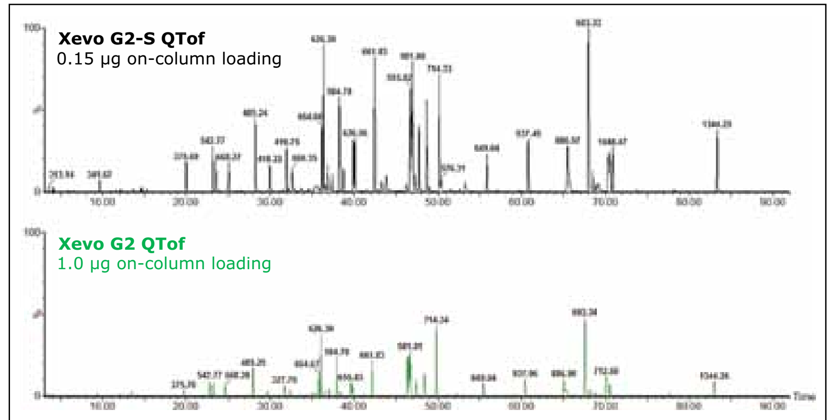


Figure 3. The UPLC/MS^E peptide maps of Trastuzumab generated from both Xevo G2-S QTof (top) and Xevo G2 QTof (bottom) systems. The peptide map acquired on the Xevo G2-S QTof, with 7-fold less digest loaded on an analytical column (2.1 x 150 mm, ACQUITY UPLC® BEH300 C18, 1.7 µm; 0.15 µg digest), shows higher sensitivity than the Xevo G2 QTof System with 1 µg on-column loading.

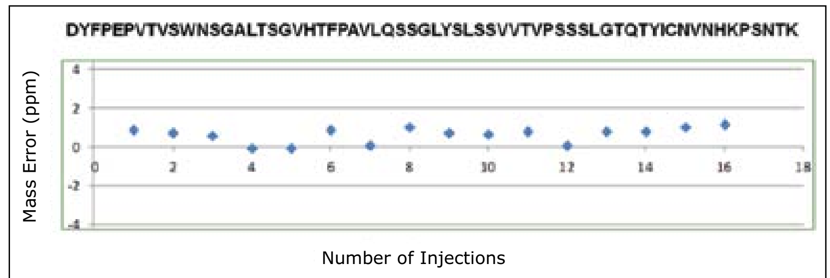


Figure 4. Mass accuracy of a heavy chain peptide T15 over 44 hours, showing system robustness. The system's mass accuracy even for a large peptide is within a narrow mass band of 1.2 ppm.

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Mass Accuracy for Biotherapeutics in the Benchtop Xevo G2 QTof with QuanTof Technology

Weibin Chen, Asish Chakraborty, St John Skilton, Hilary Major, and Scott Berger
Waters Corporation, Milford, MA U.S.

APPLICATION BENEFITS

Every aspect of the Xevo™ G2 QTof applies the design ethic of Engineered Simplicity™ to efficiently obtain the right results every time. Equipped with QuanTof™ technology, users obtain unheralded mass accuracy over a wide dynamic range of detection that leaves no room for doubt for biotherapeutic characterization studies.

WATERS SOLUTIONS

Xevo G2 QTof

BiopharmaLynx

KEY WORDS

UPLC/MS^E, QuanTof, mass accuracy, reproducibility, peptide map

INTRODUCTION

With the biopharmaceutical pipeline expanding faster than the organizations supporting biotherapeutic development, researchers have to cope with biotherapeutic protein complexity in better ways. Large biomolecules with a complex array of major and minor product variants are being developed into therapeutic products for more recalcitrant diseases. Samples are therefore complex and scientists are expected to rapidly determine fine-grained detail over a wide dynamic range of variants.

It is vital for health and safety, and for regulatory compliance, that biotherapeutic product variation is accurately measured. In a peptide map, this means knowing mass accuracy will be predictable over the full dynamic range of component detection.

Mass accuracy across the entire peptide map of a monoclonal antibody (mAb)

As demonstrated in Figure 1, mass accuracy has been established for a 148 kDa mAb on the Xevo G2 QTof by plotting the mass accuracy distribution for all 550+ antibody peptides assigned by BiopharmaLynx™ Software, an application manager for MassLynx™ Software, over a 90-min UPLC®/MS^E peptide map acquired in ESI positive resolution mode. The distribution of mass accuracy is narrow, with 89% of the peptides in a ± 3 ppm window, and 75% within 2 ppm. Detection of these tryptic, semi-tryptic, and modified peptides requires the full 4+ orders of dynamic range provided by the system's QuanTof™ Technology MS detector.

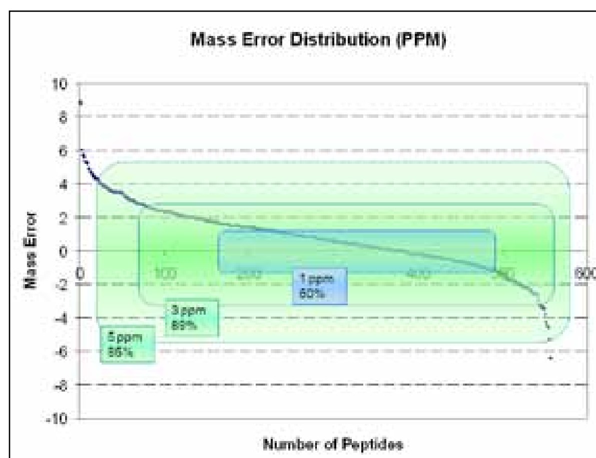

Figure 1. Mass accuracy distribution for peptides detected within an LC/MS^E monoclonal antibody peptide map.

In peptide maps, such a degree of routine mass accuracy for peptides spanning the highest and lowest masses delivered by a benchtop instrument redefines possibilities for routine applications. This level of performance, combined with the usability of both the Xevo G2 QTof System and BiopharmaLynx informatics, allows scientists who are not experts in mass spectrometry to know that their assignments are correct.

Figure 2 shows a histogram of the mass errors for a peptide map of a tryptically digested mAb of 148 kDa. The mass accuracies cluster around the 1 ppm error. The value of tight mass accuracy is that users will not need to re-check assignments, leaving them more productive in an organization testing for comparability where lot release is cost-critical.

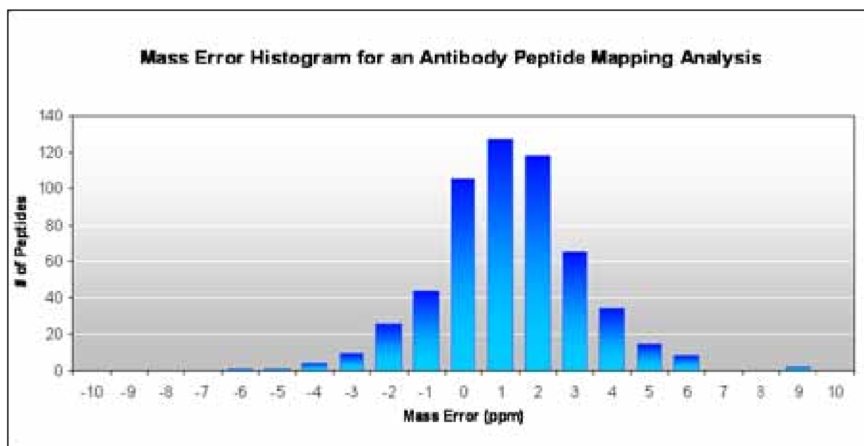

Figure 2. Mass error histogram for an antibody peptide mapping analysis.

Mass accuracy across numerous injections for a 2730 Da peptide

The Xevo G2 QTof maintains mass accuracy within 2 ppm of theoretical across days of experiment time. Figure 3 shows the narrow and consistent accuracy profile for a 2730 Da tryptic antibody peptide over 27 sequential injections (96 hours, or four days of continuous operation). Large peptide mapping studies can be accomplished with minimal effort for data acquisition, freeing scientists for higher-value activities.

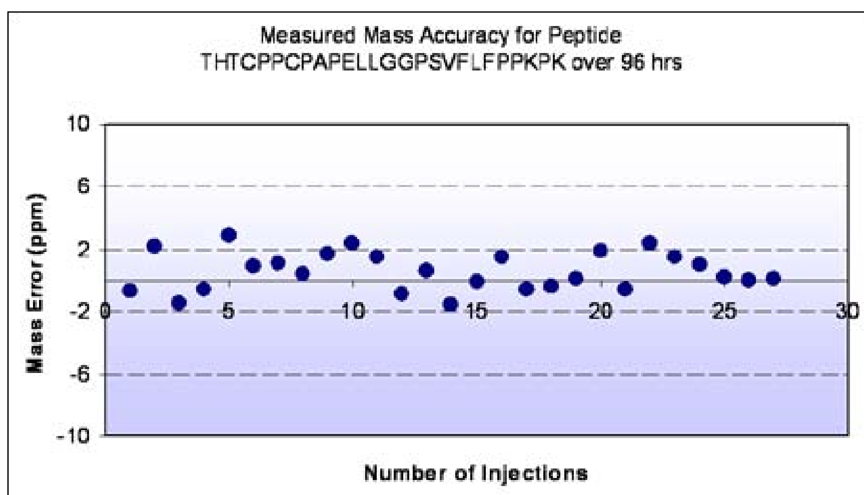

Figure 3. High mass accuracy of the peptide THTCPPCPAPELLGGPSVFLFPPKPK detected over a 96-hour series of peptide mapping experiments (27 injections). Mass accuracy remained within a narrow ±2 ppm window over this experimental series.

Mass stability across numerous injections for the same intact glycoform

The stability of mass accuracy demonstrated for the series of peptide maps is equally critical for studies of intact protein mass. Biotherapeutics organizations need to comply with regulatory authority guidelines to show that they consistently produce comparable biotherapeutics. Intact protein mass is measured as an overall indicator of protein identity and to monitor the glycoforms that account for some of the heterogeneity of the product.

Figure 4 shows the deconvoluted MaxEnt1 Spectrum in the BiopharmaLynx browser with major and minor glycoforms. Six of the most intense peaks are labeled with their attributable glycoforms according to mass.

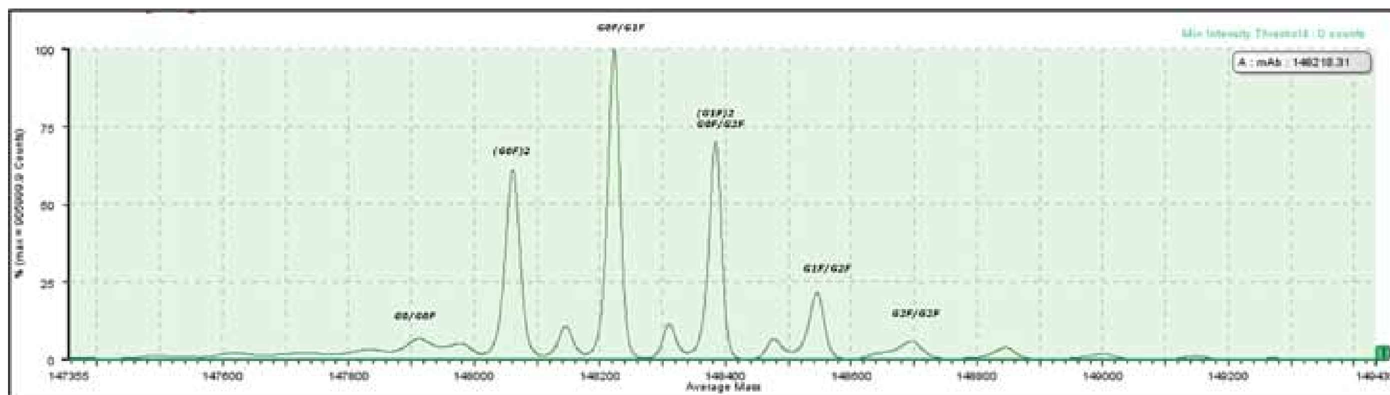

Figure 4. Deconvoluted MaxEnt1 spectrum with major and minor glycoforms.

Repeated desalting LC/MS analyses of the same antibody using the Xevo G2 QTof produced MaxEnt1 deconvoluted spectral results showing a narrow range of mass variation (Figure 5). The G0F/G0F glycoform, with an average mass of 148,061 Da, varies within a narrow 2 Da window. Having the confidence of such consistently high mass precision for species even at the intact level allows biotherapeutic manufacturers to pick up quality and comparability issues with greater clarity.

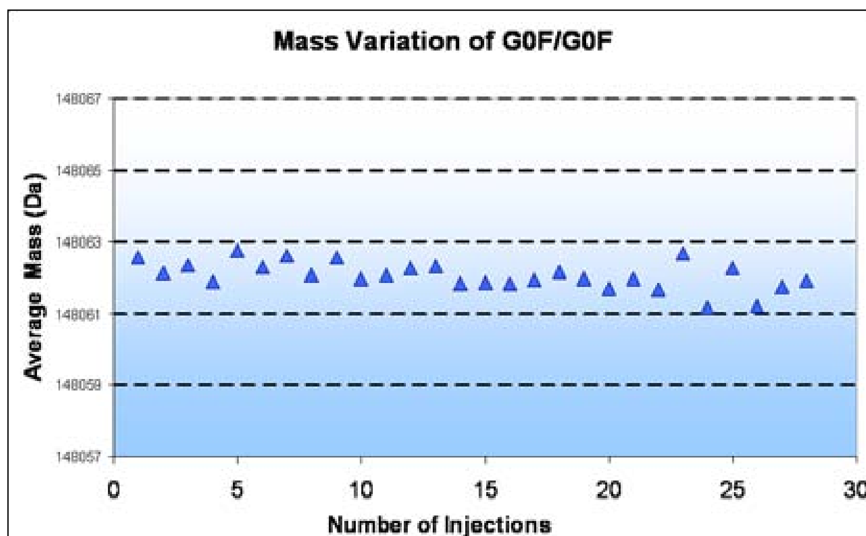

Figure 5. Consistently high mass accuracy for a glycoform of an intact mAb. Raw data was deconvoluted with MaxEnt1 as part of an automated workflow with BiopharmaLynx.

CONCLUSION

The combination of Engineered Simplicity on the Xevo G2 QTof System equipped with QuanTof Technology provides consistently dependable mass accuracy at all mass ranges relevant to biotherapeutics. The mass accuracy is provided reproducibly over extended periods without intervention by the user, allowing an organization to make the most of its capital and human investment.

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T: 1 508 478 2000
F: 1 508 872 1990
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[TECHNOLOGY BRIEF]

Enabling Greater Capability in the Characterization of Biomolecules with StepWave Ion Transfer Optics in the SYNAPT G2-S

GOAL

To demonstrate that the sensitivity benefits of the off-axis StepWave™ ion transfer technology in the SYNAPT® G2-S provides organizations with the extended capability for intact biotherapeutic characterization.

BACKGROUND

Analytical solutions that are able to provide more detailed characterization about a biomolecule (destined to become a biotherapeutic) have always been sought after by the biopharmaceutical industry. The incorporation of StepWave into a high-performance SYNAPT G2 High Defintion Mass Spectrometry™ System is another key step forward in achieving more detailed characterization of such molecules.

SYNAPT G2-S is equipped with a larger ion sampling orifice, an enhanced vacuum pumping configuration, and revolutionary StepWave ion transfer optics. This groundbreaking dual-T-Wave,™ off-axis design transfers ions from the ion source to the quadrupole MS analyzer with the highest possible efficiency, and at the same time, ensures that undesirable neutral contaminants are actively filtered out. This dramatically increases MS ion intensities while minimizing background noise – significantly improving detection limits and the repeatability of quantitative assays.

Dig deeper into the character of biomolecules with UPLC and StepWave Technology in the SYNAPT G2-S System.

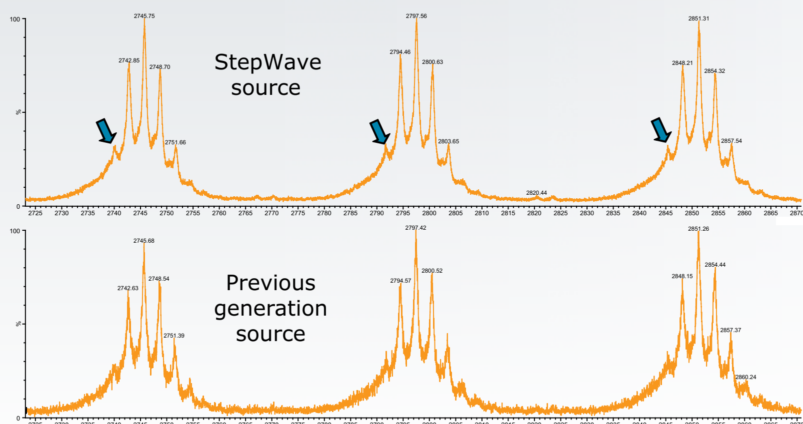

Figure 1. Electrospray positive, MS spectral data showing enhanced data quality of the for an intact mAb enabled by StepWave Technology. The three charge states shown illustrate the better defined minor glycoforms (G0/G0F) in the spectrum from SYNAPT G2-S.

This technology brief describes the performance characteristics of the UPLC®/SYNAPT G2-S System for intact mass analysis of a therapeutic monoclonal antibody and its subunit (the light chain). The data will demonstrate the superior MS sensitivity and resolution that SYNAPT G2-S offers for a rapid, in-depth characterization of proteins.

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THE SOLUTION

An experiment was performed to determine the effect of incorporating StepWave ion optics technology on the spectral data obtained when performing the intact mass analysis of an intact IgG1 monoclonal antibody (mAb), Trastuzumab. Data presented in Figure 1 were obtained during back-to-back experiments performed using two HDMS™-enabled SYNAPT instruments, one of which utilized StepWave ion transfer optics (top). The UPLC/MS analysis of 1.0 µg intact Trastuzumab was acquired in the sensitivity mode of each SYNAPT instrument. Under equal sample loading, low abundance, minor glycoforms of the mAb molecule are better defined in the spectrum from the SYNAPT G2-S instrument (with StepWave), thus providing more comprehensive data about the glycoform distribution in this batch of therpeutic protein.

Increasing ion currents entering the SYNAPT G2-S mass spectrometer enables high quality spectra to be acquired with less sample consumption. Parallel LC/MS analyses for intact Trastuzumab were undertaken with the SYNAPT G2-S (shown in Figure 2, top) and SYNAPT G2 (shown in Figure 2, bottom). Similar spectra were generated by the two instruments, even though 10-fold less material was loaded on column for the SYNAPT G2-S System, thus demonstrating the increased sensitivity of the instrument enabled with StepWave technology.

The SYNAPT G2-S is capable of achieving isotopic resolution of proteins with a size of 30 kDa and yielding low ppm mass accuracy results under routine LC/MS conditions. The clearly defined isotopic pattern of the 25 kDa Trastuzumab light chain subunit enables isotopic models to be applied for the detection of lower mass modifications, thus providing sensitive characterization with greater structural detail from the intact protein analysis.

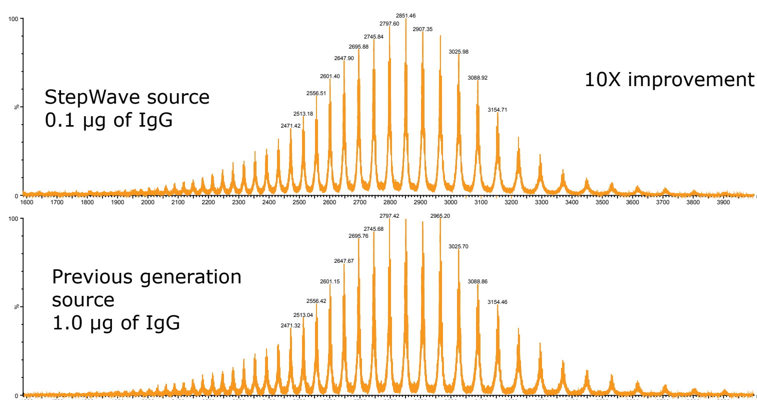

Figure 2. Summed spectra showing that data generated from the SYNAPT G2-S (x10 dilution) is equivalent to that from the SYNAPT G2 (no dilution).

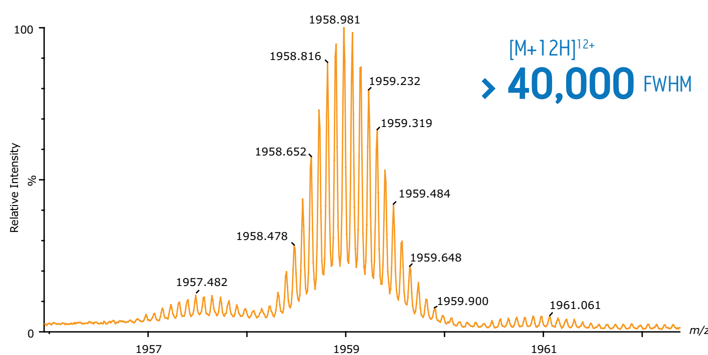

Figure 3. The isotopic distribution for the light chain (25 kDa) of a monoclonal IgG molecule with SYNAPT G2-S.

SUMMARY

- The SYNAPT G2-S has enabled high resolution LC/MS analysis of proteins with the ability to see finer structural details than previously possible.
- In this study, the performances of SYNAPT G2-S to directly analyze an intact recombinant IgG (MW~148 kDa) and its subunit light chains (~24 KDa) are demonstrated. The data show that the SYNAPT G2-S enables the analysis of a protein as large as an IgG with enhanced sensitivity to discriminate a minor glycoform in the glycosylation profiles.
- The ability to better detect minor isoforms of intact protein species provides the scientist with an increased awareness of changes in cell culture or molecular degradation that can affect biotherapeutic efficacy. This extra information enables the scientist to react quickly to such circumstances with a view to shortening the time to deliver therapeutic treatments to market.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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A Generic On-Line UPLC-SEC/UV/MS Method for the Analysis of Reduced Monoclonal Antibodies

GOAL

To demonstrate the capabilities of Waters' integrated UPLC-SEC/UV/MS system for fast and routine characterization of reduced monoclonal antibodies.

BACKGROUND

The pipeline for biotherapeutics is growing rapidly as pharmaceutical organizations shift their focus from small molecule drugs to biotherapeutic drugs. The efficient characterization of antibody drugs is increasingly important to both regulatory agencies and pharmaceutical companies to ensure the safety and efficacy of biotherapeutic products.

Although the selectivity of antibodies varies appreciably, the overall structures of antibodies are highly conserved within an antibody class. The ability to analyze the same class of antibodies using a generic method is highly desirable for improving the efficiency of analyses in the pharmaceutical industry.

Reversed phase (RP) chromatography coupled with mass spectrometry is frequently used for the analysis of reduced antibodies. RP chromatography separates proteins based on their hydrophobicity and is a popular technique for rapid, intact mass analysis. Nonetheless, proteins with different sizes may show similar hydrophobicity and are therefore difficult to separate by RP. High temperature is often used in RP chromatography to achieve better peak shape and to minimize carryover. This, however, raises questions on whether high temperature introduces changes to the protein structure.

This on-line UPLC®-SEC/MS method, accomplished by utilizing a sub-2-μm ACQUITY UPLC® BEH SEC Column, the ACQUITY UPLC System, and the SYNAPT® G2, offers a powerful solution for antibody characterization.

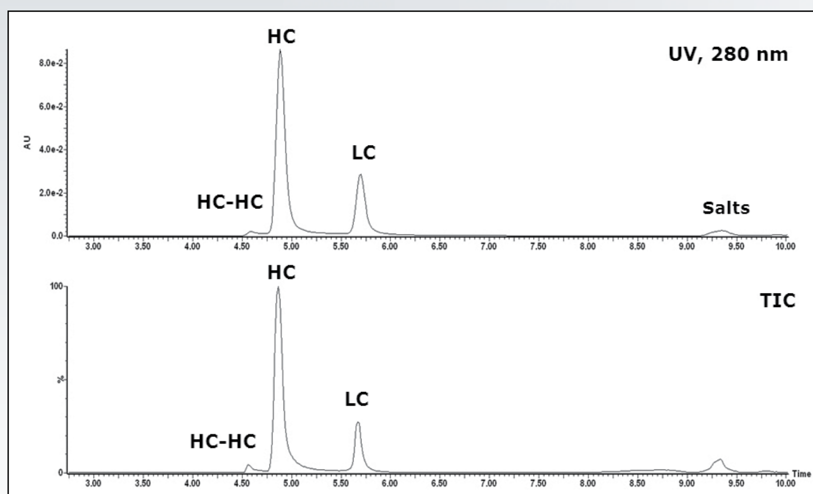

Figure 1. UPLC-SEC/UV/MS chromatogram of a reduced humanized mAb. This method is able to resolve light chain, heavy chain, and heavy chain-heavy chain clip, providing a generic way for antibody screening.

Size exclusion chromatography (SEC) separates proteins predominantly based on their size differences using an isocratic elution rather than hydrophobicity and avoids the use of high column temperature and gradient optimization. SEC is widely used in the biopharmaceutical industry to detect antibody aggregates and fragments.

However, traditional SEC mobile phases are incompatible with electrospray ionization MS, and historically optical detection methods with lower specificity have been used. Lower specificity presents a problem for organizations wishing to characterize biotherapeutics according to the well-characterized biotherapeutic product model as adopted by regulators around the world.

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This work describes an efficient on-line UPLC-SEC/MS method for the direct mass analysis of reduced humanized monoclonal antibodies (mAb), and demonstrates that rapid, high-resolution SEC separations in combination with high-performance SYNAPT G2 MS provides an efficient, generic method for routine antibody characterization.

THE SOLUTION

The successful coupling of SEC to ESI-MS was achieved using an MS-friendly mobile phase. The SEC separation was achieved using an ACQUITY UPLC BEH200 SEC Column (1.7-µm, 4.6 x 300 mm) with an ACQUITY UPLC System using isocratic elution with a flow rate of 0.46 mL/min. The mobile phase contains 30% acetonitrile, 0.1% TFA, and 0.1% FA in Milli-Q water. The flow passed through a TUV detector operated at 280 nm wavelength and then directed to the SYNAPT G2 MS.

The use of sub-2-µm UPLC BEH column packing materials along with the low-dispersion, high-pressure ACQUITY UPLC System resulted in significant improvements in size-based separation as displayed in Figure 1. The 10-minute SEC run completely resolved the earlier eluting glycosylated heavy chain (HC) from the later eluting light chain (LC), a separation not achievable in such a limited timeframe with existing techniques.

In addition, the glycosylated HC-HC fragment/clip was well separated from the heavy chain. In contrast to RP columns, the Waters UPLC BEH SEC column showed no memory effect. Consequently, no blank runs were needed between sample runs.

Figure 2 (top) depicts the summed raw mass spectrum (inset) and the resulting MaxEnt1 deconvoluted mass spectrum of the glycosylated heavy chain.

The major peaks correspond to the heavy chain containing the core fucosylated glycan (G0F), a minor nonfucosylated form (G0), and core glycan variants extending by one or two terminal galactose residues (G1F, G2F). Below, it also shows the summed raw mass spectrum (inset) and the MaxEnt1 deconvoluted spectrum of the light chain.

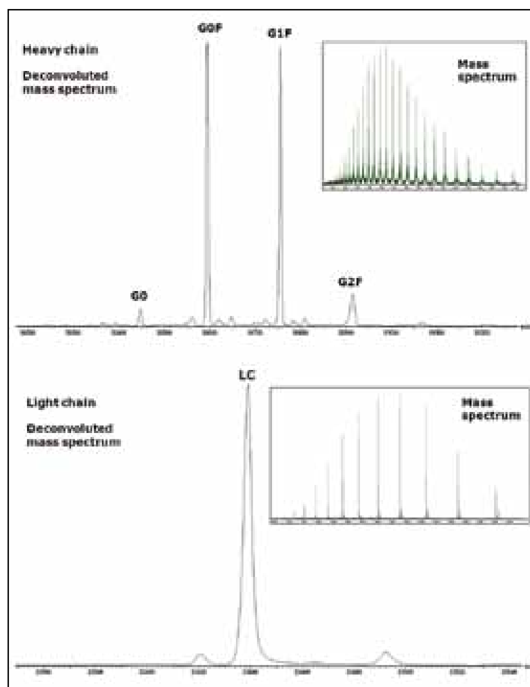

Figure 2. Combined mass spectrum (inset) and MaxEnt1 deconvoluted mass spectrum of the heavy chain (top). Combined mass spectrum (inset) and MaxEnt1 deconvoluted mass spectrum of the light chain (bottom).

SUMMARY

The optimized UPLC-SEC/UV/MS method enables the direct pairing of size exclusion chromatography with a mass spectrometer to measure the molecular weights of reduced mAbs. This well-established separation technique is now linked to mass spectrometric detection for those needing to characterize their biotherapeutics and satisfy regulators that they have sufficiently understood their biotherapeutic products.

The performance of the UPLC system and SEC column allows scientists to resolve chain, heavy chain, and heavy chain-heavy chain clip of an antibody without employing high column temperature. This optimized SEC method with separation based on size differences provides a complement to RP chromatography, and, coupled with MS, offers a powerful, routine, and generic solution for antibody characterization.

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F: 1 508 872 1990
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[APPLICATION NOTE]

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Analysis of Proteins by Size Exclusion Chromatography Coupled with Mass Spectrometry Under Non-Denaturing Conditions

Paula Hong, Stephan Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution and sensitivity with SE-UPLC as compared to traditional SE-HPLC
- Non-denaturing SEC method for MS identification of unknown biotherapeutic components
- Exact molecular weight confirmation of intact bimolecules
- BEH particles provide columns with reduced secondary interactions that allow for mobile phases with reduced salt concentrations
- SEC column with minimal MS column bleed provides improved sensitivity

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC BEH200 SEC, 1.7 µm Column

Xevo® G2 Q-Tof Mass Spectrometer

MassLynx™ Software

KEY WORDS

SE-UPLC, SEC-MS, QuanTof, monoclonal antibodies, biotherapeutics

INTRODUCTION

Ultra performance size exclusion chromatography (SE-UPLC) provides a high throughput, robust method for separation of biomolecules based on size in solution.[1] SE-UPLC is typically performed under non-denaturing conditions, which are intended to preserve the state of self-association of the biomolecule, with a UV detector for quantification. Molecular weight estimates based on this technique require the use of an appropriate set of molecular weight standards for calibration. Other methods capable of providing molecular weight information under non-denaturing conditions include on-line multi-angle light scattering (MALS) and off-line analytical ultracentrifugation (AUC), both of which do not rely on molecular weight standards. These low resolution techniques cannot always resolve minor differences in molecular weight due to post-translational modifications or degradation. The combination of SEC using non-denaturing mobile phase and mass spectrometry (MS) provides accurate on-line mass determination for biomolecular species observed by SE-UPLC, however, the form of the non-covalent self-associated species is not provided by this method.

In this application, we describe SEC-MS under non-denaturing conditions. While a similar application has been evaluated for SE-HPLC,[2] UPLC® Technology in combination with sub-2-µm SEC column packing and a time-of-flight mass spectrometer, Xevo G2 Q-Tof, allows for direct analysis with improved chromatographic resolution and sensitivity. The resulting separations are comparable in retention time to those obtained using typical SEC mobile phases that are not MS compatible. By combining these conditions with a Xevo G2 Q-Tof, SE-UPLC-MS analysis can be used as an effective complementary characterization method to low-resolution, non-denaturing mass determination methods such as MALS or AUC, and low-resolution, denaturing size separations such as capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) to confirm the identification of biomolecular species observed by size exclusion chromatography.

EXPERIMENTAL

LC Conditions

LC System:	ACQUITY UPLC H-Class Bio System with PDA detector
Flow Cell:	Titanium 5 mm (part number 205000613)
Wavelength:	280 nm
Column:	ACQUITY UPLC BEH200, SEC 1.7 µm, 4.6 x 300 mm (part number 186005226)
Column Temp.:	30 °C
Sample Temp.:	4 °C
Injection Volume:	2 µL
Flow Rate:	0.15 mL/min or 0.2 mL/min
Mobile Phase:	100 mM ammonium formate and 25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8
Additive:	Acetonitrile, 0.8% formic acid, at 0.2 mL/min
External Pump:	Waters 515 HPLC pump
Vials:	LCMS Certified Max Recovery vials (part number 600000755CV)

SAMPLE DESCRIPTION

The protein standard (obtained from Bio-Rad) containing bovine thyroglobulin (5 mg/mL), bovine γ–globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 µg/µL) and Vitamin B_{12} (0.5 µg/µL) in deionized water was analyzed. Horse heart myoglobin (Sigma) was prepared at 2 mg/mL in deionized water. A recombinant humanized monoclonal antibody, trastuzumab, was analyzed past expiry undiluted (21 µg/µL).

RESULTS AND DISCUSSION

The analysis of proteins by size exclusion chromatography (SEC) is typically performed under non-denaturing conditions which preserve the three dimensional structure and can be correlated with biological activity of the protein. Common mobile phases are 100% aqueous in a physiological pH range (6-8) and typically require non-volatile buffers and salts such as sodium phosphate and sodium chloride.[3] In order to obtain MS characterization of sample fractions separated under these conditions, the most common solution is to desalt the sample prior to analysis; however, this approach can result in sample speciation and can be cumbersome.

Another strategy is to perform SEC under denaturing conditions, so that species are efficiently ionized for detection by MS.[4,5] These methods typically require the use of mobile phases containing acetonitrile, formic acid and trifluoroacetic acid (TFA) for direct coupling of SEC to MS. While TFA does cause ion suppression in MS, it is required to minimize secondary interactions between the column packing material and the biomolecule. This application provides a useful tool for desalting of a sample without the need for column re-equilibration and has been used for the analysis of reduced and alkylated monoclonal antibodies as well as other smaller proteins.[5,6] This method does not typically preserve the self-associated state of the protein.

An alternative approach to SEC-MS has been the use of aqueous mobile phases that are MS compatible such as ammonium formate and ammonium acetate at low concentrations (<100 mM). While these mobile phases may not completely preserve the native structures for biomolecules,[3] they have been found to provide MS sensitivity while best preserving protein self-association and size-based chromatographic separation.

METHOD DEVELOPMENT

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column was evaluated at varying ammonium formate concentrations (5-200 mM) for resolution and MS sensitivity. Initial screening by UV evaluated the effect of salt concentration on both peak shape and resolution. A protein standard (Bio-Rad Laboratories) was used for the analysis. At low ammonium formate concentrations (<100 mM), secondary interactions result in poor peak shape and increased tailing for most of the proteins compared to phosphate buffers. These interactions can be due to either an "ion exchange" or

MS Conditions

MS System:	Xevo G2 QTof
Ionization Mode:	ESI+
Analyser Mode:	Sensitivity
Acquisition Range:	500-5000
Capillary Voltage:	3.00 kV
Cone Voltage:	40.0 V
Source Temp.:	150 °C
Desolvation Temp.:	450 °C
Cone Gas Flow:	0.0 L/Hr
Desolvation Gas Flow:	800.0 L/Hr
Calibration:	NaI 2 µg/µL from 1000-4000 m/z

Data Management

MassLynx software

MaxEnt™1 software

”ion-exclusion” effect between the free silanols on the packing material and the biomolecules.[7] While peak shape and resolution improved at higher ammonium formate concentrations, ion suppression in the ESI process was also observed with lower intsenity counts. The final mobile-phase conditions were selected to balance resolution and ion suppression. At 100 mM ammonium formate, no tailing significant was observed and the MS signal was adequate for peak identification.

Comparison of the UV chromatograms with 100 mM ammonium formate and PBS (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8) mobile phases show similar retention and peak shape (Figure 1). For this example, ammonium formate provides an adequate SEC separation. However, not all biomoelcules exhibit the same degree of secondary interactions. In instances in which there are greater secondary interactions, the ammonium formate concentration can be altered to improve peak shape.

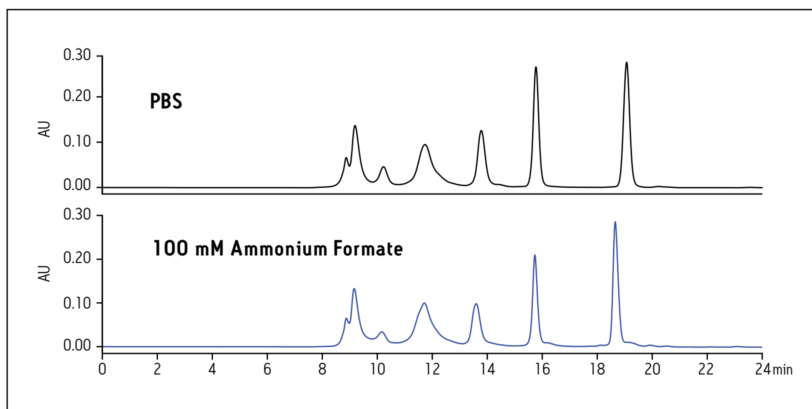
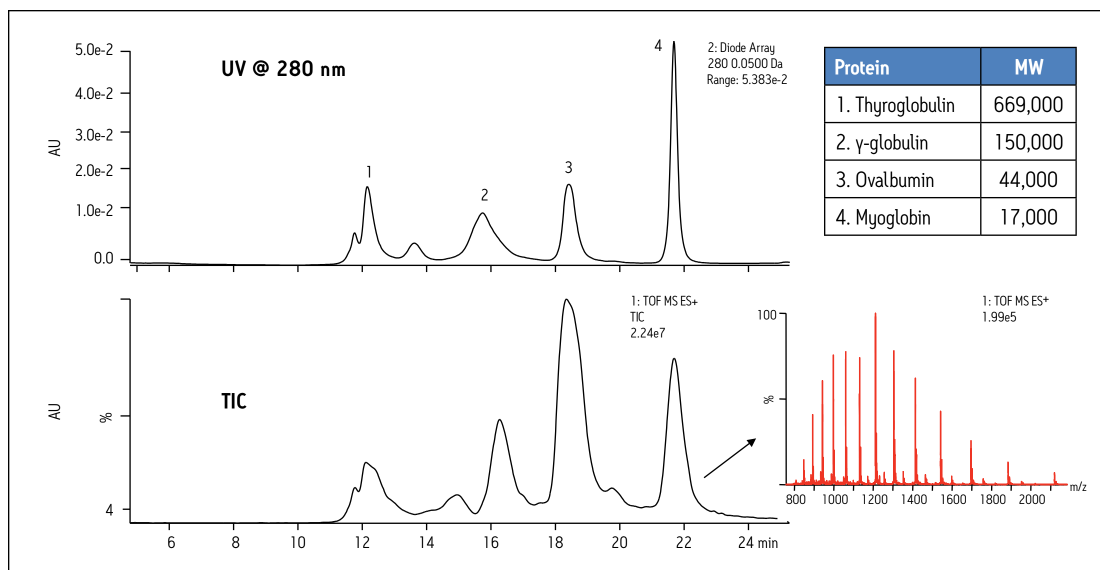


Figure 1. Influence of mobile-phase composition on the SEC separation of a protein standard.

As described above, ammonium formate was selected because of its volatility and MS compatibility. Since the use of non-denaturing mobile phases such as ammonium formate can reduce MS signal by a factor of 10 or greater,[8] a denaturing modifier (formic acid in acetonitrile) was added to the eluent post-column. The post-detector tubing and external pump were connected with a tee just prior to the MS inlet valve. Differences in resolution between the UV and TIC were minimal (Figure 2). As expected, there were significant differences in relative peak area ratios of the proteins in the TIC and UV chromatograms due to differential ionization efficiencies of the protein species. In these experiments the ESI-MS TIC was used solely for identification purposes, and the UV traces for quantification, where relevant.



Protein	MW
1. Thyroglobulin	669,000
2. γ-globulin	150,000
3. Ovalbumin	44,000
4. Myoglobin	17,000

Figure 2. SEC-UV-MS analysis of a protein standard.

Analysis of Myoglobin Aggregates

The ACQUITY UPLC BEH200 SEC, 1.7 μm Column provides adequate resolution and MS sensitivity of the myoglobin size variants, including the monomer (peak 1), dimer (peak 2) and higher order aggregates (peak 3) (Figure 3). The ESI mass spectrum of the myoglobin monomer and dimer show multiple charged ion signals (Figure 4). The spectrum for the monomer reveals multiple-charge states from m/z approximately 800 to 2000 corresponding to charge states from $[M+8H]^{+8}$ to $[M+21H]^{+21}$. The deconvoluted spectrum of the monomer mass spectrum confirms the intact mass of myoglobin at 16,951. The MS signal for the dimer is a factor of 10 weaker than that of the monomer. The ESI mass spectrum of the dimer shows multiple charge states from $[M+20H]^{+20}$ to $[M+40H]^{+40}$. The deconvoluted spectrum shows the presence of both myoglobin monomer and the dimer (m/z 16,951 and 33,886).

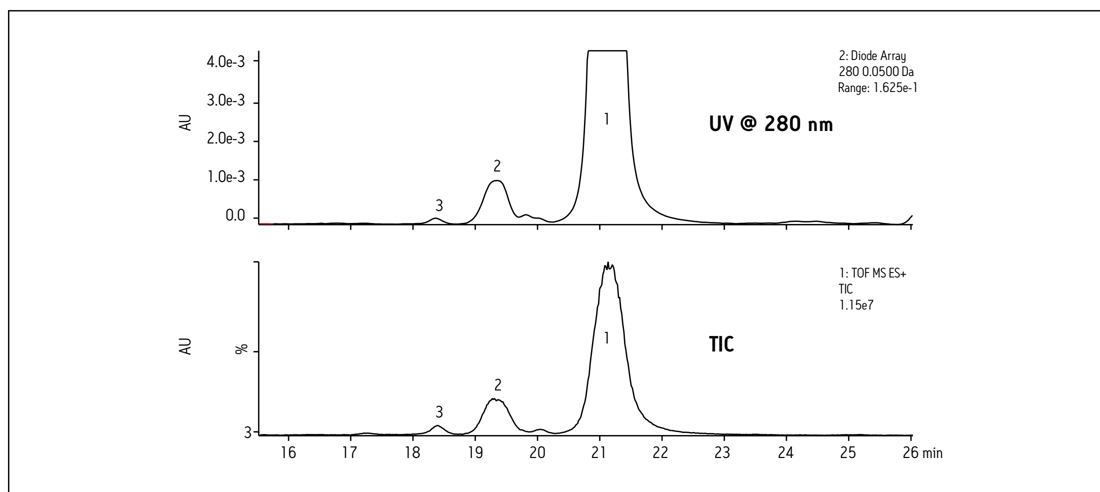


Figure 3. SEC-UV-MS anslysis of myoglobin monomer and aggregates.

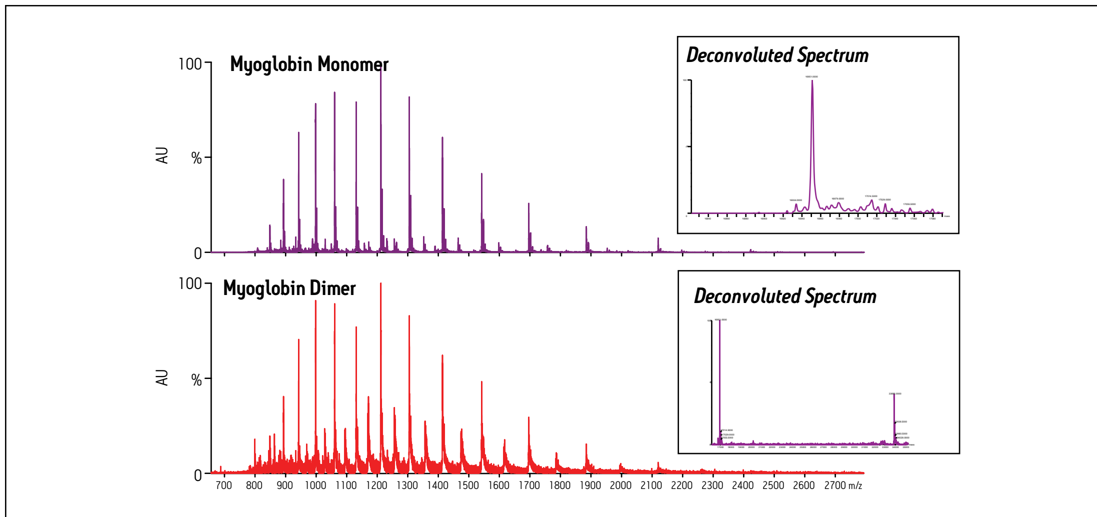


Figure 4. ESI mass spectrum and deconvoluted spectrum (inset) of myoglobin monomer and dimmer.

The simultaneous presence of monomer and dimer in the deconvoluted spectrum may be due to a variety of factors including dissociation of the non-covalent dimer in source, and/or presence of additional size variants. As described above, an acidic organic modifier is required post-column to provide adequate ionization of the proteins. These sample conditions can cause the proteins to denature, thus disrupting protein-protein interactions including non-covalent interactions.[2] An additional factor may be due to the presence of misfolded forms of myoglobin. While separation of the myoglobin monomer and dimer is achieved, a minor peak is present between the two peaks, possibly due to misfolded proteins or other size variants. These forms may be one factor for the appearance of the monomer mass in the deconvoluted spectrum of the dimer. Nevertheless, the presence of only myoglobin monomer and dimer indicates that the aggregation is primarily related to self-association of myoglobin.

Identification of Unknown Components in a Biotherapeutic

An intact monoclonal antibody biotherapeutic, which was past expiry, was analyzed by SEC (Figure 5) using MS-friendly, non-denaturing conditions. In the UV chromatogram, not only are the mAb aggregate and monomer observed, but a low molecular weight (LMW) peak eluting after the intact mAb is partially resolved as well. In addition to these peaks, the UV chromatogram reveals two other LMW species.

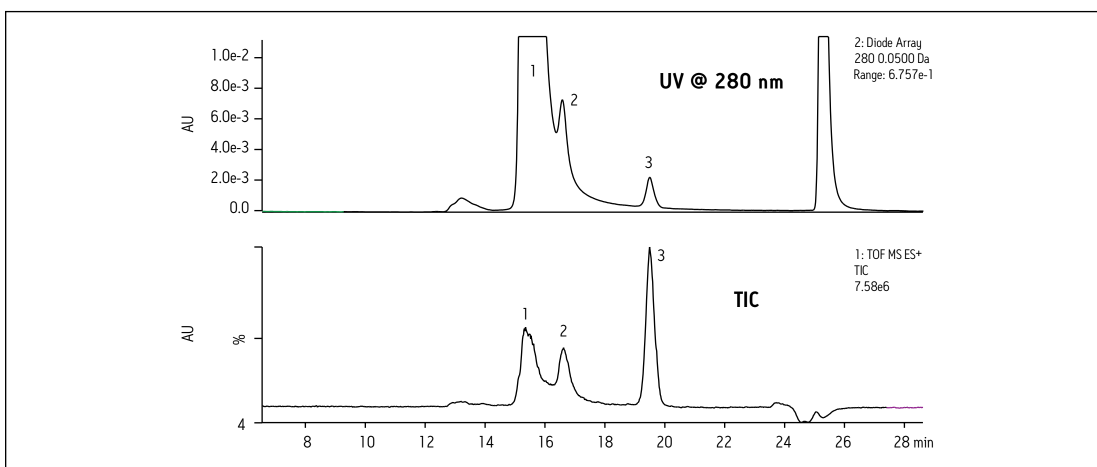


Figure 5. SEC-UV-MS of a recombinant humanized monoclonal antibody.

The ESI-mass spectrum of the monoclonal antibody (1) shows charge-states from $[M+34H]^{+34}$ to $[M+70H]^{+70}$ (Figure 6). The sensitivity of the method is illustrated by the high TIC satellite peaks of the $[M+39H]^{+39}$ and $[M+40H]^{+40}$ charge-states of the monomer . The deconvoluted spectrum of the monomer peak confirms the presence of the major glycosylated forms of the intact antibody with values corresponding to previously published results.[9] The exact masses can be assigned to G0F/G0F (148,058 m/z), G0F/G1F (148,219 m/z) and (G01F)2 or G0F/G2F (142,379 m/z).

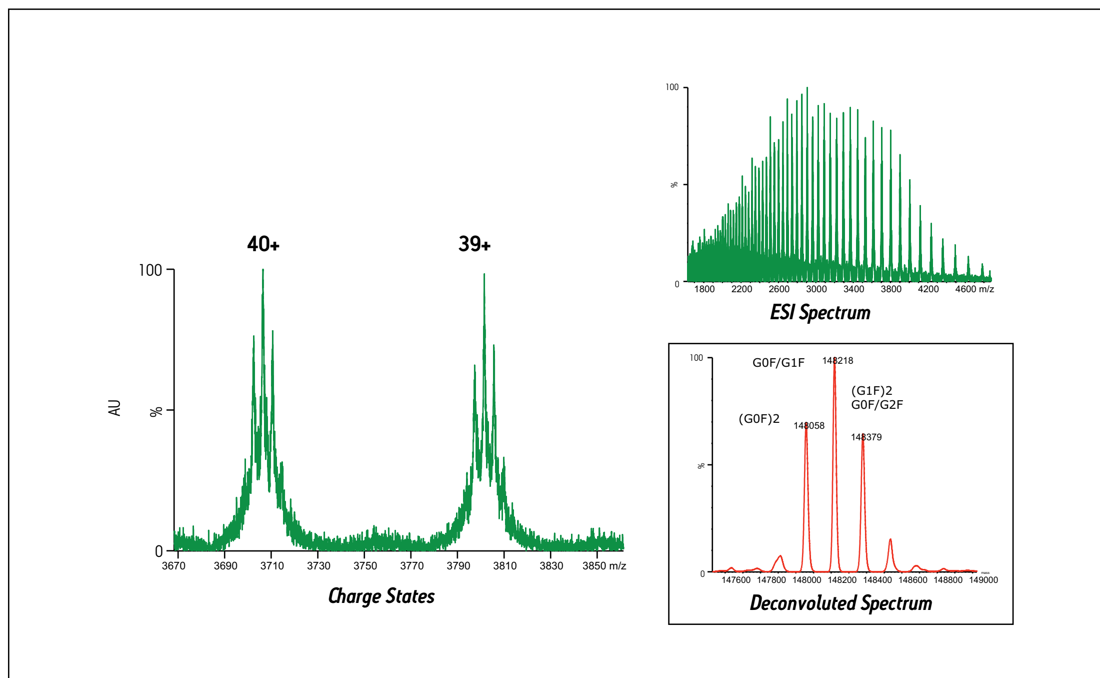


Figure 6. ESI mass spectrum of an intact monoclonal antibody. Deconvoluted spectrum (inset) shows intact mAb as well as gylcosylated forms.

The LMW peak (peak 3) eluting at 19 minutes also provides an adequate MS signal for molecular weight confirmation. Analysis of the ESI spectrum shows the presence of two different charge envelopes from 1100-2400 m/z (Figure 7). This is evident in the magnified view in which the satellite peaks for both sets of charge-states are resolved. The deconvoluted spectrum shows multiple peaks (Figure 8 inset), with 47,269 m/z (F1) and 47,636 m/z (F2) having the highest intensities. These intact masses correspond to the two prominent multiply charged ion states in the ESI mass spectrum: the charge states from $[M+19H]^{+19}$ to $[M+31H]^{+31}$ are shown in the zoomed spectrum. Based on the sequence of the protein, the main peaks in the deconvoluted spectrum can be assigned to Fab fragments resulting from hydrolytic cleavage of the heavy chain: the mass of F1 (47,270 m/z) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Asp^{224} while the mass of F2 (47,637 m/z) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Thr^{228}.

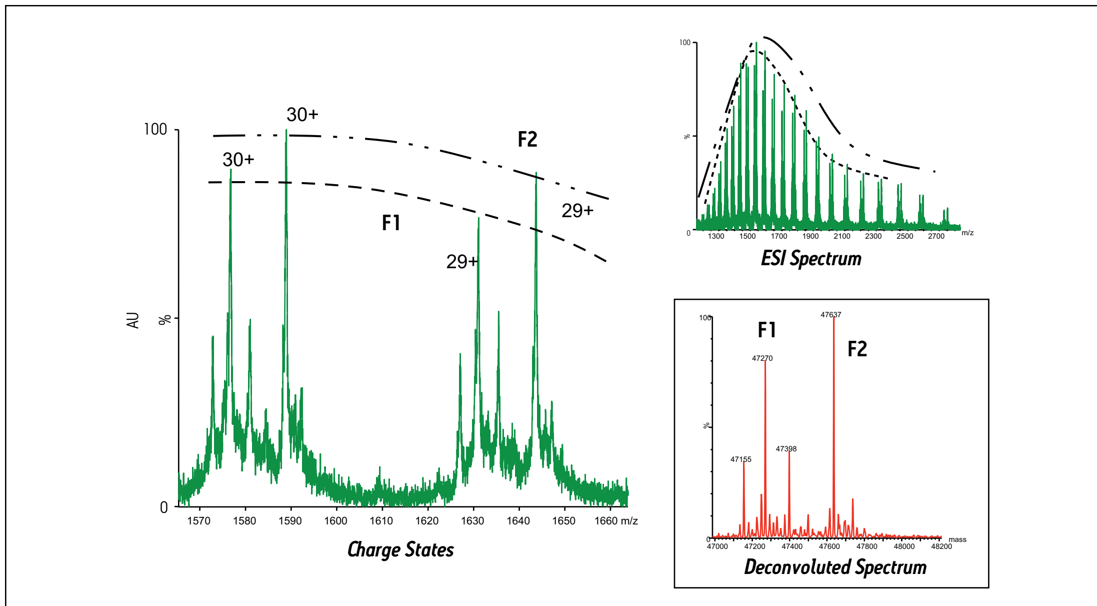


Figure 7. ESI mass spectrum of low molecular weight species (peak 3) in a recombinant humanized monoclonal antibody.

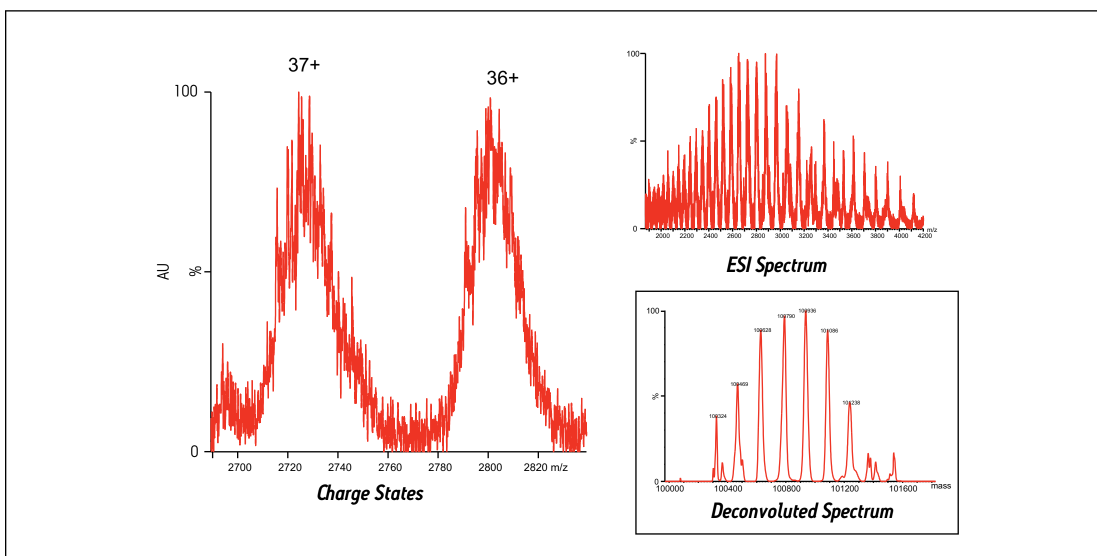


Figure 8. ESI mass spectrum of fragment (peak 2) in a recombinant humanized monoclonal antibody.

A similar analysis can be performed for the partially resolved low molecular weight species (peak 2 in Figure 5). The ESI mass spectrum of the fragment shows charge states from $[M+32H]^{+32}$ to $[M+48H]^{+48}$ at 2200-4000 m/z. While the satellite peaks are not well-resolved for the lower molecular weight species (Figure 7), the charge states are evident. The deconvoluted spectrum (Figure 8) shows molecular weights consistent with antibodies that have a missing Fab arm with fragments ranging from 100,468 to 101,237 m/z (Figure 8 inset). The species observed at 100,468 is consistent with an antibody without one of the Fab arms cleaved at the N-terminal side His^{229}. The confirmation of other, minor fragments that appear to be present is beyond the scope of this application note.

The SEC-MS analysis of the recombinant humanized monoclonal antibody allows for identification of not only the intact monoclonal antibody, but also the lower molecular weight fragments. Deconvolution of the ESI mass spectrum provides intact molecular weight information for the monomer and fragment species.

CONCLUSIONS

Size exclusion chromatography under non-denaturing conditions is a standard method for testing biomolecules and their aggregates. MALS and AUC are established detectors but cannot provide exact mass for unknown species with a sufficient accuracy. The presence of an unexpected peak requires further investigation and/or confirmation of molecular weight, and SE-UPLC-MS under aqueous, non-denaturing conditions can provide valuable information that would more rapidly solve an organization's issues with characterization or quality.

While SEC-MS does not typically preserve protein self association, it can assist in identification. The analysis of myoglobin illustrates the utility of an SEC-MS approach by confirming that the HMW forms observed in the myoglobin sample are related to the protein. The SEC-MS analysis of a humanized monoclonal antibody under non-denaturing conditions provides exact masses for LMW antibody fragments. By efficiently combining the ACQUITY UPLC BEH200 SEC, 1.7 µm Column and the benchtop Xevo G2 Q-Tof with an extended m/z range, the intact antibody and its associated fragments can be identified, providing a rapid method for exact molecular weight determination of intact biomolecules.

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T: 1 508 478 2000
F: 1 508 872 1990
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[TECHNOLOGY BRIEF]

Intact Protein Electron Transfer Dissociation Analysis

GOAL

To evaluate the performance of SYNAPT® G2-S incorporating StepWave™ Technology for the rapid analysis of intact proteins using Electron Transfer Dissociation (ETD).

BACKGROUND

ETD is a fragmentation method that can be applied for the structural characterization and subsequent identification of intact proteins. This so-called 'top-down' proteomics approach is fast, because no enzymatic digestion step is necessary, and the method has been shown to require only low attomole to femtomole amounts of sample.

The ETD method is known to provide vast N-Cα backbone cleavage (c and z ions), preserves weakly bonded post-translational modifications, and it has the potential for cleaving disulphide bonds. Taken together, the attributes of the ETD technique combined with the enhanced sensitivity and speed of Waters® SYNAPT G2-S may offer a suitable platform for intact protein analysis, particularly hydrophobic proteins, compared to the more routinely used 'bottom-up' approach for protein identification.

Rapid top-down ETD analysis using travelling wave ion guide technology.

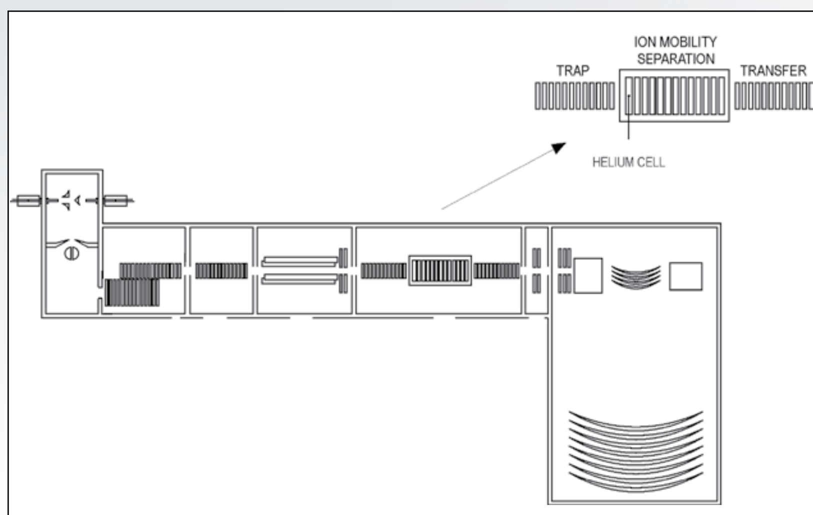

Figure 1. Schematic diagram of the SYNAPT G2-S hybrid quadrupole – ion mobility - orthogonal acceleration time-of-flight mass spectrometer.

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THE SOLUTION

Bovine Carbonic Anhydrase II (BCA) was dissolved to a concentration of 100 fmol/µL (50% aqueous acetonitrile containing 0.2% formic acid). The solution was infused into the source region of the instrument at a flow rate of 4 µL/min. The total acquisition time was 1 minute. Tandem mass spectra were recorded on an ETD enabled SYNAPT G2-S Mass Spectrometer. Gas phase ion-ion chemistry for protein fragmentation using ETD was induced by selecting both BCA precursor cations retaining 35 charges (*m/z* 830), and precursor anions formed from 1,4-dicyanobenzene (*m/z* 128) using the quadrupole analyzer (1 s polarity switch during acquisition). Following quadrupole selection, shown in Figure 1, these ions interacted within the trap travelling wave ion guide of the instrument. The ETD fragmentation process is illustrated in Figure 2 for peptide and protein type analysis. Figure 3 shows the analysis of intact BCA using ETD. The spectrum is relatively complex and interpretation was simplified through the use of BioLynx™ Software for dedicated ETD product ion annotation. This short acquisition allowed for the annotation of approximately 40 N-terminal (c ions) and 40 C-terminal (z ions) product ions following ETD.

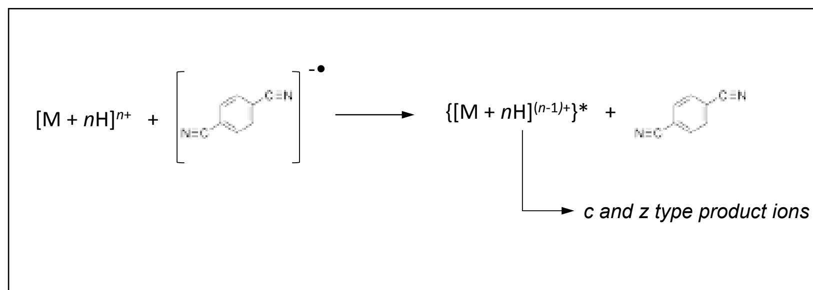

Figure 2. Illustration of the ETD process.

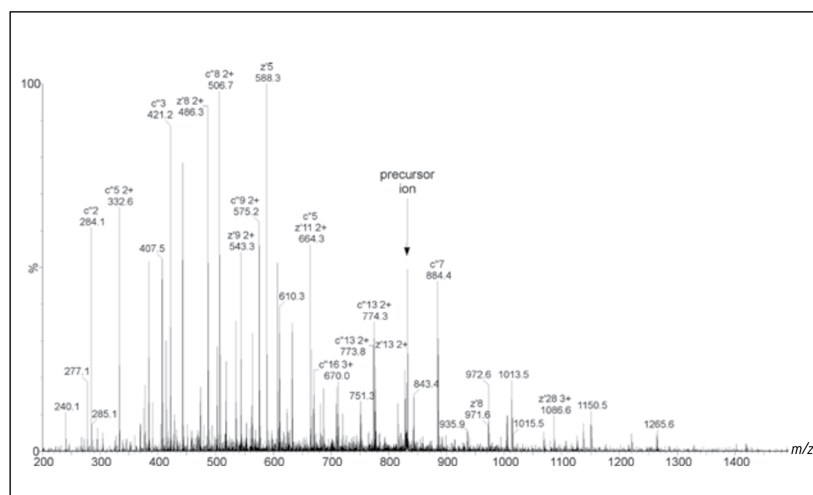

Figure 3. Top-down analysis of intact BCA using ETD.

SUMMARY

This technology brief shows that the implementation of ETD on the SYNAPT G2-S provides a beneficial means for analyzing intact proteins using a 'top-down' approach. ETD affords extensive N-Cα backbone cleavage for sufficient structural characterization and subsequent identification of intact BCA on a rapid timescale.

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F: 1 508 872 1990
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[TECHNOLOGY BRIEF]

Routine Generation of Biotherapeutic Antibody Glycoprotein Profiles by Waters SQ Detector 2

GOAL

Demonstrate use of Waters® SQ Detector 2 for LC/MS glycoprofiling of an intact 145 kD biotherapeutic monoclonal antibody.

BACKGROUND

The ability to derive semi-quantitative glycosylation profiles at the intact protein level permits rapid screening and monitoring of antibodies, with minimal sample preparation, and reduces the need for more granular but time-consuming approaches such as reduced antibody mass profiling or released glycan analysis.

Glycosylation is known to play a critical role in stability and function of therapeutic antibodies. The abundance of the singly defucosylated glycovariant (Figure 2, labeled G0/G0F) has dramatic impact on resulting antibody-dependent cell-mediated cytotoxicity (ADCC) immune response, and is routinely screened for (or against) depending on the desired mode of action for the therapeutic. Other examples now populate the literature where the presence of extended sialic acid bearing glycovariants or relative proportionality of the neutral glycovariant structures can have demonstrable effects on antibody therapeutic stability, safety, and efficacy.

Nominal mass quadrupole-based systems can be used for routine monitoring of biotheraputics, including protein mass confirmation and mAb glycovariant profiling.

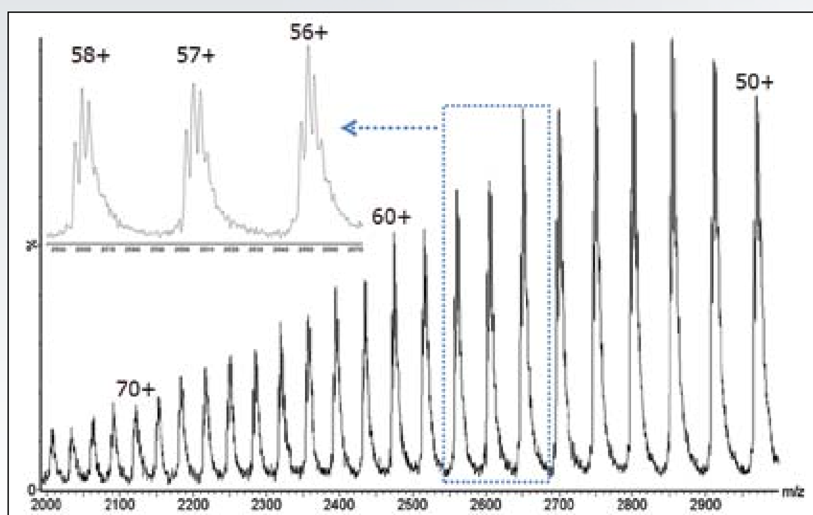

Figure 1. Mass spectrum of a therapeutic mAb acquired on the SQ Detector 2.

While exact mass Tof-MS has come to dominate the discovery workflows used for biopharmaceutical protein characterization, nominal mass quadrupole mass detectors have remained a workhorse for routine protein intact mass analysis. Here we explore the capabilities of the Waters SQ Detector 2 quadrupole mass analyzer for confirming the mass of an intact biotherapeutic antibody, and displaying the glycovariant profile for that protein.

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THE SOLUTION

LC/MS analysis of a therapeutic monoclonal antibody standard (50 µg, Waters Part No. 186006552) was acquired using a Waters ACQUITY UPLC® H-Class System coupled to a SQ Detector 2. The antibody was loaded onto a 2.1 x 50 mm Protein Separations Technology (BEH 300Å C4, 1.7 µm) column (80 °C), desalted with a 0.5 mL/min flow of 5% acetonitrile in 0.1% formic acid for 1.5 min to waste, and then eluted with a 1.5 min linear gradient (5-95% acetonitrile, 0.1% formic acid, 0.2 mL/min) directed to the mass detector. Spectra were acquired (1 Hz, 500-3000 *m/z*) in the ESI+ mode of acquisition.

Spectra summed over the chromatographic peak of the desalted antibody (Figure 1) gave rise to the MaxEnt1 deconvoluted results shown in Figure 2. The major peaks in the deconvoluted mass spectrum corresponded to the antibody containing pairs (one per heavy chain) of the fucosylated biantennary glycan structures typically associated with monoclonal antibody N-glycoproteins, and an N-terminal pyroglutamic acid modification on each of the heavy chain subunits. The observed mass of the first major glycoform (G0F/G0F, containing two fucosylated N-glycan core structures without galactose extensions) was 148,227 Da, in good agreement (< 50 ppm error) with the calculated mass of the modified antibody (148,220 Da).

Notable in the raw (*m/z*) data, is that the mass spectral charge state envelope (roughly representing charge states 50+ through 70+) makes full use of the extended *m/z* range (up to 3000 *m/z*) of the SQD 2 platform. This is typical of larger proteins, and illustrates that measurement of ions at high mass-to-charge ratio is a fundamental requirement for quadrupole-based systems intended for intact biomolecule analysis.

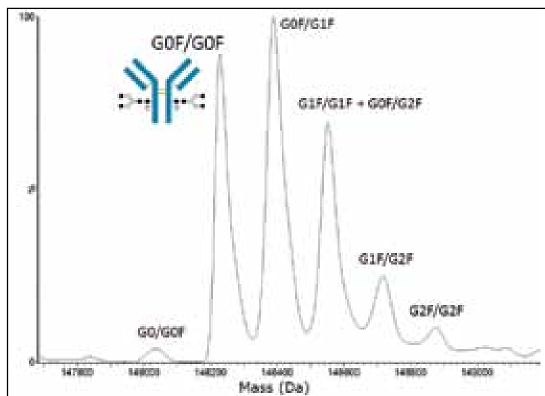

Figure 2. MaxEnt1 deconvoluted mass spectrum of a therapeutic mAb.

As shown in Figure 1 (inset), tuning the SQ Detector 2 for unit resolution is sufficient to distinguish glycovariant structures within individual charge states. The exceptional performance of the MaxEnt1 deconvolution algorithm to infer a more highly resolved deconvolution spectrum from this multitude of charge states, represented over the larger spectral window, enables both qualitative glycan assignments and relative glycoform distribution determinations.

Work at the intact level can indicate the requirement for additional monoclonal antibody profiling. While generally applicable to most therapeutic antibodies, some with more extensive microheterogeneity (*e.g.*, high levels of unprocessed C-terminal Lysines, inefficient N-terminal pyroglutamic acid formation, extensive oxidation) generally prove more amenable to profiling using chromatographically resolved light and heavy chains within a reduced antibody LC/MS analysis. Such information can often be inferred from intact antibody screening, and confirmed using the more granular reduced subunit analysis.

SUMMARY

- The extended *m/z* range (up to 3000) of the SQ Detector 2 facilitates routine LC/MS analysis of large proteins such as monoclonal antibodies (~148 kD).
- Use of the SQ Detector 2 for LC/MS intact protein studies can enable both protein mass confirmation and mAb glycovariant profiling.
- Organizations charged with biotherapeutic analysis can effectively deploy nominal mass quadrupole based systems for routine monitoring of biotheraputics, and dedicate higher resolution MS platforms for the more challenging tasks of primary structural characterization and unknown variant identification.

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PROTEIN SEPARATIONS

The successful development and commercialization of biopharmaceuticals and diagnostic reagents depends on the adequate characterization of variability within these complex biomolecules. Consequently, detailed protein characterization studies are necessary that incorporate orthogonal analytical techniques, including chromatographic assays applying separation modes such as size exclusion (SEC), ion exchange (IEX), hydrophobic interaction (HIC), and reversed phase (RP) chromatography.

Waters Protein Separation Technology (PrST) and BioSuite™ bioseparations chemistries for UPLC® and HPLC offer outstanding performance for the separation and analysis of modern therapeutic and diagnostic proteins.

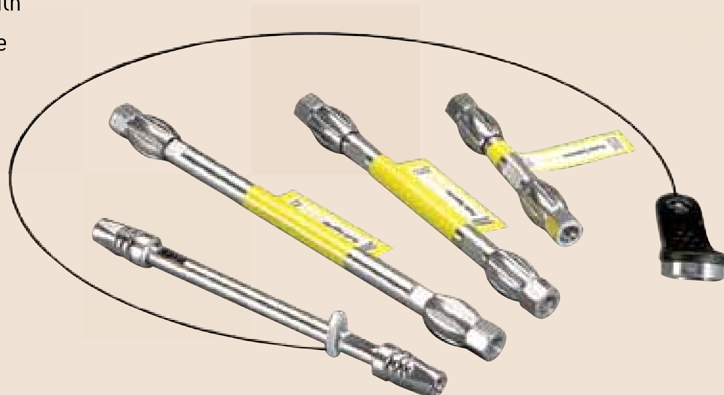
SIZE EXCLUSION CHROMATOGRAPHY (SEC)

The apparent size and shape of proteins in solution is the basis for separation in size exclusion chromatography. This simple separation mechanism relies on protein size differences, column packing material, pore size, and particle size as well as operating conditions, such as sample concentration and volume, for the successful resolution of protein mixtures. SEC column packing materials have evolved from large-particle polysaccharide soft gels to small-particle rigid packings that offer enhanced resolution and significantly faster separations.

SEC is used to measure protein aggregates, product truncations, and other size variants present in biopharmaceuticals. Soluble protein aggregates, in particular, can contribute to immunogenicity; accurate analysis and quantitation of biotherapeutic protein aggregates is, therefore, often required. Waters ACQUITY UPLC® Columns for SEC are enabled by 125 Å, 200 Å, and 450 Å pore-size ethylene bridged hybrid (BEH) particles with a diol coating that provides a stable particle with minimal secondary interactions. The packing material is more resistant to chemical and mechanical degradation over time.

These attributes combine to provide an SEC column that is stable over more than 600 injections and requires lower buffer and salt concentrations than traditional silica-based columns.

ACQUITY UPLC BEH125 SEC 1.7-µm Columns prove effective separations for peptides and lower molecular weight proteins between 1000 and 80,000 MW. ACQUITY UPLC BEH200 SEC 1.7-µm Columns optimally resolve proteins in the 10,000 to 450,000 MW range, and are capable of determining aggregation levels in therapeutic monoclonal antibodies up to 10 times faster than HPLC-based SEC methods. The larger ACQUITY UPLC BEH450 SEC 2.5-µm Columns can resolve proteins in the 100,000 to 1,500,000 MW range, and are suitable for larger proteins and analysis of some higher-order antibody aggregates.



ION EXCHANGE CHROMATOGRAPHY (IEX)

Ion exchange chromatography is utilized to assess the distribution of protein charge variants resulting from alterations in primary structure, or the presence of charge modifying post-translational modifications (e.g., terminal modifications, deamidation, sialated glycan addition).

During ion exchange separations, the distribution and net charge on the protein's surface determines the strength of protein interactions with charged groups on the column particle surface. Charges on the protein and packing materials must be opposite for interaction to occur. Variables that can be manipulated – including pH, buffer composition, gradient slope, and gradient forming salt – offer a wide range of options for optimizing a separation.

Waters Protein-Pak™ Hi Res IEX Columns support UPLC characterization of recombinant proteins and monoclonal antibodies destined to be used as therapeutics. The non-porous, high-ligand density particles provide superb resolution for high molecular weight proteins compared to traditional porous IEX particles. In addition, the particles in Protein-Pak Hi Res IEX columns are surface-modified with a multi-layered network of ion exchange groups (sulfopropyl, carboxymethyl, or quaternary ammonium), that assists in the effective binding of charged proteins. This allows for high sample loading capacities and component resolution while minimizing column fouling.

For protein IEX analyses, adjustments in mobile phase pH are often the most useful parameter for method development. Such experiments are, traditionally, time consuming and cumbersome. The ACQUITY UPLC H-Class Bio System and its quaternary solvent manager take advantage of Auto•Blend Plus™ Technology to simplify IEX method development. Auto•Blend Plus Technology enables scientists to independently manipulate pH and ionic strength through automated system blending and delivery of mobile phase, using simple buffer, salt and water stock solutions. This approach coupled with the robustness of the ACQUITY UPLC H-Class platform provides an efficient tool for method development and assay execution for protein IEX separations.

REVERSED PHASE CHROMATOGRAPHY (RP)

Reversed phase chromatography is a hydrophobic separation technique that relies on the interaction between the protein's non-polar amino acid side chains with hydrophobic ligands on the chromatographic sorbent. This mechanism provides high resolution protein separations for separating subunits of multi-chain recombinant proteins and complex subunit vaccines. RP analysis represents an excellent tool for assessing purity, protein quantification, and isolating proteins for sequencing or other compositional analysis.

ACQUITY UPLC PrST Columns provide enhancements in both resolution and analysis speed compared to traditional HPLC separations. The BEH300 C4 large pore/short chain ligand configuration is typically employed for protein separations, and availability in two particle sizes (1.7 and 3.5 µm) enables efficient method transfer between HPLC and UPLC systems. In addition, BEH300 C4 material can be effectively run with mass spectrometry compatible eluents (such as formic acid) for protein mass profiling studies.

For a full listing of PrST chromatography columns and products, go to **www.waters.com/proteins** or for the Waters bioseparations products catalog, **www.waters.com/biosep**.

[APPLICATION BRIEF]

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Analysis of Biomolecules by Size Exclusion UltraPerformance Liquid Chromatography

Kenneth J. Fountain, Paula Hong, Susan Serpa, Edouard S. P. Bouvier, Damian Morrison

Waters Corporation, Milford, MA, USA

INTRODUCTION

In the production of biopharmaceuticals, there may be different analytical requirements for groups performing clone section, formulations and stability, and quality control (QC). Depending on the goal of the separation, methods may be optimized for fast analysis time, highest possible resolution, and/or reproducibility. Size exclusion (SEC) chromatography is often used throughout the biopharmaceutical production process for the analysis of proteins and their aggregates. While SEC has traditionally been used in conjunction with low pressure HPLC instrumentation, the advent of UPLC® Technology and new sub-2 µm packing materials allows for substantial improvements in chromatographic resolution and throughput. This application note will demonstrate the use of the new ACQUITY UPLC® SEC solution for the improved detection and/ or faster analysis of protein aggregates in biopharmaceuticals.

EXPERIMENTAL

UPLC System:	ACQUITY UPLC System with TUV (with stainless steel flow cell)
HPLC System:	Waters 2796 Separations Module with 2487 dual λ detector
UPLC Column:	ACQUITY UPLC BEH200 SEC, 1.7 µm, 4.6 x 300 mm (P/N 186005226) and 4.6 x 150 mm (P/N 186005225)
HPLC Column:	Traditional silica diol-coated SEC, 7.8 x 300 mm, 5 µm
Mobile Phase A:	25 mM sodium phosphate, pH 6.8, 0.15 M NaCl
Flow rate:	0.4 mL/min
Temperature:	30 °C
Detection:	UV 280 nm (sampling rate 10 Hz, 0.2 sec filter time constant)
Sample Diluent:	25 mM sodium phosphate, pH 6.8, 0.15 M NaCl

Other conditions are specified in the figure captions.

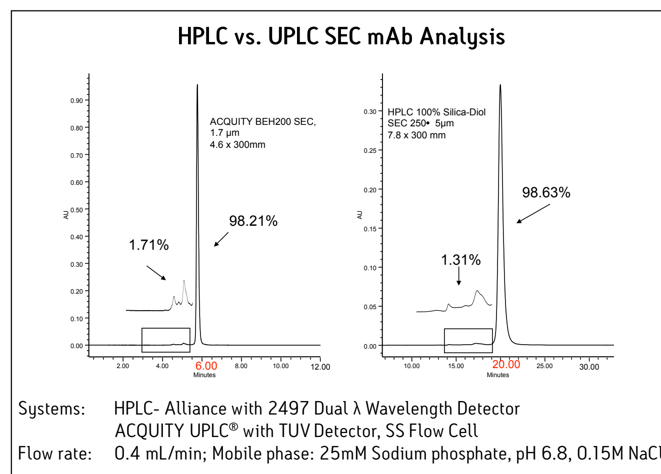

Figure 1. Comparison of traditional HPLC and ACQUITY UPLC SEC for the separation of a humanized monoclonal antibody (IgG). Injection volumes: 20 µL for HPLC and 5 µL for ACQUITY UPLC.

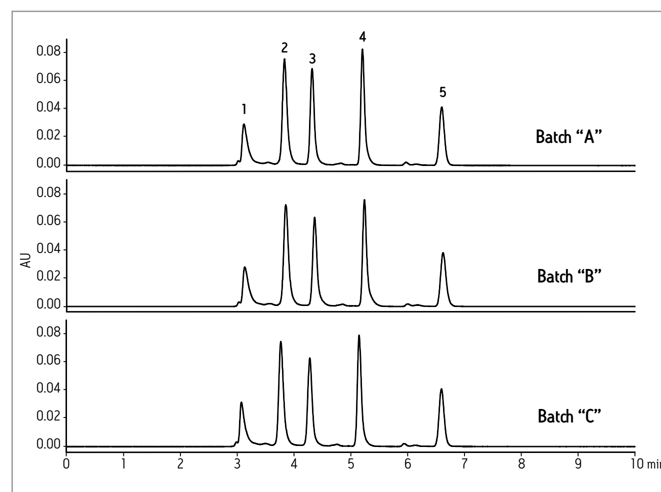

Figure 2. Batch-to-batch reproducibility for a mixture of protein standards. Buffer: 100 mM sodium phosphate, pH 6.8. Flow rate is 0.3 mL/min. Peaks: (1) thyroglobulin, (2) IgG, (3) bovine serum albumin, (4) myoglobin, (5) uracil.

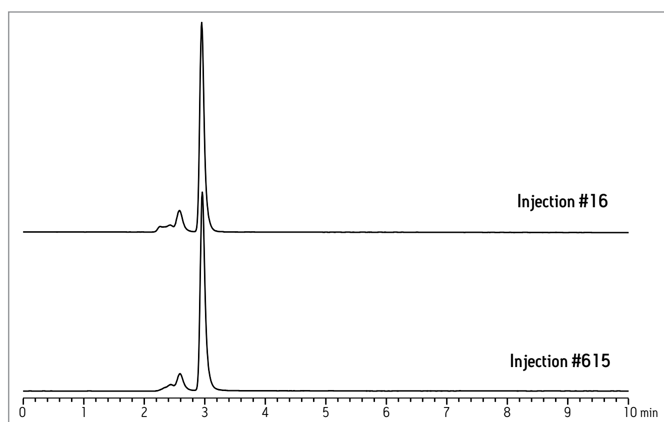

Figure 3. Lifetime study for a humanized IgG sample on the ACQUITY UPLC BEH200 SEC, 1.7 µm, 4.6 x 150 mm column.

RESULTS AND DISCUSSION

Figure 1 shows the chromatographic comparison of traditional HPLC and ACQUITY UPLC for the separation of a humanized monoclonal antibody. Equivalent aggregate quantification in significantly shorter run times is possible with the ACQUITY UPLC SEC solution as compared to traditional SEC. This is especially important for those scientists performing clone selection who may need increased throughput for large numbers of samples.

In regulated environments, the use of SEC is often required for the characterization of biopharmaceutical therapeutics. Given these demands, columns are expected to be reproducible from batch-to-batch and have long lifetimes. Figure 2 shows the batch-to-batch performance for three different batches of 1.7 µm, BEH200 SEC packing material. These data show the consistent performance of the BEH200 SEC columns regardless of the batch of material being used, which provides confidence for those performing aggregate determination in biopharmaceutical drugs. Figure 3 demonstrates the lifetime of the ACQUITY UPLC BEH200 SEC columns with the same humanized IgG sample shown in Figure 1. No deterioration in peak shape or retention was observed, which ensures accurate identification and quantitation of the antibody monomer and dimer over several hundred injections.

CONCLUSIONS

The Waters ACQUITY UPLC SEC solution provides the speed, resolution, and sensitivity of UPLC for proteins (antibodies) in a molecular weight range of 10,000 to 450,000 Daltons. It can be adapted to varying requirements of run time and resolution that are often needed in clone selection and/or quality control testing. This work extends UPLC technology to a wider range of bioseparations.

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

Reliability of Size Exclusion Chromatography Measurements on ACQUITY UPLC H-Class Bio System

GOAL

To demonstrate reliability of the quaternary-based ACQUITY UPLC® H-Class Bio System and the ACQUITY UPLC BEH200 SEC Column for the analysis of proteins by size exclusion chromatography (SEC).

BACKGROUND

The complete characterization and analysis of biopharmaceuticals includes the application of size exclusion chromatography (SEC) to measure protein aggregates and other size variants. Soluble protein aggregates, in particular, can contribute to immunogenicity; accurate analysis and quantitation of biotherapeutic protein aggregates is, therefore, often required.

Current HPLC/silica-based SEC methods can be time-consuming and unreliable. These uncertain results may be due to changes in retention time, peak shape, or spacing between peaks as well as irreproducibility between columns and changes in columns within a few runs.

With the introduction of the ACQUITY UPLC H-Class Bio System and sub-2-µm ACQUITY UPLC BEH200 SEC Column chemistry, SEC separations can be obtained reproducibly, reliably, and in shorter analysis time with minimal development. Methods can be easily developed with the system's quaternary solvent manager utilizing Auto•Blend Plus™ Technology. This new implementation of instrument control functions removes the need for buffer pH adjustment and reduces time spent in buffer preparation.

The ACQUITY UPLC H-Class Bio System along with an ACQUITY UPLC BEH200 SEC Column deliver reliable and reproducible SEC separations for biomolecules.

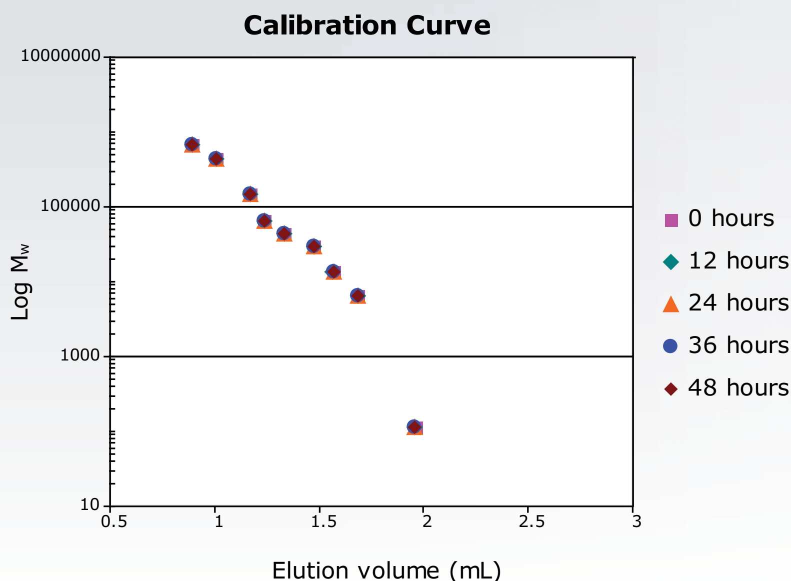

Figure 1. Protein calibration curve, ACQUITY UPLC BEH200 SEC 1.7-µm, 4.6 x 150 mm Column. Recommended molecular weight range is 10,000 to 450,000. Overlay of five calibration curves over 48 hours. Buffer: 20 mM Sodium Phosphate, 0.15 M NaCl, pH 6.8. Flow rate: 0.4 mL/min.

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The superior performance of this UPLC® SEC method relies on both the inert, low-dispersion system and the chemically-stable BEH column. The combination of these components allows users to obtain more accurate and reproducible results over a larger number of samples than is observed with current SEC methodologies.

THE SOLUTION

The SEC separation of biomolecules combines the ACQUITY UPLC H-Class Bio System with a 1.7-µm ACQUITY UPLC BEH SEC Column that provides the biochemist with a reliable separation. The low-dispersion, high-pressure system contains an inert flow path, that, when combined with four-solvent mixing and Auto•Blend Plus Technology, facilitates easy buffer preparation without pH adjustment.

The ACQUITY UPLC BEH200 SEC particle has an effective diol coating that provides a stable particle with minimal secondary interactions. The packing material is more resistant to chemical and mechanical degradation over time. These attributes combine to provide an SEC column stable more than 600 injections and requiring lower buffer concentrations than traditional silica-based columns.

In a series of experiments, protein standards and monoclonal antibody biotherapeutics were analyzed with UPLC-based SEC. Repeated analysis of the same sample was performed at regular intervals over a two-day period. Reproducibility of the calibration was tested by analysis of proteins standards over the molecular weight range of 10,000 to 450,000 Da.

The elution volume for each protein standard was found to be within 0.2% RSD. The calibration curve points do not fall on a perfect straight line because

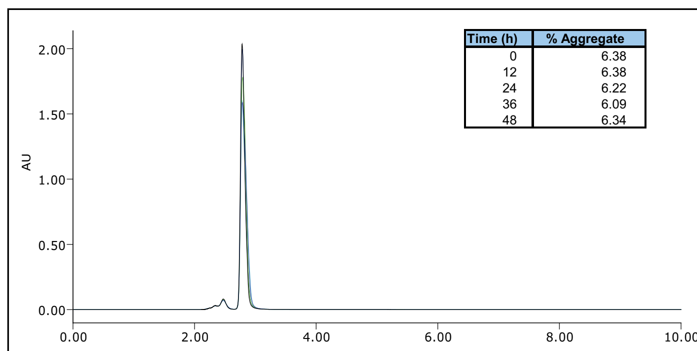

Figure 2. SEC separation of humanized IgG, 20 mg/mL. Injection of undiluted humanized IgG over 48 hours showed aggregate quantitation relative to the monomer of 6.09% to 6.38% with a RSD of 0.2%. Buffer: 20 mM Sodium Phosphate, 0.15 M NaCl, pH 6.8. Flow rate: 0.4 mL/min.

the elution volume reflects both size and shape of protein standard. The consistency of the calibration curve is, however, indicative of both the column life and instrument control of flow rate and injection volume.

To test the reliability of quantitation, a humanized monoclonal antibody was analyzed. The sample shown was found to have an average aggregate quantitation of 6.82% +/- 0.3% of the monomeric species over the time period. The reliability of this analysis is demonstrated by the reproducibility of this measurement. The SEC separations demonstrate the accuracy and reproducibility of UPLC SEC technology, which, in turn, ensures accurate identification and aggregate determination.

SUMMARY

The ACQUITY UPLC H-Class Bio System with an ACQUITY UPLC BEH200 SEC Column combine to provide reliable separations of proteins and their aggregates. As previously described, the analysis of both protein standards and monoclonal antibodies demonstrates the reliability of the calibration over a period of days. This reproducibility ensures accurate identification and quantitation of proteins and their aggregates, which can minimize analysis delays due to irreproducible results or incorrect peak identification. This, in turn, can increase throughput, thereby saving time and money. With the introduction of the ACQUITY UPLC H-Class Bio System and the new ACQUITY UPLC BEH200 SEC Column, reliable and reproducible SEC separations can be obtained for biomolecules.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Advances in Size Exclusion Chromatography for the Analysis of Small Proteins and Peptides: Evaluation of Calibration Curves for Molecular Weight Estimation

Paula Hong, Stephan Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution and throughput of peptide separations and small proteins with SE-UPLC compared to SE-HPLC
- Optimized conditions for true size-based separations of peptides
- Organic mobile phase compatibility for reliable molecular weight estimation of hydrophobic peptides

WATERS SOLUTIONS

ACQUITY UPLC® BEH125 SEC, 1.7 µm Column

ACQUITY UPLC H-Class Bio System

Auto•Blend Plus™ Technology

KEY WORDS

Size exclusion chromatography, SEC, peptides, proteins, SE-UPLC, Gel-Filtration Chromatography, calibration curves

INTRODUCTION

In 2010 over 60 therapeutic peptides were available in the US, Europe and/or Japan.[1] Recent trends indicate this number will only increase: the decline in development of small molecule pharmaceuticals, combined with improvements in peptide synthesis, have renewed interest in the research and development of peptide biotherapeutics a class of compounds that includes synthetic peptides such as vasopressin analogues and enfuvirtide.[2,3]

However, the complex nature of biotherapeutics requires a number of different analytical techniques for complete characterization, with each technique providing information on a different physical property of the biomolecule. One such technique, size exclusion chromatography (SEC) can be used to provide molecular weight characterization for the both the biomolecule and any process related species.[2,3] In this chromatographic mode, apparent molecular weight, based on hydrodynamic radius, is determined by comparing the elution volume of the unknown biomolecule with the elution profile of a known set of standards. However, these results can only provide useful information if the separation is solely size-based and not influenced by non-ideal or secondary interactions.

We have previously described the benefits of Ultra Performance Liquid Chromatography (UPLC®) combined with 200 Å sub-2 µm SEC packing materials for the analysis of monoclonal antibodies; however, these packing materials are not ideal for small biomolecules (<80,000 Da).[4,5] In the following application, the impact of a 125 Å pore sub-2 µm packing material on the separation and resolution of small proteins and peptides will be demonstrated. We will also show the impact of both physical and chemical properties of SEC packing materials on SEC calibration curves used for molecular weight estimation.

EXPERIMENTAL

SAMPLE PREPARATION: All samples were prepared in 25 mM sodium phosphate, 150 mM sodium chloride pH 6.8 buffer. Proteins and peptides were purchased as individual standards (Sigma-Aldrich). Sample concentrations ranged from 1–5 mg/mL. All samples were tested as individual standards unless otherwise noted.

LC Conditions

LC System:	ACQUITY UPLC H-Class Bio System with Column Manager or 30 cm Column Heater
Detection:	TUV detector with 5 mm Titanium Flow Cell
Wavelength:	280 and 214 nm
Columns:	ACQUITY UPLC BEH125 SEC, 1.7 µm Column, 4.6 x 150 mm and 4.6 x 300 mm (Part Number: 186006505); ACQUITY UPLC BEH200 SEC, 1.7 µm Column, 4.6 x 150 mm (Part Number: 186005225); BioSuite™ 125 UHR, 4 µm Column, 4.6 x 300 mm (Part Number: 186002161)
Column Temp.:	30 °C
Sample Temp.:	10 °C
Injection Volume:	2–8 µL
Flow Rate:	0.4 mL/min
Mobile Phases:	25 mM sodium phosphate, 150 mm sodium chloride, pH 6.8, 25 mM sodium phosphate, 250 mm sodium chloride, pH 6.2 and 30% ACN, 0.1% TFA (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Vials:	Maximum Recovery Vials (Part Number: 186002802)

Data Management

Chromatography Software:	UNIFI™ v 1.5 Software

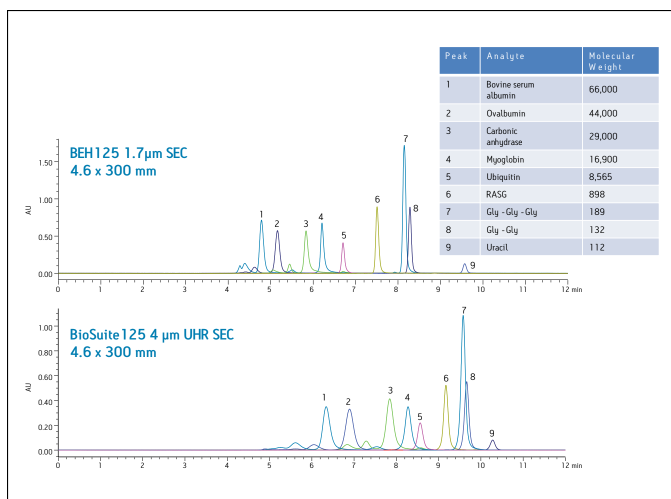
RESULTS AND DISCUSSION

Size-based separation calibration curves are based on known molecular weights of each protein as a function of elution volume or retention time. These curves, typically linear or third order polynomial, provide a means to get an approximate molecular weight of an unknown protein or peptide. While the linear portion of the calibration curve provides the highest resolution, non-linear behavior can also be observed since elution is dependent on the hydrodynamic radius of the molecule. While pore size is the predominant determining factor for the linear range of an SEC calibration curve, other factors include total pore volume of the column and pore size distribution.

Effect of Particle Size

The benefits of smaller particles for size exclusion chromatography have been well documented demonstrating improvements in efficiency and resolution.[5] Until recently, most studies have evaluated packing materials consisting of particle sizes greater than 3 µm. The advent of sub-2 µm SEC column packing materials allows for further improvements in resolution and efficiency.

A set of proteins and peptides were analyzed on both a UPLC-based BEH SEC column (1.7 µm) and an HPLC-based silica SEC column (4 µm) using the same ACQUITY UPLC H-Class Bio System (Figure 1) and aqueous mobile phase conditions (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8). The elution volume of the peptides and proteins was lower for the ACQUITY UPLC BEH125 SEC, 1.7 µm column as compared to the HPLC-based silica column. In addition, improved sensitivity and narrower peak widths were observed on the sub-2 µm packing material. USP resolutions for the main constituents were also calculated for both the UPLC-based BEH SEC (1.7 µm) column and the HPLC-based silica SEC (4 µm) column (Figure 2). While resolution in SEC with respect to the particle used is primarily a function of pore size and pore volume, the particle size of the separation medium also affects the ability to resolve closely related, molecular weight species. As shown in Figure 2, the calculated USP resolutions for peaks 2–8 in the test mixture showed resolution gains from 24–200% as compared to the 4 µm SEC column. As predicted, the greatest improvements in resolution on the 125 Å SEC-UPLC column were obtained in the molecular weight range less than 20,000 Da.



Peak	Analyte	Molecular Weight
1	Bovine serum albumin	66,000
2	Ovalbumin	44,000
3	Carbonic anhydrase	29,000
4	Myoglobin	16,900
5	Ubiquitin	8,565
6	RASG	898
7	Gly - Gly - Gly	189
8	Gly - Gly	132
9	Uracil	112

Figure 1. Effect of particle size on SEC separation of proteins and peptides.

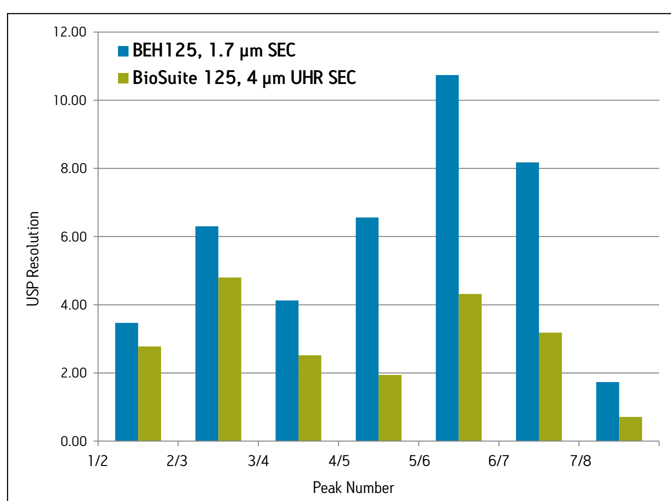
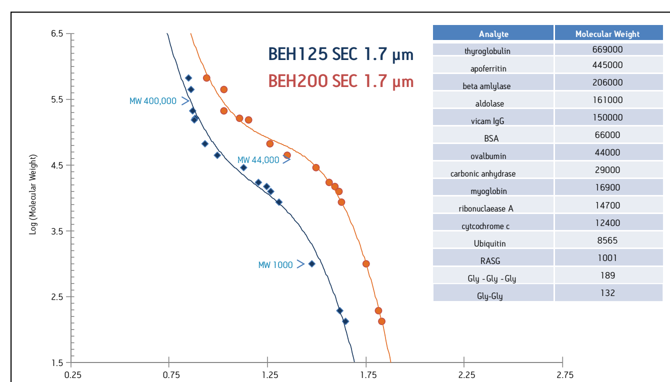


Figure 2: USP resolution of protein and peptides in Figure 4. USP Resolution was calculated by $2(t_{R2} - t_{R2})/(w_2+w_1)$, where t_R = retention time and w = peak width at 50% peak height.

Effect of Pore Size

For the analysis of small proteins and peptides, SEC packing materials typically contain pores of with a diameter <200 Å. These pore diameters have been shown to provide optimum resolution for solutes with less than 100,000 molecular weight. To evaluate the effect of pore size, a set of proteins and peptides were analyzed on both the 125 and 200 Å BEH sub-2 µm SEC columns under aqueous conditions (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8).

The calibration curve for each column was also evaluated to verify the effect of pore size on the molecular weight range (Figure 3). As described above pore size has a significant impact on the linear portion of an SEC calibration curve. Comparison of the 125 and 200 Å BEH sub-2 µm SEC columns illustrates this phenomenon. The calibration curve for the ACQUITY UPLC BEH200 SEC, 1.7 µm column showed greatest linearity and highest resolution, in the molecular weight range of 400,000 to 44,000 Da. Likewise, ACQUITY UPLC BEH125 SEC, 1.7 µm column provided highest resolution from 44,000 to 1,000 Da, the molecular weight range of most peptide biotherapeutics. As expected, pore size of the packing material had a significant impact on the useable molecular weight range of the column. The 200 Å packing material produced a separation with highest resolution over the molecular weight of 1,000,000 to approximately 44,000 Da, while the separation on the 125 Å packing material had greatest resolution from 44,000 to approximately 1,000 Da.



Analyte	Molecular Weight
thyroglobulin	669000
apoferritin	445000
beta amlylase	206000
aldolase	161000
vicam IgG	150000
BSA	66000
ovalbumin	44000
carbonic anhydrase	29000
myoglobin	16900
ribonuclaease A	14700
cytcochrome c	12400
Ubiquitin	8565
RASG	1001
Gly - Gly - Gly	189
Gly-Gly	132

Figure 3: Calibration curves: Effect of pore size. ACQUITY UPLC BEH125 SEC, 1.7 µm and ACQUITY UPLC BEH200 SEC, 1.7 µm columns, 4.6 x 150 mm comparison.

Effect of Mobile Phase Composition

SEC separations based on the hydrodynamic radius of the biomolecule rely on minimal adsorption between the analyte and packing material. These secondary interactions can be due to a number of different mechanisms including ionic interactions between the solute and the free silanols of the packing material or hydrophobic interactions between the solute and the hydrophobic sites on the packing material. While ion exchange effects can be minimized by the addition of buffers and salts and/or pH adjustments of the mobile phase, hydrophobic effects are commonly minimized by the addition of organic solvents or other additives. Given these considerations, careful evaluation of mobile phase conditions must be conducted to ensure a predominantly size-based separation for peptides

As described above, the ACQUITY UPLC BEH125 SEC, 1.7 µm column provided improved component resolution in molecular weight range less than 20,000. To explore the SEC separations within a defined molecular weight range, a series of peptides less than 9,000 Da were analyzed under aqueous conditions. Method development experiments evaluated the effect of mobile phase pH and salt concentration. The results showed minimal effect of salt concentrations (150–350 mM) and mobile phase pH (6.2–7.4) on retention time (data not shown). All of the aqueous mobile phases resulted in later than expected elution for most small peptides and proteins (<17,000 Da) as well as elution order that did not correspond to published molecular weight values. For example, bradykinin fragment 1–7 (MW 757) eluted before greater molecular weight peptides such as angiotensin I (MW 1296) and bradykinin (MW 1,060). Figure 4a. These results also suggest the non-ideal interactions of the tested peptides with the media is not solely due to an "ion exchange" mechanism since increasing salt concentration had no significant impact on retention time.

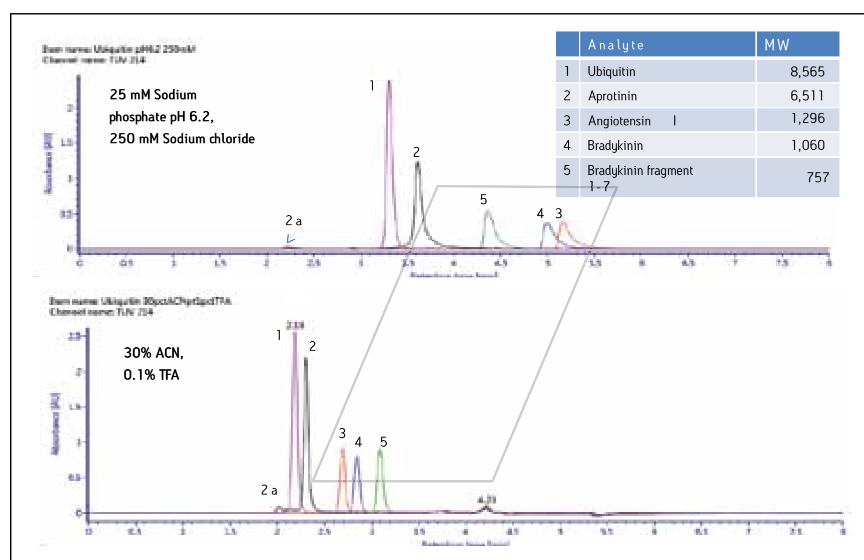
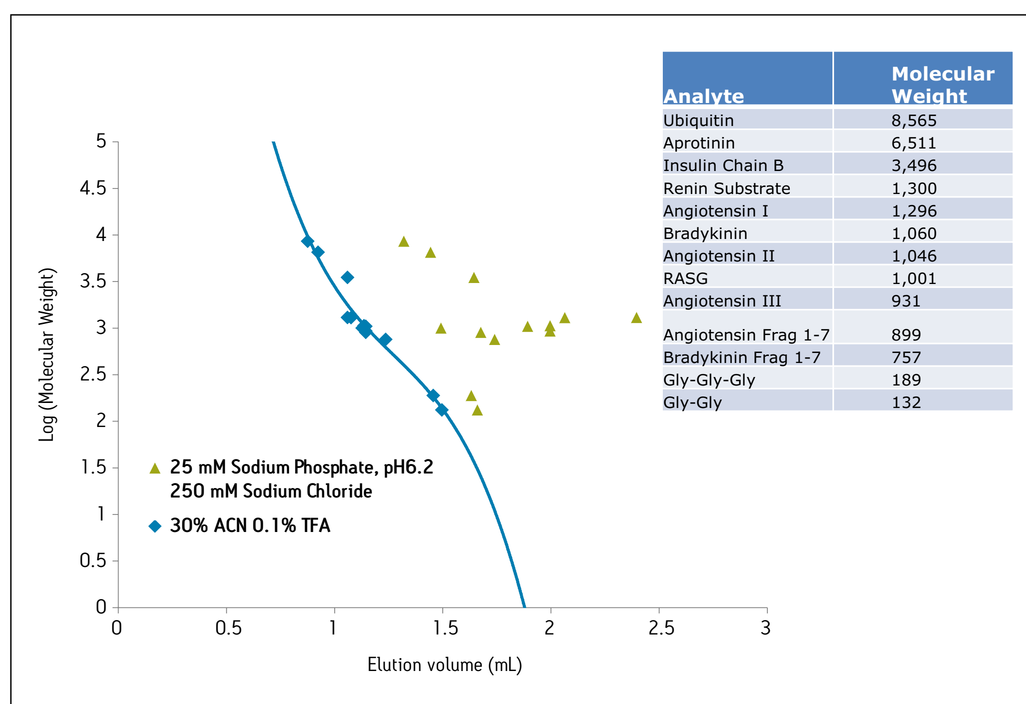


Figure 4. Effect of mobile phase on SEC separation of peptides.

In order to optimize the SEC separation for these peptides, evaluation of mobile phase was conducted. Mobile phases commonly used for SEC analysis of biotherapeutic peptides are denaturing and often contain organic solvents, acids and denaturants/charge additives such as arginine. These mobile phases minimize non-ideal (hydrophobic and/or ionic) interactions and thus are often needed to obtain a size-based separation for some peptides.[6] Additionally, these mobile phases can also affect retentivity by changing the structural conformation of the peptides. Under native conditions peptides may form stable secondary structures, while in the presence of denaturants these same polypeptides form random coil structures. These confirmation changes can increase the hydrodynamic radius of the biomolecule resulting in changes in elution volume.

The ACQUITY UPLC BEH125 SEC, 1.7 µm column was tested under similar conditions with organic/ion-pairing mobile phases (Figure 4). Acetonitrile was used to minimize hydrophobic interactions and trifluoroacetic acid was used as an ion pairing reagent to reduce “ion exchange” or charge-charge interactions. As expected, this mobile phase (30% acetonitrile and 0.1 % trifluoroacetic acid [TFA]) produced earlier retention times and more symmetrical peak shapes for the peptides analyzed. Furthermore, in contrast to the SEC separation of peptides under 100% aqueous mobile phases conditions, the use of organic and ion-pairing mobile phases resulted in the expected elution order for bradykinin fragment 1–7, angiotensin I and bradykinin, based on their molecular weights (Figure 4b). These elution order changes could be due to reduction of secondary interactions and/or changes in the confirmation and hydrodynamic radii of the peptides.

Comparison of the SEC calibration curves more clearly illustrates the effect of mobile phase formulation on the SEC separation of small biomolecules (Figure 5). Under aqueous condition (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8), the elution order of the peptides appears random. However, the use of acetonitrile and TFA in the mobile phase produced a 3rd order polynomial calibration curve, as predicted in size exclusion chromatography. This allows for reliable molecular weight estimation based on the linear region of the calibration curve. For example, the high molecular weight species of aprotinin (peak 2a) was calculated to be within 11% (or 14,370 Da) of the expected molecular weight (13,022 Da). This same estimation could not be performed under aqueous conditions because of the non-linearity of the calibration curve.



Analyte	Molecular Weight
Ubiquitin	8,565
Aprotinin	6,511
Insulin Chain B	3,496
Renin Substrate	1,300
Angiotensin I	1,296
Bradykinin	1,060
Angiotensin II	1,046
RASG	1,001
Angiotensin III	931
Angiotensin Frag 1-7	899
Bradykinin Frag 1-7	757
Gly-Gly-Gly	189
Gly-Gly	132

Figure 5. Effect of mobile phase on calibration curve of BEH125 SEC, 1.7 µm column.

CONCLUSIONS

Size exclusion chromatography has been the preferred method for the analyses of biomolecules based on size. By combining 125 Å sub-2 µm packing materials with a low dispersion ACQUITY UPLC H-Class System, improved resolution and high-throughput of SE-UPLC can be realized for small biomolecule separations. However, secondary interactions may need to be minimized in the development of a size-based separation for reliable molecular weight estimation.

The ACQUITY UPLC BEH125 SEC, 1.7 µm column combined with the ACQUITY UPLC H-Class Bio System provides:

- Improved resolution and higher throughput as compared to traditional SE-HPLC packing materials
- Improved resolution for 80,000–1,000 Da as compared to larger pore sized packing materials
- Compatibility with denaturing mobile phase used to reduce secondary interactions between peptides and packing materials for molecular weight characterization and impurity testing in the production of peptide biotherapeutics.

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T: 1 508 478 2000
F: 1 508 872 1990
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Size Exclusion UPLC for the Analysis of Covalent High Molecular Weight Insulin

Stephan Koza, Paula Hong, and Kenneth J. Fountain
Waters Corporation, 34 Maple Street, Milford, MA, USA

APPLICATION BENEFITS

- Improved separation of insulin monomer and covalent high molecular weight forms
- Faster analysis times and high throughput SEC separation
- Increased resolution of low molecular weight insulin degradant
- Reduced acetonitrile containing waste-stream volumes

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC BEH125 SEC, 1.7 µm Column

Empower™ 2 Software

KEY WORDS

Size exclusion chromatography, UPLC, insulin, USP, EP, method development, aggregates

INTRODUCTION

According to the World Diabetes Foundation there were an estimated 285 million people world-wide with diabetes in 2010, which is predicted to grow to 438 million people by 2030 and insulin is the primary treatment for this affliction. In 1982, insulin was the first recombinant biopharmaceutical, and by 2009, world-wide insulin and insulin analog sales were $13.1 billion.[1] As part of the US and European Pharmacopoeia (USP and EP), one of the critical quality control attributes for injectable insulin is the control of covalent high molecular weight (HMW) proteins.[2,3] The current USP 34 (p. 3134) and EP (Volume 5, p. 1800) monograph methods for this HMW determination are based on HPLC size exclusion chromatography (SEC). The USP method prescribes an L20 packing (dihydroxypropane groups chemically bonded to porous silica particles, 3 to 10 micro-m in diameter) in a 7.8-mm x 30-cm geometry and the EP method prescribes the use of a "hydrophilic silica gel for chromatography R (5-10 µm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers" with a length of 30 cm and a minimum internal diameter of 7.5 mm.

Presented in this application are the advantages that may be realized using a 125Å pore size, sub-2-µm ethylene-bridged hybrid (BEH) silica packing material and Waters UltraPerformance Liquid Chromatography (UPLC®) instrumentation for this traditional analysis. Among these advantages are faster run times, higher sensitivity, and higher resolving separations of insulin and covalent insulin HMW, while at the same time greatly reducing acetonitrile containing waste-stream volumes.

EXPERIMENTAL

LC Conditions

System: ACQUITY UPLC H-Class Bio with TUV and titanium flow cell

Wavelength: 276 nm

Column Temp.: 25 °C

Sample Temp.: 10 °C

Injection Volume: 10 µL (unless specified otherwise)

Flow Rate: 0.4 mL/min (unless specified otherwise)

Mobile Phase: L-arginine (1.0 g/L)/acetic acid (99%)/acetonitrile; 65/15/20 (v/v/v)

Wash and Injector Needle Purge: 10% acetonitrile

Seal Wash: 10% methanol

Sample Diluent: 0.01 N hydrogen chloride

Columns

ACQUITY UPLC BEH125 SEC, 1.7 µm, 4.6 x 150 mm (part number 186006505)

ACQUITY UPLC BEH125 SEC, 1.7 µm, 4.6 x 300 mm (part number 186006506)

ACQUITY UPLC BEH200 SEC, 1.7 µm, 4.6 x 300 mm (part number 186005226)

BioSuite™ 125 UHR SEC, 4 µm, 4.6 x 300 mm (part number 186002161)

Insulin HMWP HPLC, 10 µm, 7.8 x 300 mm (part number WAT201549)

Data Management

Software: Empower 2 with Auto•Blend Plus™

SAMPLE PREPARATION

The insulin control sample (Sigma, I2643) was reconstituted and diluted to 4.0 mg/mL in a 0.01 N hydrochloric acid solution. The injectable insulin product samples were analyzed past expiry.

RESULTS AND DISCUSSION

The focus of this application is an evaluation of the performance of the ACQUITY UPLC BEH125 SEC, 1.7 µm, 4.6 x 300 mm column (part number 186006506) under the conditions provided by the USP and EP monographs for the analysis of HMW protein in therapeutic insulin samples. The acidic mobile phase prescribed by both of these pharmacopoeial methods is comprised of 0.65 g/L L-arginine, 15% acetic acid, and 20% (v/v) acetonitrile. This mobile phase provides an assessment of the levels of covalent HMW present in these preparations while disrupting non-covalent insulin self-association and column interactions. The column factors that were evaluated for this method include pore-size, particle diameter, and column length.

Selected columns, all of which posses a hydrophilic coating to minimize undesired secondary effects between analyte and particle surface chemistry, were evaluated based on resolution and HMW quantitation. All columns were configured on the same ACQUITY UPLC H-Class Bio System using Waters Empower 2 Software. The resolution results were determined based on the tangent peak widths USP determination and by peak-to-valley ratio (P/V) as directed by both the EP and USP monographs as part of the determination of system suitability. The P/V ratio is obtained by dividing H_p (height above the baseline of the peak due to the covalent dimer) by H_v (height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer). The requirement for this system suitability parameter is not less than (NLT) 2.0 for a sample containing more than 0.4% HMW in both the EP and USP monographs.

Pore Size

The insulin size exclusion HMW determination method in the EP monograph prescribes the use of an SEC pore size of 120Å to 125Å (12 - 12.5 nm), however, the USP monograph does not specifically state a pore size requirement. The ACQUITY UPLC BEH125 SEC, 1.7 µm particles have a pore size of 125Å and, therefore, meet the EP monograph requirements. Figure 1 shows the comparison between the BEH125 SEC, 1.7 µm particle column and the 200Å pore diameter BEH200 SEC 1.7 µm particle column in order to demonstrate the importance of using the appropriate pore diameter for a SEC separation. The principal benefit that can be realized by selecting the optimal pore-size is increased resolution. The critical separation shown in these chromatograms is between the HMW species and the insulin monomer and an 8% improvement in that resolution is observed for 125Å pore-size particles as compared to the 200Å pore-size particles for the standard sample. The improvement observed in the two therapeutic samples,

which were analyzed past expiry, is lessened due to the increased extent of peptide degradation. In addition to an improvement in resolution, the average P/V determined for the two therapeutic samples, which had HMW peak area percentages above 0.4%, was higher for the 125Å pore-size particles (P/V=37) versus the 200Å pore-size particles (P/V=11). However, both columns were able to surpass the US and EP monograph system suitability criterion of a P/V of NLT 2.0 for a sample containing more than 0.4% HMW. Additional improvements in the separation between the covalent insulin dimer HMW form and the multimeric HMW forms, and between the insulin monomer and an insulin fragment, are also observed in the therapeutic samples analyzed using the 125Å pore-size particles that are not observed using the 200Å pore-size particles, thereby providing an additional assessment of sample quality.

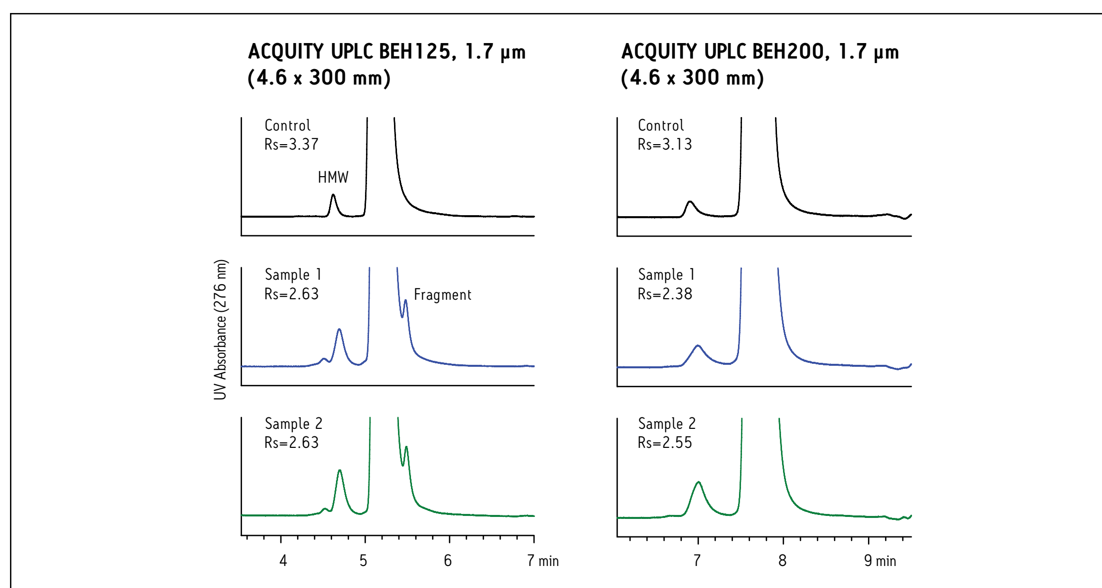

Figure 1. Zoomed view of insulin HMW, monomer, and low molecular weight fragment SEC separations using a 125Å pore-size ACQUITY UPLC BEH125 column and a 200Å pore-size ACQUITY UPLC BEH200 column. Samples 1 and 2 were therapeutic insulin samples analyzed past expiry.

Particle Size

The insulin SEC HMW determination method in the EP monograph prescribes an SEC particle size of 5 to 10 µm while the USP monograph specifies a particle size of 3 to 10 µm. As part of this study, a comparison was performed among three SEC columns with particle sizes of 1.7, 4, and 10 µm that are of comparable 125Å pore size and equivalent 300 mm length (Figure 2). The 1.7-µm column demonstrates a significant increase (>35%) in resolution as compared to the 4- and 10-µm columns. The average HMW P/V system suitability criterion for the two therapeutic samples was also higher for the 1.7-µm column (P/V=37) than for the 4-µm (P/V=9) and 10-µm (P/V=8) columns. All three columns met the EP and USP HMW P/V system suitability criterion of NLT 2. In addition to increased resolution, the 1.7-µm column also provides a smaller total elution volume (~5 mL) than the 4-µm (~6 mL) and 10-µm (~13 mL) columns. This decrease in total elution volume provides the analyst with increased sample throughput in addition to a decrease in mobile-phase use. Shown in Figure 3 is an expanded base-line view comparison of the chromatograms obtained from the 1.7- and 10-µm particle size columns that highlights the dramatic improvements in resolution that are observed for the sub-2-µm UPLC column configuration.

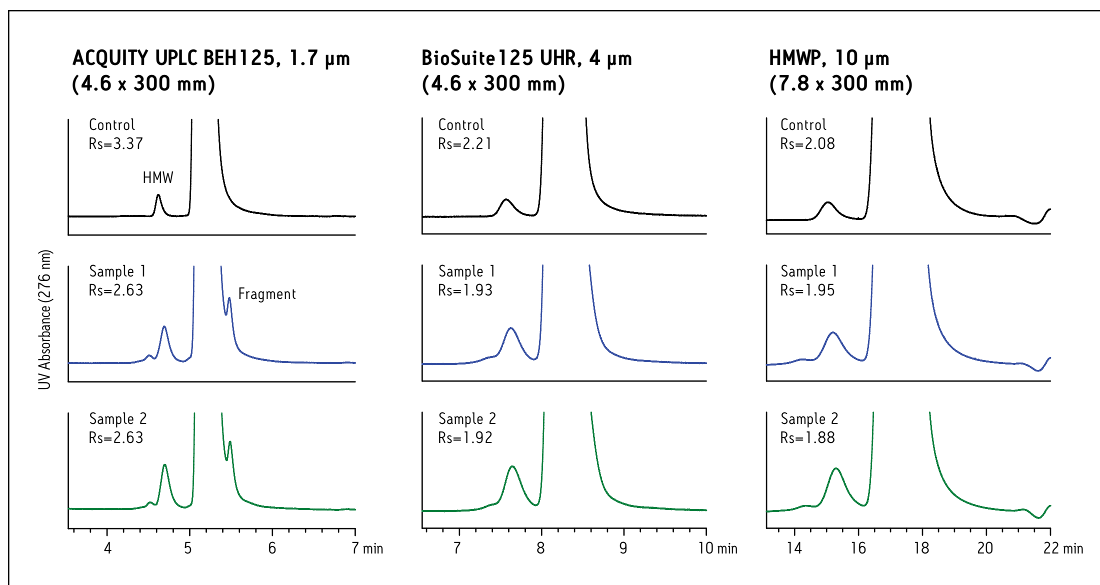

Figure 2. Zoomed view of insulin HMW, monomer, and low molecular weight fragment SEC separations using 125Å pore-size columns with particle sizes of 1.7, 4 and 10 µm. The sample load volume and flow rate for the 10-µm particle size column were 100 µL and 0.5 mL/minute in accordance with the EP monograph method. Samples 1 and 2 were therapeutic insulin samples analyzed past expiry.

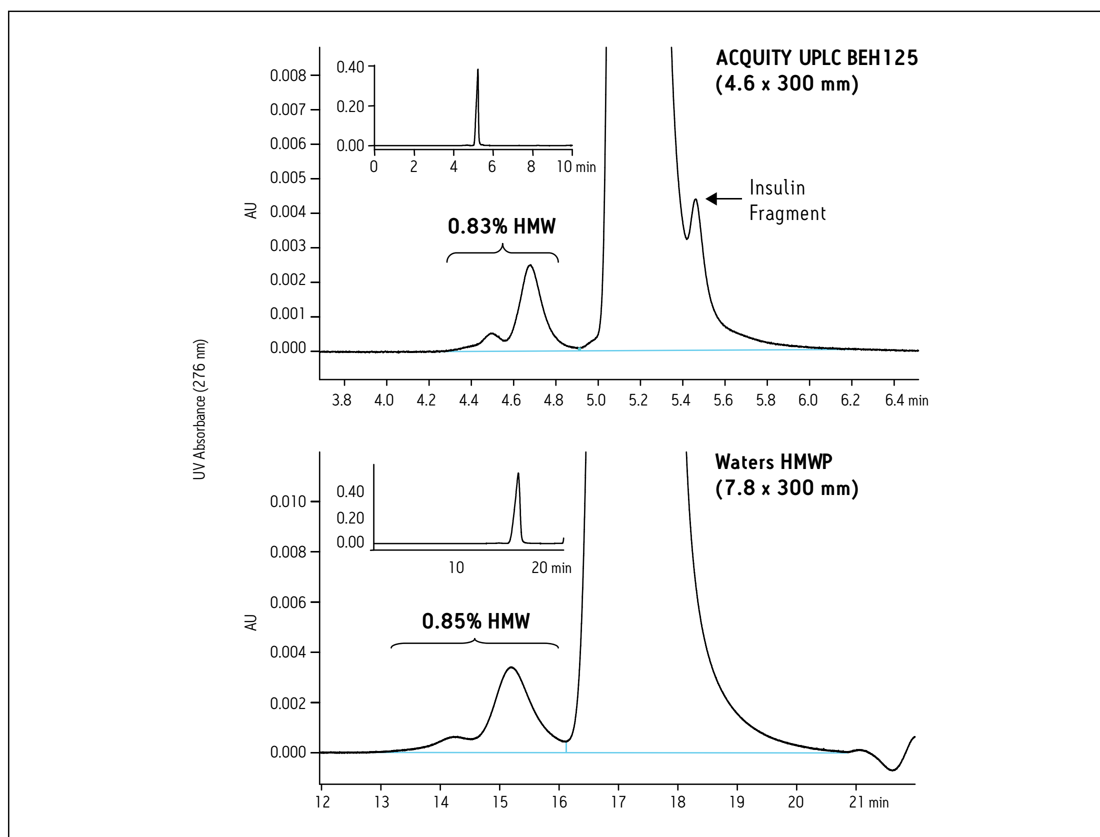

Figure 3. Zoomed view of chromatograms from Figure 2 of a biotherapeutic insulin sample (analyzed past expiry) generated by the ACQUITY UPLC BEH125 and the Waters HMWP columns. The complete chromatographic profiles are presented in the insets. The sample load volume and flow rate for the Waters HMWP column were 100 µL and 0.5 mL/minute in accordance with the EP monograph method. The ACQUITY UPLC BEH column provides improved resolution, improved sensitivity, significantly shorter analysis time, and reduced mobile-phase use.

Column Length

The effect of column length was also evaluated by comparing the insulin separation on both a 4.6 x 150 mm and a 4.6 x 300 mm ACQUITY UPLC BEH125 column (Figure 4). Chromatographic principles predict that resolution should be linearly proportional to the square-root of column length and that is what was observed. The 300 mm column provided a 41% to 43% increase in resolution as compared to the 150 mm column, consistent with the predicted increase of 41% (√2). The average HMW peak-to-valley ratio system suitability criterion for the two therapeutic insulin samples was also higher for the 300 mm column (P/V=37) than for the 150 mm column (P/V=5). Both columns met the EP and USP HMW peak-to-valley ratio system suitability criterion of NLT 2. The improved resolution is also apparent in the monomer peak tail, in which a small, lower molecular weight fragment peak is partially resolved on the 300 mm but not on the 150 mm column. However, it should also be noted that the improved resolution is accompanied by a two-fold increase in analysis time and mobile-phase use.

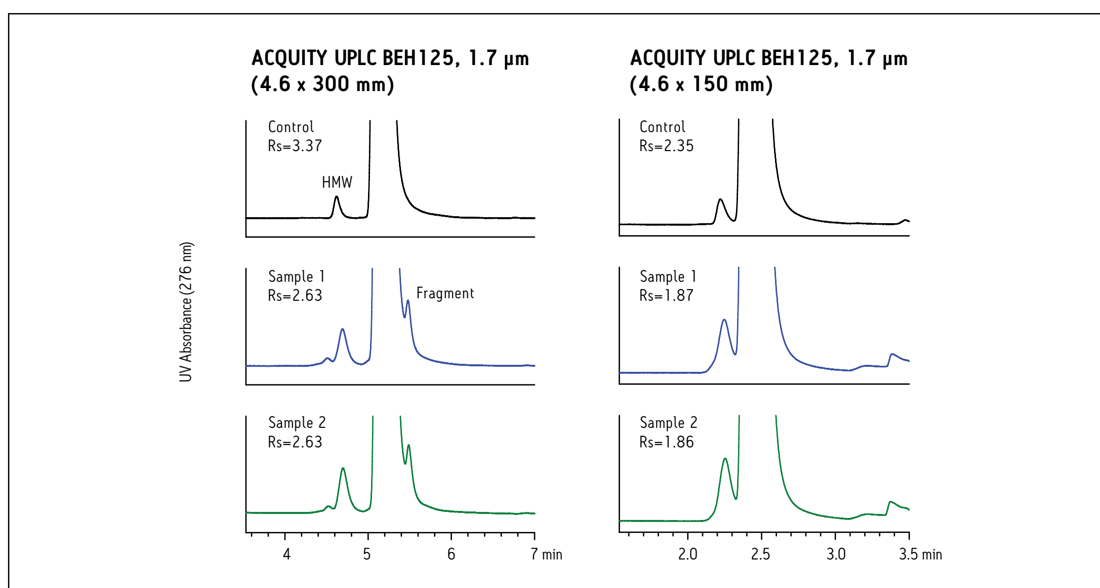

Figure 4. A comparison of two ACQUITY UPLC BEH125 columns with lengths of 300 mm and 150 mm. The flow rates for this study were equivalent (0.5 mL/minute). The injection volumes were 10 µL and 5 µL for the 300 mm and 150 mm columns, respectively.

Depending on the method requirements, column length can be chosen to either provide improved resolution or higher sample throughput. For example, for a registered quality test a longer column provides improved resolution that can result in better quantitative reliability. While in discovery, development, or during real-time process monitoring, a shorter column allows for faster analysis time and higher sample throughput.

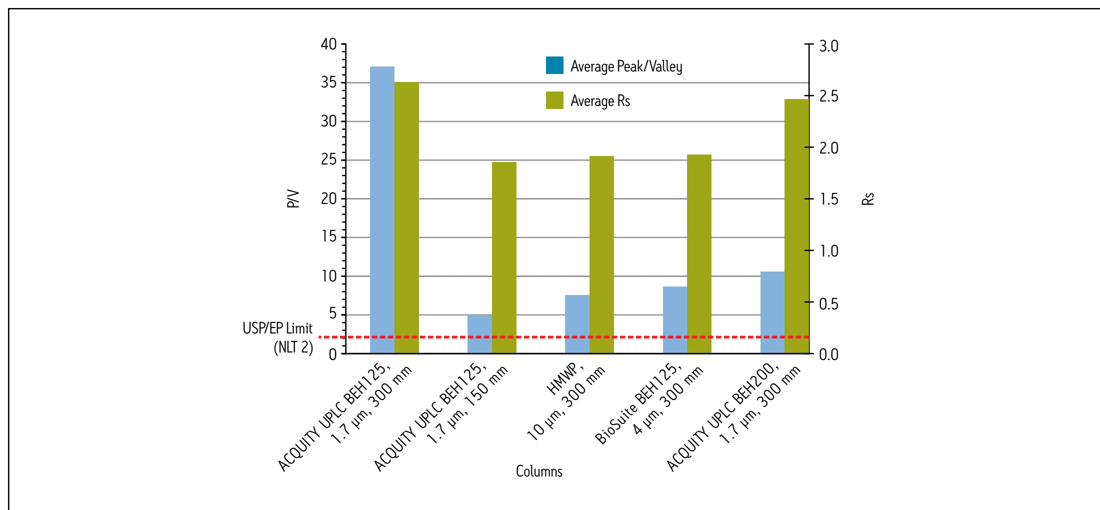

Figure 5. A summary of the performance with respect to HMW peak-to-valley ratio (P/V) and USP resolution (Rs) for the columns evaluated. Shown for reference is the minimum P/V required by the EP and USP monograph methods.

CONCLUSIONS

Size exclusion chromatography is the USP and EP standard method for the analysis of covalent HMW insulin in therapeutic preparations. The chromatographic profiles demonstrating the performance of this method using SEC columns of different pore size, particle size, and length have been presented and these data are summarized in Figure 5. Based on these results the use of 125Å pore size, sub-2-µm ethylene-bridged hybrid (BEH) silica packing material and Waters Ultra Performance Liquid Chromatography (UPLC) instrumentation for this traditional SEC-based analysis provides significant improvements in resolution compared to traditional SE-HPLC methods while reducing analysis time and mobile-phase use.

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

[TECHNOLOGY BRIEF]

Improving the Lifetime of UPLC Size Exclusion Chromatography Columns using Short Guard Columns

GOAL

To demonstrate the improved long-term stability of the ACQUITY UPLC® BEH200 SEC, 1.7 µm column with the use of a guard column.

BACKGROUND

Size exclusion chromatography (SEC) is commonly used to measure aggregates and other size variants in protein-based therapeutics. Of particular interest are soluble aggregates, which are thought to play a role in immunogenicity. Studies have found a variety of chemical and physical conditions can affect the amount of aggregates in biotherapeutics. To minimize these effects, excipients or stabilizers are typically added to protein-based therapeutics. Additives can decrease long-term column stability in SEC, resulting in inaccurate quantitation due to changes in retention, peak shape or spacing between peaks. While a variety of cleaning protocols may be used to try to restore the column, this approach can be time consuming and ultimately ineffective. As a last resort, column replacement is often required, resulting in higher costs and further delays in analysis time. With the introduction of the ACQUITY UPLC BEH200 SEC, 1.7 µm guard column, long-term stability for the SEC column can be increased, thereby saving time and money.

THE SOLUTION

The UPLC® SEC separation for biomolecules combines the ACQUITY UPLC H-Class Bio System with a sub-2 µm BEH SEC column. While the ACQUITY UPLC BEH200 SEC packing material is resistant to chemical and mechanical degradation for hundreds of injections, various sample components may adversely affect the column. With the use of a guard column in combination with the BEH200 SEC column, the column stability can be extended for hundreds of injections in the presence of these excipients.

In this set of experiments, protein standards, uracil and a murine monoclonal antibody (mAb) were analyzed on both a BEH200 SEC, 1.7 µm, 4.6 x 150 mm column alone and a BEH200 SEC, 1.7 µm, 4.6 x 150 mm column equipped with a guard column with the same packing material (4.6 x 30 mm). The mAb was diluted in a representative biotherapuetic formulation containing polysorbate 80, sucrose and sodium phosphate. The samples were analyzed on a single BEH200 SEC column over the course of 500 injections and on a BEH200 SEC column equipped with a guard column for over 900 injections. The guard column was replaced approximately every 200 injections. Evaluation of the mAb monomer efficiency on the BEH200 SEC column alone shows a decrease in plate count (from 5000 to 3000) after 550 injections. The same sample tested on the column and the guard combination shows no significant change in mAb monomer efficiency over 900+ injections (Figure 1). Any drops in monomer efficiency were restored after replacement of the guard column.

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Before replacement of each guard column, the BEH200 SEC, 1.7 µm column was tested alone to check column efficiency. Comparison of this test to the initial column testing demonstrates the long-term stability achievable with a guard column (Figure 2). The mAb separation on the column alone shows a decrease in peak height and an increase in peak tailing over the course of 480 injections. Furthermore, the mAb monomer/dimer USP resolution decreases 26%. In contrast, the separation on the column protected by a guard shows no significant change in peak height or peak tailing over the course of 900 injections. The mAb monomer/dimer USP resolution remains relatively unchanged, demonstrating the effectiveness of the guard column.

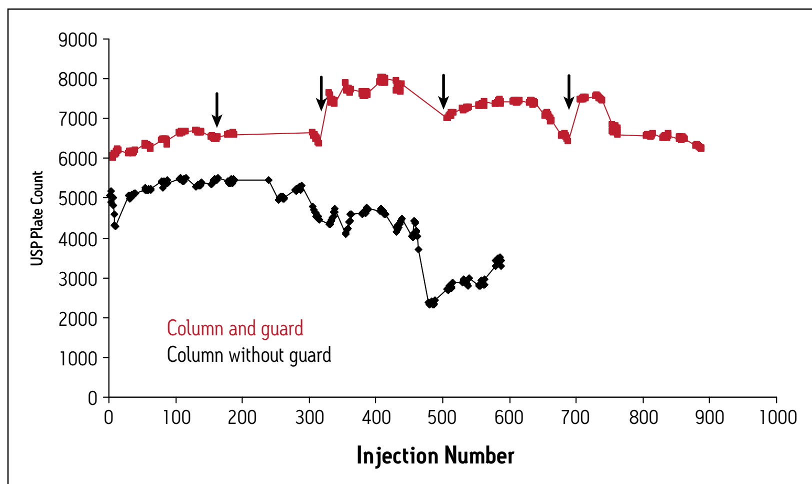
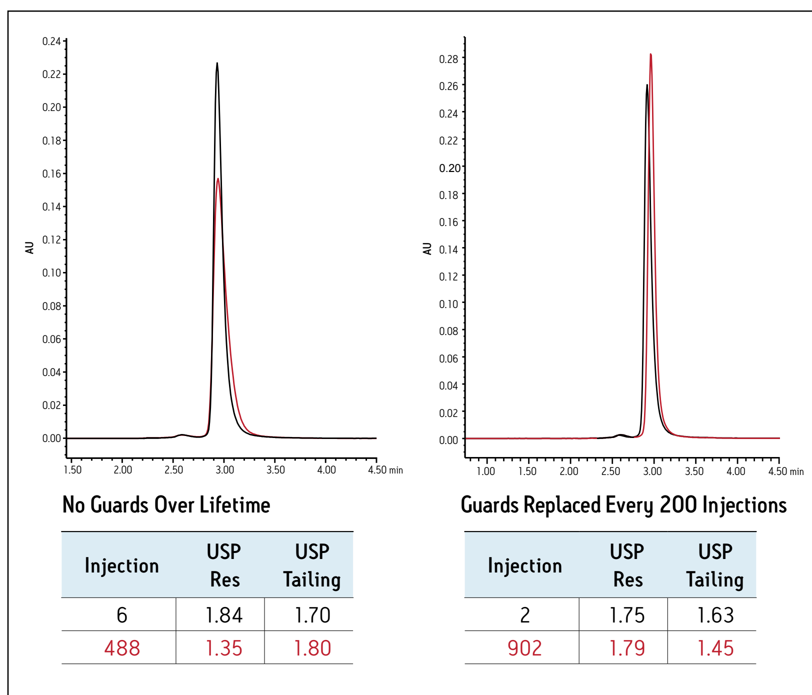


Figure 1. Effect of using a 30 mm guard column on column efficiency. The arrows indicate where the guard column was changed.



No Guards Over Lifetime

Injection	USP Res	USP Tailing
6	1.84	1.70
488	1.35	1.80

Guards Replaced Every 200 Injections

Injection	USP Res	USP Tailing
2	1.75	1.63
902	1.79	1.45

Figure 2. Effect of a 30 mm guard column on mAb separation performance. Chromatograms of mAb on BEH200 SEC column before and after multiple injections of a formulated mAb sample. Note: Chromatograms at right were run on column without guard to confirm column efficiency, i.e. column check. Conditions: 0.025 M sodium phosphate pH 6.8, 0.15 M sodium chloride, 0.4 mL/min at 30 °C.

SUMMARY

The combination of the BEH200 SEC analytical column and guard column allow for a stable analysis and quantitation of a monoclonal antibody in the presence of excipients. While the presence of excipients or other matrix components may adversely affect a SEC separation over time, the regular replacement of the guard column preserves the performance of the analytical column for over 900 injections. This allows the biochemist to reduce costs and minimize down time, thereby resulting in more stable analyses for monoclonal antibodies and their soluble aggregates.

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

[TECHNOLOGY BRIEF]

Ion Exchange Chromatography Method Development on ACQUITY UPLC H-Class Bio System

GOAL

To simplify ion exchange chromatography (IEX) methods development for the analysis and characterization of proteins using the quaternary-based ACQUITY UPLC® H-Class Bio System in combination with Auto•Blend Plus™ Technology.

BACKGROUND

The complete analysis and characterization of proteins requires orthogonal analytical techniques focusing on different physical and chemical properties. Ion exchange chromatography is often utilized to assess the distribution of proteins or the presence of protein variants formed by post-transitional modifications (*e.g.*, deamidation) that can be recognized by charge differences. For these analyses, adjustments in mobile phase pH are the most useful parameter for method development. Such experiments are, however, time-consuming and cumbersome. The ACQUITY UPLC H-Class Bio System and its quaternary solvent manager takes advantage of UPLC® Technology and Auto•Blend Plus Technology to simplify IEX method development.

Auto•Blend Plus Technology allows users to manipulate pH and ionic strength by calculating and delivering the proportions of buffer stocks required for the desired conditions. The introduction of this new system provides users a robust, efficient tool for method development for IEX separations of proteins.

The ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology together help IEX users streamline the development of methods for the analysis of proteins and their charge variants.

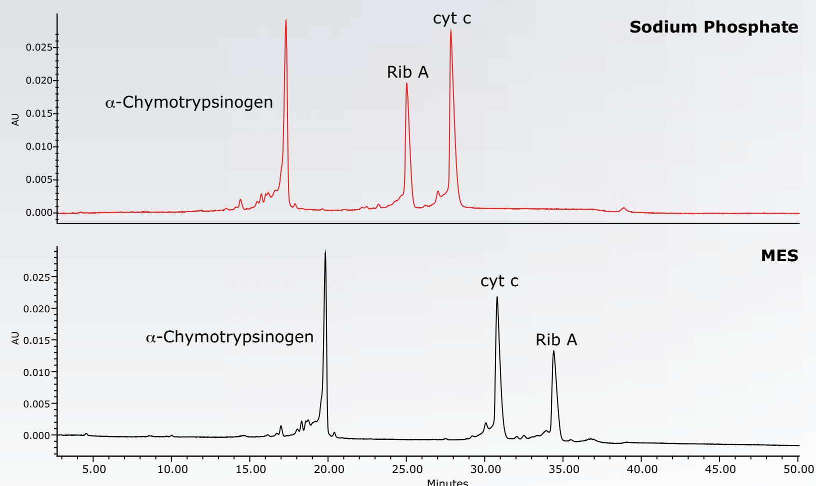

Figure 1. Effect of buffer composition on the IEX separation of proteins.
Sample: Bovine, α-Chymotryspinogen, Bovine Ribonucelase A, Equine cyctochrome c.
Column: Protein-Pak Hi Res CM 7-µm, 4.6 x 100 mm.
Conditions: 20 mM buffer (MES or Sodium Phosphate) pH 6, 1 mL/min, 0 to 0.2 M NaCl in 34 min at 30 °C.

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THE SOLUTION

Ion exchange chromatography of proteins combines the ACQUITY UPLC H-Class Bio System with Auto•Blend Plus Technology and Protein-Pak™ Hi Res Columns for simplified method development. The ACQUITY UPLC H-Class Bio System is an inert system that provides stability in the aqueous, high ionic strength buffers used for IEX separations, while also giving the highest recovery of the sample.

Auto•Blend Plus Technology takes advantage of the system's four-solvent blending capabilities to prepare and adjust chromatographic mobile phases using pure solvents and concentrated stocks of acid, base, salt, and water. In the newest implementation, its user interface allows for expressing the chromatographic method in parameters that are most familiar to the biochemist, specifically pH and ionic strength.

Using Auto•Blend Plus Technology, a series of experiments were performed to demonstrate the effect of buffer composition and pH on IEX separations of proteins. To illustrate the effect of buffer composition, a mixture of proteins was separated using weak cation-exchange chromatography. Two common cation-exchange buffers, Sodium phosphate and MES ((N-Morpholino) ethanesulfonic acid), were compared. At a pH of 6, different selectivity was observed for the most basic proteins, as shown in Figure 1.

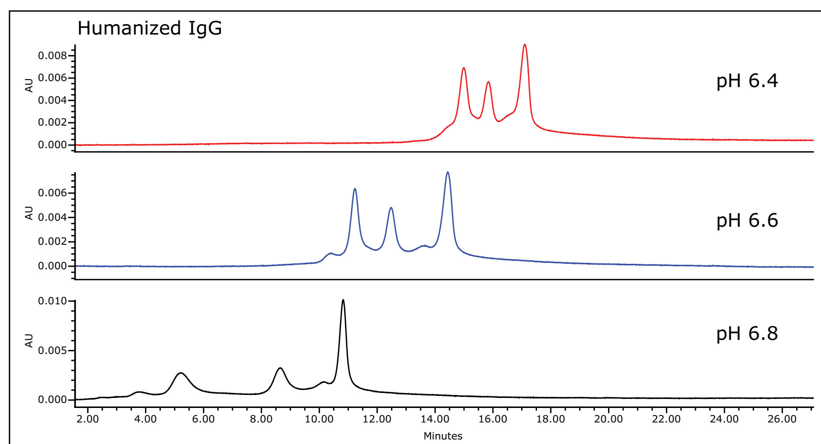

Figure 2. Effect of pH on IEX separation of humanized IgG.
Sample: Humanized IgG, 1.5 mg/mL.
Column: Protein-Pak Hi Res CM 7-µm, 4.6 x 100 mm.
Conditions: 20 mM Sodium Phosphate, 0.5 mL/min, 0 to 0.1 M NaCl in 40 min at 30 °C.

The buffer system also influenced overall retention: MES buffers resulted in longer retention times for the proteins. In a second experiment, a monoclonal antibody containing lysine variants was separated with phosphate buffer at different pHs. The monoclonal antibody separations (Figure 2) demonstrate the influence of pH on retention time and selectivity for a humanized IgG and its variants. In these experiments, the use of Auto•Blend Plus Technology and a four-buffer blending system allowed for simple, fast method development for IEX separation of proteins.

SUMMARY

The ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology together provide IEX users with a fast and easy method development system for the analysis of proteins and their charge variants. Adjusting pH is simplified with the use of a four-solvent blending system (acid, base, salt, and water) in combination with Auto•Blend Plus Technology software. These improved protocols translate to savings in both time and reagent costs, increasing overall productivity.

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IEX Method Development of a Monoclonal Antibody and its Charge Variants

Paula Hong, Kenneth J. Fountain, Thomas E. Wheat, Damian Morrison

APPLICATION BENEFITS

- Robust method for confirmation of monoclonal antibody charge variants
- Reproducible and simplified method development without the need to prepare additional buffers
- Ideal for monitoring biopharmaceutical charge variants throughout manufacturing process

WATERS SOLUTIONS

Protein-Pak™ Hi Res IEX columns

ACQUITY UPLC® H-Class Bio system

Auto•Blend Plus™ technology

KEY WORDS

IEX, monoclonal antibody, Protein-Pak Hi Res, lysine variants

INTRODUCTION

The use of biologic-based therapeutics, including monoclonal antibodies, has grown rapidly over the past twenty years. The complexity of these macromolecules requires the use of orthogonal analytical techniques for complete analysis and characterization. One technique, ion exchange chromatography (IEX), is used for analysis of charge heterogeneity biotherapeutics. Since each protein has a unique charge distribution, chromatographic selectivity can be adjusted by pH; however, these separations often lack resolution and consistency making method development more difficult. To address these issues, a solvent management system using pure solutions and concentrated stocks was developed. This system, capable of four solvent blending to prepare and adjust chromatographic mobile phases, can be combined with a high-resolution ion exchange column to develop a separation for the lysine truncation variants of a chimeric monoclonal antibody. The weak-cation exchange column allows for method development over a range of pH's and with multiple buffers to obtain the optimum separation of these charge variants. This will be illustrated with a specific method for a chimeric antibody.

EXPERIMENTAL

SAMPLE DESCRIPTION: A chimeric monoclonal antibody sample containing C-terminal lysine truncation variants was prepared at 1.25 mg/mL in 20 mM MES buffer, pH 6.

C-terminal lysine cleavage was performed using Carboxypeptidase B (CpB) (Worthington Biochemical Corp., p/n LS005304) prepared at 1 mg/mL. The monoclonal antibody (1000 µL, 1.25 mg/mL) and CpB (12.2 µL, 1 mg/mL) were combined. At predetermined time intervals (t= 0, 1, 2.5, 5, 7.5, 10, 12.5, 15, and 20 min), a 100 µL aliquot of the mixture was removed and combined with glacial acetic acid (1.7 µL) to halt the reaction.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System with Auto•Blend Plus Technology
Detector:	PDA Detection with Titanium Flow Cell
Wavelength:	280 nm
Sampling rate:	20 pts/sec
Filter time constant:	Normal
Column:	Protein-Pak Hi Res IEX CM, 4.6 x 100 mm, 7 µm (P/N 186004929)
Column temp.:	30 °C
Sample temp.:	4 °C
Injection volume:	10 µL
Flow rate:	0.5 mL/min
Mobile phase A:	100 mM Sodium Phosphate, monobasic, or 100 mM MES monohydrate
Mobile phase B:	100 mM Sodium Phosphate, dibasic, or 100 mM MES sodium salt
Mobile phase C:	1000 mM Sodium Chloride (NaCl)
Mobile phase D:	Water
Purge and wash solvents:	20mM Sodium Phosphate, pH 6.0 or 20mM MES, pH 6.0
Gradient:	0-10% C in 60 min, (pH specified in figures)

Data management

Software:	Empower™ 2 with Auto•Blend Plus Technology

RESULTS AND DISCUSSION

Charge heterogeneity of a monoclonal antibody may be caused by several structural changes including C-terminal lysine processing.[1] When present in biopharmaceuticals, these charge variants are often monitored throughout manufacturing to ensure control of the process. In the following study, an IEX method was developed to confirm and quantify the presence of C-terminal lysine truncation variants in a chimeric monoclonal antibody therapeutic. Method development was performed on a weak-cation exchange column (Protein-Pak Hi Res CM, 4.6 x 100 mm, 7 µm) by manipulating pH and ionic strength. A four solution blending system was used to make pH buffer adjustments by using a weak acid (line A) and the cognate base (line B). Sodium chloride (NaCl, line C) and water (line D) were used to adjust the ionic strength of the buffer. These adjustments were performed using Auto•Blend Plus Technology, which allowed the gradient to be expressed directly in terms of pH and ionic strength. The operating software automatically calculates the percentage of acid and base required for the specified pH from the known or measured pKa of the selected buffer system.

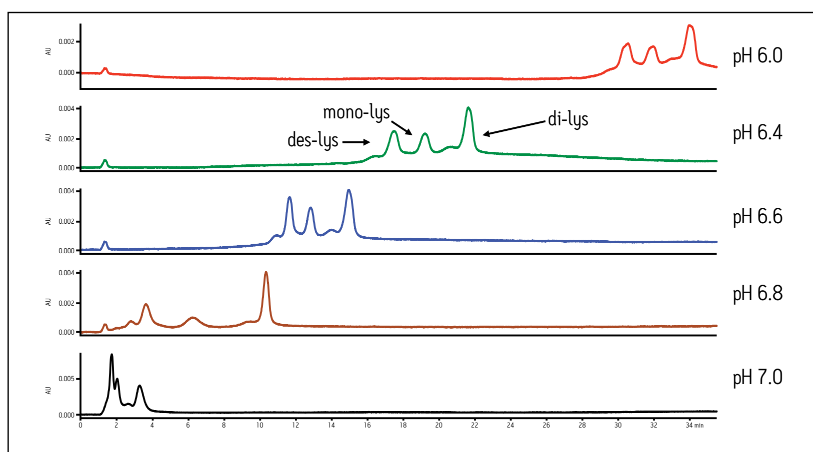

Figure 1. Analysis of a chimeric antibody and its truncated C-terminal lysine variants on a Protein-Pak Hi Res CM column with sodium phosphate buffer. Separations were performed over a pH range of 6.0-7.0 using Auto•Blend Plus Technology.

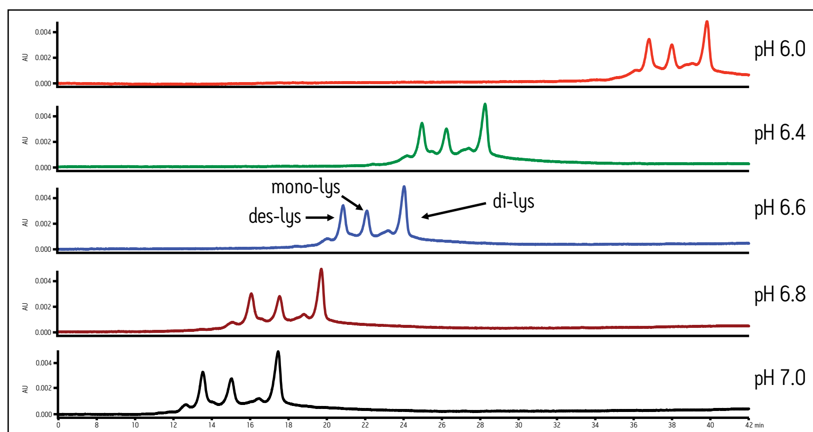

Figure 2. Analysis of a chimeric antibody and its truncated C-terminal lysine variants on a Protein-Pak Hi Res CM column with MES buffer. Separations were performed over a pH range of 6.0-7.0 using Auto•Blend Plus Technology.

Two common cation-exchange buffers, sodium phosphate and MES ((N-Morpholino) ethanesulfonic acid), were compared. For each buffer system, the effect of pH was studied (Figures 1 and 2). A single salt gradient (0-100 mM NaCl in 60 minutes) was tested over a pH range of 6.0-7.0.

With a sodium phosphate buffer system, the resolution of the monoclonal antibody (des-lys) and the C-terminal lysine truncation variants varies with pH. Higher pH corresponds to earlier elution of the chimeric antibody and the lysine truncation variants. This behavior is typical of cation-exchange chromatography since the overall positive charge of a protein decreases as pH increases, thereby resulting in elution of the analytes at a lower ionic strength. The non-truncation species (des-lys) elutes at retention times ranging from 3.4 minutes at pH 6.8 to 29.0 minutes at pH 6.0 (Table 1). This corresponds to NaCl concentrations ranging from 4 mM at pH 6.8 to 68 mM at pH 6.0 (Table 2). A similar trend is observed for the antibody with a single C-terminal lysine truncation (mono-lys). However, under the same conditions, the doubly C-terminal lysine truncation variant (di-lys) exhibits a smaller shift in both retention time and NaCl concentration. This difference in retentivity of the variants as pH is increased yields higher resolution between the mono-lys and di-lys variants at pH 6.8 (Figure 1) as compared to lower pH. A mobile phase pH of 7 results in almost no retention for the antibody and the charged variants.

The same study with an MES buffer system demonstrates the effect of buffer composition on an IEX separation. As similar to the sodium phosphate buffer system, a higher pH results in lower retention for the antibody and the charge variants. However, resolution is not significantly affected (Figure 2). Overall, the MES buffer results in later elution of the analytes when compared to the sodium phosphate buffer system. Varying pH with the MES buffer produces changes in retention. Selectivity is not significantly altered.

Differences in the retention observed between the two buffers can be attributed to the different ionic

Retention Time (min)

pH	des-lys	mono-lys	di-lys	des-lys	mono-lys	di-lys
6.0	36.6	37.8	39.6	29.0	30.3	32.1
6.4	25.0	26.3	28.3	16.3	17.9	20.2
6.6	21.0	22.2	24.1	10.9	12.0	14.0
6.8	16.3	17.8	19.9	3.4	5.8	9.7
7.0	13.8	15.3	17.7	1.6	1.9	3.1

Table 1. Retention times of a chimeric antibody (des-lys) and its truncated C-terminal lysine variants (mono-lys and di-lys) at pH 6.0-7.0 with MES and sodium phopsphate buffer systems.

Concentration (mM)

pH	des-lys	mono-lys	di-lys	des-lys	mono-lys	di-lys
6.0	87	90	95	68	71	76
6.4	58	61	66	37	41	46
6.6	48	51	56	23	26	31
6.8	37	40	45	4	10	20
7.0	30	34	40	0	1	3

Table 2. NaCl concentration in mM corresponding to the retention times of a chimeric antibody (des-lys) and its truncated C-terminal lysine variants (mono-lys and di-lys) at pH 6.0-7.0 with MES and sodium phosphate buffer systems. Calculations are based on a 1 minute gradient hold and gradient delay volume of 0.350 mL.

strengths of each buffer system. The ionic strength of a buffer is based on the total number of ions contributed by both the sodium chloride and the buffering agent. For the two buffering agents used in this screening, this difference is largely due to the different number of sodium ions present. The sodium phosphate buffer system combines the mono- and di-basic forms of phosphate. Therefore, when the weak acid and cognate base are in equal proportion, three sodium ions are contributed by the buffer. In contrast, the MES buffer system is comprised of the weak acid form and the cognate sodium base. When both acid and base are in equal proportion in the MES buffer system, an amount of sodium equimolar to the cognate base (10 mM) is contributed by the buffer. Thus, when the ionic strength of NaCl is held constant and both buffering agents are at the same concentration and pH, the phosphate buffer system will have a greater ionic strength when compared to the MES buffer due to the additional sodium ions present. In quantitative terms, at a pH of 6.0 the sodium phosphate buffer system contributes an additional 22.7 mM of sodium ions to the mobile phase while the MES buffer system adds an additional 8.9 mM of sodium ions. This difference in contributing ions results in earlier elution of the antibody and the C-terminal lysine truncation variants with a sodium phosphate buffer (Table 1). As pH is increased, sodium phosphate

contributes even more sodium ions in the form of the base (32.3 mM at pH 7) as compared to MES buffer system (17.8 mM sodium ions), resulting in a greater ionic strength at a constant NaCl concentration, and thus a greater retention time shift with pH as compared to MES buffer system (Table 1).

The separation method was used to confirm the identity of the peaks. To confirm the C-terminal lysine truncation variants, the antibody biotherapeutic was treated with carboxypeptidase B following previously published protocols.[2,3] The separation was performed with MES buffer system at a pH of 6.6. These buffer conditions allow for separation of the antibody and the C-terminal lysine truncation variants in addition to the analysis of smaller eluting acidic or basic variants. The reaction was monitored over a period of 20 minutes. At predetermined time points, an aliquot of the sample was removed and combined with acetic acid to halt the reaction.

The time course study of the reaction demonstrates both the reproducibility of the IEX method and the conversion of the di-lys and mono-lys forms to the des-lys form. The retention time reproducibility was less than 0.1% RSD for all of the major components over the time course study (Table 3). The conversion of the mono-lys and di-lys variants to the des-lys antibody is also confirmed by analysis of % peak area. Within the first 2.5 minutes of the reaction, the latest eluting peak (di-lys) shows the greatest decrease in % peak area (Figure 3). In that same time period, the des-lys and mono-lys both show an increase in % peak area (Figures 3 and 4). Subsequent time point analyses show a continual increase in % peak area for des-lys, while both mono-lys and di-lys variants continue to exhibit a decrease in % peak area and are almost undetectable at 20 minutes (Figure 3). These results are consistent with conversion of the di-lys and mono-lys variants to the des-lys antibody.

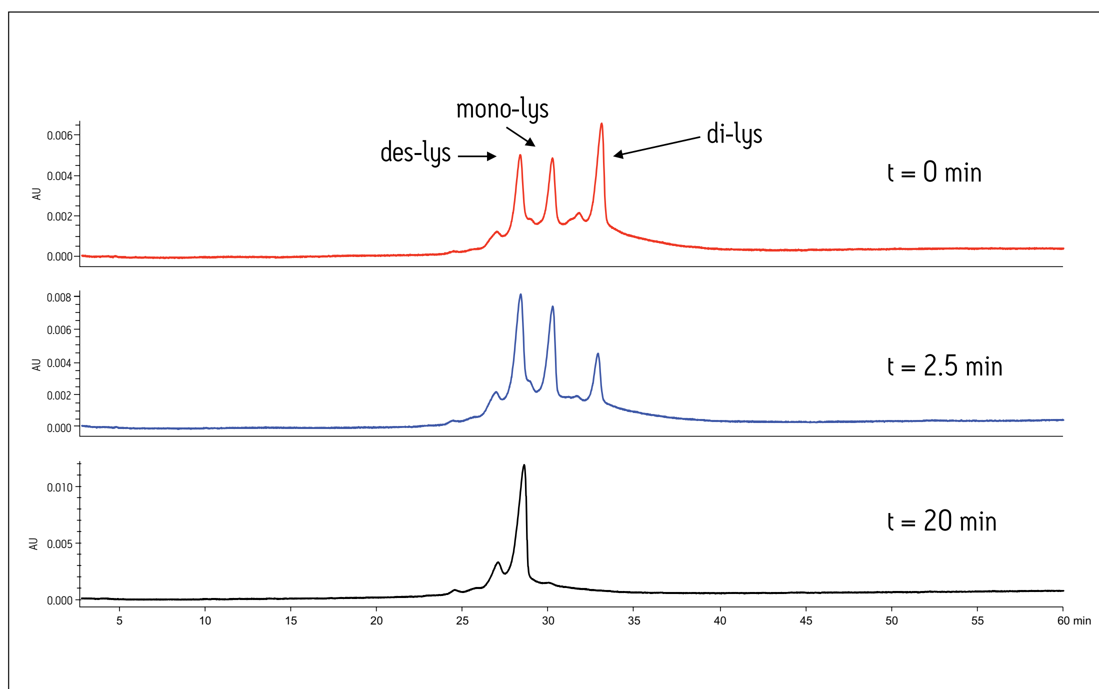

Figure 3. Treatment of a chimeric antibody with carboxypeptidase B over 20 minutes in MES buffer at pH 6.6. Analysis at intervals of 0, 2.5 and 20 minutes confirms the presence of truncated C-terminal lysine variants..

Retention Time (min)	des-lys	mono-lys	di-lys
0.0	27.77	29.63	32.50
1.0	27.74	29.65	32.45
2.5	27.79	29.65	32.28
5.0	27.82	29.55	32.12
7.5	27.88	29.49	32.08
10.0	27.95	29.48	31.81
12.5	27.93	29.44	N/A
15.0	27.96	29.40	N/A
20.0	27.98	29.42	N/A
Mean	27.87	29.53	32.21
Std Deviation	0.09	0.10	0.26
%RSD	0.33	0.32	0.80

Table 3. Retention time reproducibility for the IEX separation of a chimeric antibody and its truncated C-terminal lysine variants. Samples were treated with carboxypeptidase B and tested at set time intervals.

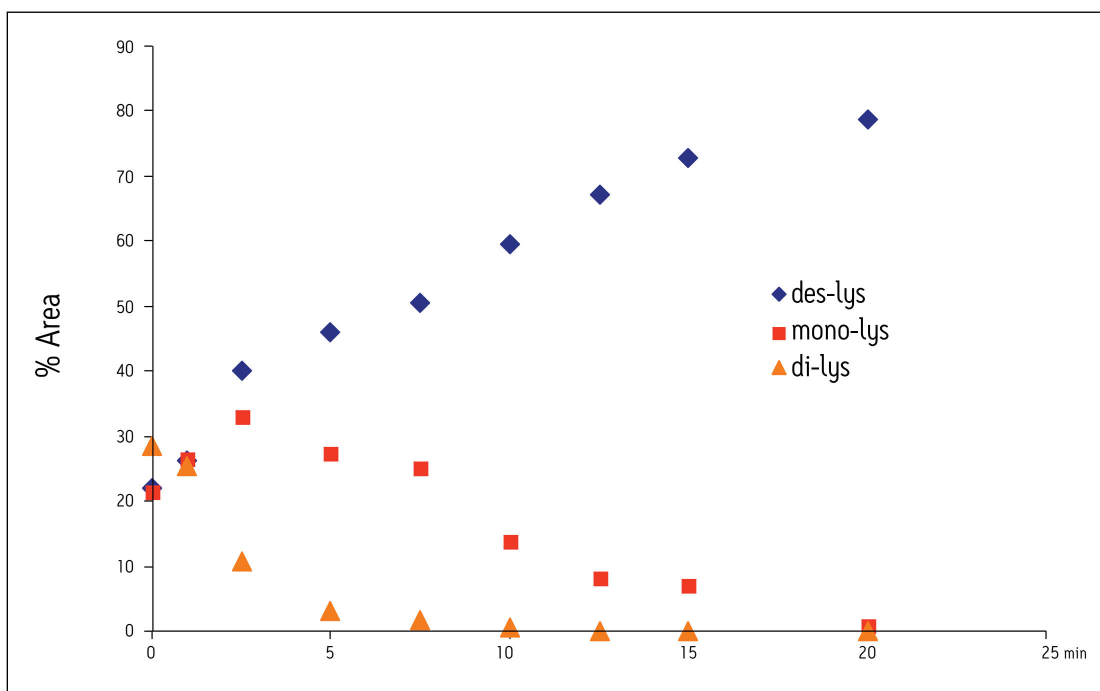

Figure 4. Analysis of the chimeric antibody (des-lys) and its truncated C-terminal lysine variants (mono-lys and di-lys) over 20 min after treatment with carboxypeptidase B. Total % peak area is not equal to 100 due the presence of additional variants.

CONCLUSIONS

The heterogeneity of a biopharmaceutical monoclonal antibody from C-terminal lysine truncation is typically monitored throughout manufacturing to ensure process stability and insure quality control. For these charge variants, the Protein-Pak Hi Res CM column provides a tool for the analysis and confirmation of a chimeric antibody and its C-terminal lysine truncation variants. The column, in combination with the ACQUITY H-Class Bio System and Auto•Blend Plus Technology, allows for simplified pH screening and evaluation of multiple buffer systems. Method development studies for the chimeric antibody demonstrate the dramatic affect of pH with a sodium phosphate buffer, which is partially due to the ionic strength of the buffering agent. In contrast, minimal resolution effects are observed with varying pH in a MES buffer system. All of the screening studies performed are simplified with the use of a four-solvent blending system and Auto•Blend Plus Technology. The resulting separation provides a robust method for analysis and confirmation of a monoclonal antibody and its C-terminal lysine truncation variants.

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Solutions in Practice

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

[TECHNOLOGY BRIEF]

Guidelines for Routine Use and Maintenance of UPLC Size Exclusion and Ion Exchange Chromatography Systems

GOAL

To outline good practices for the routine maintenance and use of UltraPerformance LC® Systems in conjunction with high ionic strength, 100% aqueous mobile phases, typical eluents used in size exclusion and ion exchange chromatography.

BACKGROUND

Given the complexity of biotherapeutics, full characterization typically requires a variety of orthogonal methods. While many chromatographic techniques are conducted under reversed phase conditions, others are conducted under native separation conditions, requiring high ionic strength, 100% aqueous eluents. For high performance liquid chromatography systems, these conditions can be problematic: in the absence of bactericides, lack of proper maintenance can lead to bacterial contamination within hours. The presence of high salt concentrations increases the potential of particulates in the mobile phases. The presence of bacteria and particulates in the LC system can affect chromatography quality and column lifetime.

The components of the chromatographic system are equally important. If the chromatographic system is not inert or bio-compatible, metal-protein adducts or undesired protein interactions can occur. Long-term use of high ionic strength, 100% aqueous mobile phases can also lead to rust formation if the chromatographic system contains steel components in the wetted path. However, with proper set-up and care of a chromatographic system, robust and reproducible chromatography can be achieved with minimal down time.[1,2]

Proper Set-Up and Maintenance of an ACQUITY UPLC System Allows for Robust and Reliable SEC and IEX Separations.

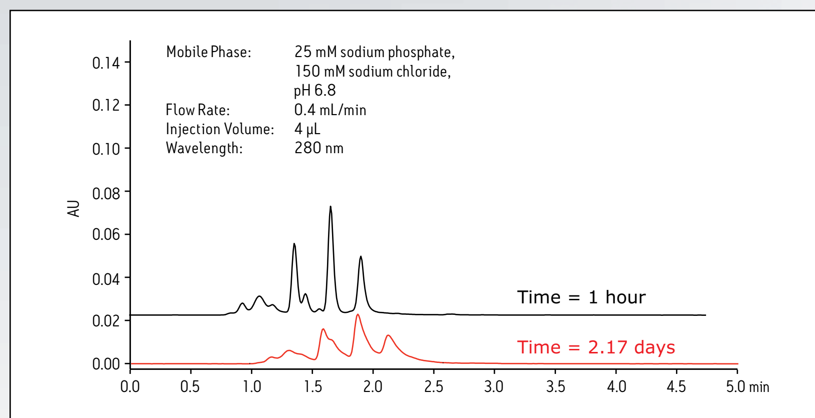

Figure 1. Effect of microbial growth on a SEC chromatogram of protein mix. Contamination confirmed by analytical analysis of column frit.

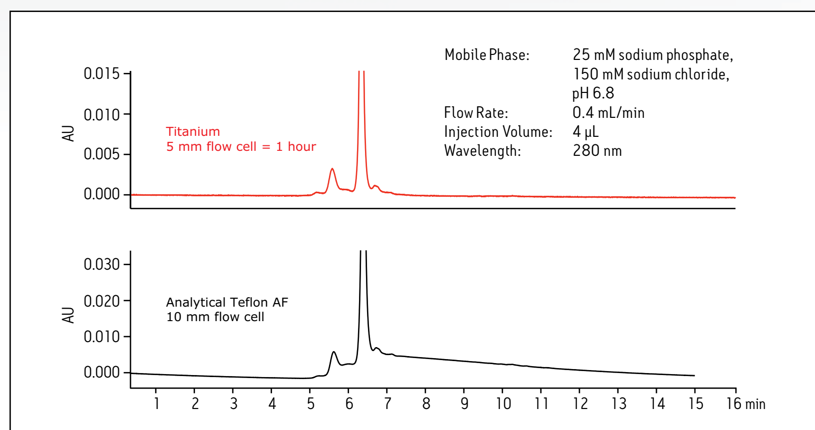

Figure 2. SEC-PDA chromatogram of bovine serum album (BSA) (5 mg/mL in water) shows the effect of flow cell material on peak shape. BSA monomer exhibits extensive peak tailing.

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THE SOLUTION

The care and use of a size exclusion and/or ion exchange chromatographic system requires many of the same standard practices as any other system. However, there are some additional protocols that are required for high salt, aqueous mobile phases. While the practices outlined in this document are described for ACQUITY UPLC® Systems, the principles apply to any chromatographic system. Overall system recommendations include:

- If using a steel system, modify according to manufacturer's recommendations. For use with a Waters UPLC® System, detailed guidelines are available.
- Clean laboratory glassware properly.
- If chronic loss of prime, check valve problems, reproducibility of retention time, pressure or quantitative reproducibility are observed, clean the system following a standard protocol.[3]
- If possible, use mobile phases containing a bacteriostat (i.e., 0.02% sodium azide) to prevent microbial growth.
- Retention time or pressure fluctuation can be indicative of problems with the pump.
- Area or peak variability without retention time variation can be indicative of an injector problem.

Additional recommendations are listed below by component. These considerations are for microbial growth, system suitability and/or protein stability.

Solvent Delivery System:

The buffers used in SEC and IEX can favor microbial growth leading to contamination of the column and system (Figure 1). Recommendations include:

- Always filter aqueous mobile phase through compatible 0.22 µm or smaller membrane filters. The use of sterile filters and containers is also recommended.
- Use only high purity water (18.2 MΩ cm). Bottled water should be opened the day of use.
- Never 'top-off' mobile-phase bottles. Always change bottles when replacing mobile phase.
- High-ionic-strength eluents (> 100 mM) should be replaced every two weeks.
- Low-ionic-strength eluents (< 100 mM) should be replaced every 2-3 days.
- Water (100%) bottles should be replaced daily.
- Higher salt concentrations, which inhibit microbial growth, would reduce the frequency of solvent replacement. While it is not always practical to change mobile phases, a quaternary solvent mixing system can accomplish the same effect by combining high concentration buffers (> 100 mM) to produce SEC and IEX mobile phases.[5]
- All eluent bottles should be visually inspected daily for microbial growth and/or particulates. Microbial growth can be a film on the bottle surface or may be observed by swirling the bottle.
- If microbial growth has occurred in the eluent bottle, replace the mobile-phase filter or flush it with a 70% isopropanol solution. Microbes can contaminate mobile-phase filters.
- Use compatible mobile-phase filters, such as titanium. Clean mobile-phase filters weekly to reduce microbial contamination. Sonicate or flush with 70% isopropanol solution.
- Recommended seal wash cycle time of 0.10 minutes (6 seconds).
- Seal wash recommendation of 90/10 water/methanol.
- The solvent manager should never be left idle in either high salt mobile phases or 100% water.
  - For short-term storage, maintain 0.1 mL/min of drawing an equal percentage of mobile phase from all lines in use.
  - If system will be idle for more than 2 days, prime each line for 10 minutes with high purity water. Thoroughly flush system. Repeat steps with 90/10 water/methanol.

Sample Manager:

SEC and IEX conditions often require high-ionic-strength solutions in the sample manager wash lines (wash/purge, strong and weak needle). However, these eluents may have a detrimental affect on the sample syringe and/or needle. For variable flow through needle systems, remove salt deposit on a regular basis to minimize maintenance and repairs. Other recommendations include:

- Ensure the sample is soluble in the mobile-phase and sample manager washes.
- Follow the manufacturer's recommendations for wash solvents. For example, washes containing less than 500 mM salt(s) are recommended for the ACQUITY UPLC H-Class Bio System.
- If visible salt deposits appear, clean the surfaces. If salt deposits reappear, check connections and system for problems.[4]
- If the sample manager is idle for more than 2 days, purge needle and/or wash lines with high purity water (minimum of 20 cycles or 200 seconds). Repeat steps with 90/10 water/methanol. The sample manager should never be left idle for longer than 2 days in lines containing high salt washes (>100 mM).

UV Detectors:

Waters recommends titanium or stainless steel optical flow cells when performing SEC or IEX under aqueous conditions. The standard ACQUITY® optical flow cell contains Teflon AF in the fluidic path. Some proteins, under native conditions, may interact with the flow cell surface, resulting in peak tailing and sloped baseline (Figure 2). Recommendations for detectors include:

- Use titanium or stainless steel flow cells to reduce protein-surface interactions. Other flow cell material (i.e., Teflon) may cause peak tailing.
- Never leave the detector idle in high salt eluents. Flush thoroughly with water (60 minutes at 0.2 mL/min) followed by 90/10 water/methanol or higher organic eluent.

Column Storage:

To maintain long column lifetimes and minimize the risk of microbial contamination, the following recommendations should be followed:

- Columns should never be stored in high salt, aqueous mobile phase or 100% water.
- Before switching to recommended storage conditions, flush columns with 10-20 column volumes of water.
- Flush and store columns following the manufacturer's recommendations. Typical recommendations are 10-20% methanol or with a bactericide (i.e., 0.1% sodium azide).
- Consider the use of guard columns to extend column lifetimes. Regular replacement may be required. Frequency of guard column replacement may be dependent on sample cleanliness.
- Size exclusion columns can typically be stored at 4-8 °C to reduce microbial growth. Ion exchange columns are usually stored at room temperature. Check the manufacturer's recommendations for details.

SUMMARY

SEC and IEX chromatography are performed under native conditions, requiring high-ionic strength, 100% aqueous eluents. To minimize protein-surface interactions these conditions may require the use of a bio-compatible chromatographic system specifically designed for these applications. Precautions must be taken to prevent and minimize bacterial contamination. Signs of such contamination, which can occur within hours include: deteriorating peak shape, resolution and column lifetime. Unfortunately, once the column has been contaminated, regeneration is difficult. To decrease the frequency of system repairs and contamination, a series of steps have been outlined for maintenance and care of a chromatographic system and columns used for the analysis of biomolecules. These recommendations include maintenance for the solvent manager, sample manager, detector and column. Using these procedures in combination with good laboratory practices ensures a robust, reproducible system for ultra-performance-size exclusion and ion exchange chromatography.

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

[TECHNOLOGY BRIEF]

Reversed Phase Analysis of Proteins using ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology

GOAL

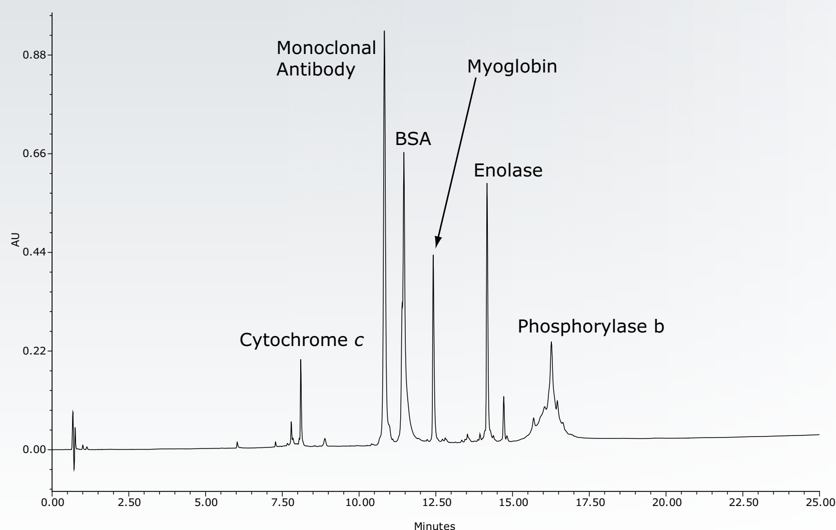
To demonstrate rugged, high-resolution separation of proteins using reversed phase techniques, four-solvent blending, UPLC® Protein Separation Technology columns, and the ACQUITY UPLC® H-Class Bio System.

BACKGROUND

The characterization and analysis of protein samples must be sensitive to chemical differences in a molecule that, while but a small fraction of the large molecule, can have large biological effects. The general approach to this analytical challenge is to use an array of separation techniques, each sensitive to a specific physical or chemical property. The ACQUITY UPLC H-Class Bio System was designed to be suitable for use with all the common protein analysis techniques, including ion exchange, size exclusion, HILIC, and reversed phase. The experiments described here focus on reversed phase analysis of large, intact proteins.

Reversed phase separations of proteins typically use wide pore columns with short chain bonded phases. The ACQUITY UPLC BEH 300 C_4, 1.7-µm column was selected for these experiments. The samples are eluted with a gradient of increasing organic solvent in the presence of a polar acid modifier. There are several choices in identity and concentration for both the solvent and the modifier. We can manage these choices

A robust separation of intact proteins is readily achieved with this biocompatible UPLC system that streamlines use of desired mobile phase combinations.



The Auto•Blend Technology method blends the required mobile phase from reservoirs of pure solvents and concentrated modifier stocks. The reversed phase chromatogram generated from this convenient method shows a wide range of proteins as a test of robustness.

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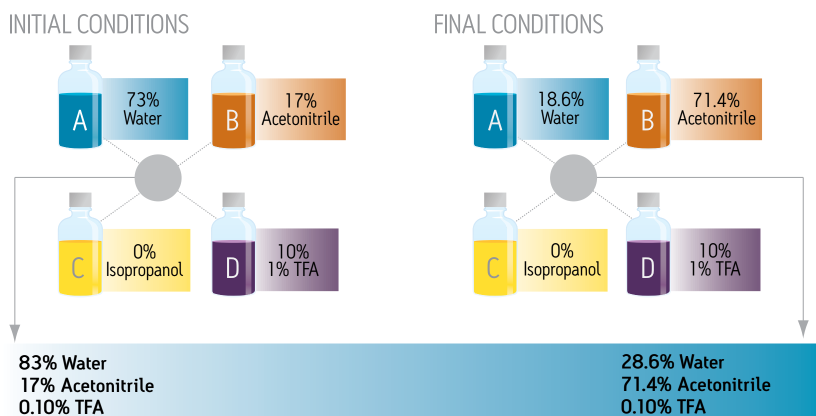
by using the four-solvent blending capability of the ACQUITY UPLC H-Class Bio System's quaternary solvent manager to prepare the different desired mobile phase combinations on-demand from bottles of pure solvents and concentrated modifiers. This Auto•Blend Plus™ Technology is only useful if it reliably produces the programmed mobile phase gradient accurately and precisely. The experiments described here focus on that system performance.

THE SOLUTION

A robust method for protein analysis must include an instrument, a column chemistry, and the set of operating conditions. For developing the separation discussed here, UPLC technology was chosen as the underlying principle. The use of sub-2-μm particle packing materials on instruments that minimize dispersion ensures the best resolution, sensitivity, and sample throughput. The ACQUITY UPLC family of instruments provides the required performance.

The ACQUITY UPLC H-Class Bio System was chosen for these experiments due to its biocompatible materials of construction, and because its four-solvent blending combines with Auto•Blend Plus Technology for easy methods development and execution. The UPLC Protein Separation Technology BEH300 C_4, 1.7-μm column was selected for the separation because its combination of large pores, short chain bonded phase, and inert base particle gives the best resolution and recovery. Auto•Blend Plus Technology was used to find the best combination of modifier concentration and organic solvent. The convenience of method development with Auto•Blend Plus requires a high degree of accuracy and precision in solvent blends over a long series of runs. The selected four-solvent method and the resultant chromatogram are shown in the accompanying figure.



Injection	cytochrome c	Chimeric Antibody	BSA	Myoglobin	Enolase	Phosporylase b
1	8.169	10.898	11.532	12.497	14.252	16.353
2	8.16	10.882	11.523	12.482	14.237	16.359
3	8.18	10.909	11.548	12.505	14.259	16.356
4	8.176	10.897	11.546	12.497	14.251	16.358
5	8.179	10.898	11.536	12.501	14.244	16.354
6	8.179	10.909	11.548	12.504	14.257	16.358
mean	**8.174**	**10.899**	**11.539**	**12.498**	**14.250**	**16.356**
std dev	**0.008**	**0.010**	**0.010**	**0.008**	**0.008**	**0.002**
%RSD	**0.096**	**0.091**	**0.089**	**0.067**	**0.058**	**0.015**

The accompanying table shows the reproducibility of retention times for this protein mixture. The proteins represent a wide range of properties and sizes, providing a good test of separation consistency. The reproducibility of the separation is suitable for routine use. The application of Auto•Blend Technology for preparation of blended mobile phases reduces labor and the possibility of error while providing reliable results.

SUMMARY

The requirements for a reversed phase analysis of intact proteins are satisfied by the system solution that combines ACQUITY UPLC H-Class Bio instrumentation, UPLC column chemistry, and software. The robustness of the system includes simplified mobile phase preparation, and is demonstrated with reproducibility of the separation.

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Waters Corporation
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T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[APPLICATION NOTE]

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Method Development Considerations for Reversed Phase Protein Separations

Hillary B. Hewitson, Thomas E. Wheat, Paula Hong, Kenneth J. Fountain

APPLICATION BENEFITS

- The BEH300 C_4 chemistry is available in sub-2 µm particles for maximum resolution
- Changes in operating conditions alter selectivity and resolution to meet the requirements for a particular sample
- The stability of the BEH300 C_4 chemistry allows use at high temperature for improved recovery and for selectivity modification
- The physical strength allows use of a range of organic solvents
- The inert surface allows use of different acids and concentrations
- The range of operating conditions permits automated, unattended method development
- The flexibility in creating conditions reduces the need for screening multiple columns

WATERS SOLUTIONS

- ACQUITY UPLC® System, fitted with peptide needle and peptide mixer, or ACQUITY® H-Class System, TUV detection at 220 nm
- ACQUITY UPLC BEH300 C_4, 2.1 x 50 mm, 1.7 µm column, Part number 186004495; ACQUITY UPLC BEH300 C_4, 2.1 x 150 mm, 1.7 µm column, Part number 186004497; ACQUITY UPLC BEH300 C_4, 4.6 x 150 mm, 3.5 µm column, Part number 186004504
- MassPREP™ Protein Standard Mix, Part number 186004900

KEY WORDS

Monoclonal antibodies, reduced monoclonal antibody, gradient slope, protein, BEH300 C_4, MassPREP protein standard mix, method development

INTRODUCTION

Due to the resolving power that reversed phase chromatography provides, it has long been a preferred analytical technique to characterize and quantify various products. With an ever increasing emphasis on protein biopharmaceuticals, there is a need to develop reversed phase separations of these macromolecules. Reversed phase separations for proteins are not as powerful as they are for small molecules. Changes to the protein are often small in proportion to the structure of the large molecule. Variant forms, therefore, have similar chromatographic properties. There are still many factors that can be used to optimize the separation of a particular sample. The requirements of this specific application will dictate the best approach for method development. This paper will consider each of these factors, including particle size, column length, flow rate, modifier concentration, organic solvent, column temperature, and gradient slope. The evaluation of each of these method variables will be demonstrated on a variety of proteins, including monoclonal antibodies, covering a wide range of properties. These include different isoelectric points, hydrophobicities, and molecular weights.

EXPERIMENTAL

Sample description

Protein Mixture: Prepared in 5% acetonitrile with 0.1% CF_3COOH

Protein	mg/ml
Ribonuclease A, bovine pancreas	0.08
Cytochrome C, horse heart	0.11
Albumin, bovine serum	0.40
Myoglobin, horse heart	0.25
Enolase, baker's yeast	0.43
Phosphorylase B, rabbit muscle	1.18

Intact Murine IgG1, prepared in 0.1% CF_3COOH, 0.5 µg/µL. Reduced/Alkylated Murine IgG1, prepared in 0.1% CF_3COOH, 0.5 µg/µL. Intact IgG Mixture: Humanized IgG_4, Chimeric IgG1, and Murine IgG1, prepared in 0.1% CF_3COOH, 0.5 µg/µL each.

Vials: Waters Certified Total Recovery, Part number 186000384c

LC conditions
(unless otherwise specified in figure captions)

System: ACQUITY UPLC (fitted with peptide needle and peptide mixer) with ACQUITY TUV detector, at 220 nm;
Column: BEH300 C_4, 2.1 x 50 mm, 1.7 µm, Part number 186004495
Column temp.: 40, 60, 80, or 90 °C (as indicated in figures)
Sample temp.: 10 °C
Injection volume: 3.3 µL
Flow rate: 0.2 mL/min
Mobile phase A: 0.1% CF_3COOH in water
Mobile phase B: 0.1% CF_3COOH in acetonitrile
Weak needle wash: 0.1% CF_3COOH in 5% acetonitrile
Strong needle wash: 0.1% CF_3COOH in 75% acetonitrile
Seal wash: 50/50 acetonitrile/water
Gradient: 20 – 71.4% in 29.6 min; 1 min regeneration at 90%; 17 min re-equilibration at initial conditions

Data management
Empower™ 2 CDS

RESULTS AND DISCUSSION

Column Chemistry and Particle Size

The advent of sub-2 µm particles along with UPLC® Technology has shown benefits for samples of all types. This technology was applied for the separation of biological macromolecules with the development of BEH300 C_4, a column chemistry that combines appropriate pore volume and chain length on a hybrid particle.[1] This stationary phase is available in 3.5 µm and 1.7 µm particle sizes, so methods can be directly transferred between HPLC and UPLC with the same chromatographic selectivity. Figure 1 shows the comparison of the effect of particle size, using a reduced and partially alkylated IgG sample. The relative positions of the peaks are exactly the same for both particle sizes. Since both separations were tested on the same UPLC system with the same mobile phases and conditions, the improvement in resolution observed for the 1.7 µm separation is directly attributable to the smaller particle size. The rigorous manufacturing control in the particle synthesis ensures scalability and constant selectivity across particle sizes. The improvement in resolution can only be fully realized with use of a system designed to minimize band-broadening during the separation.

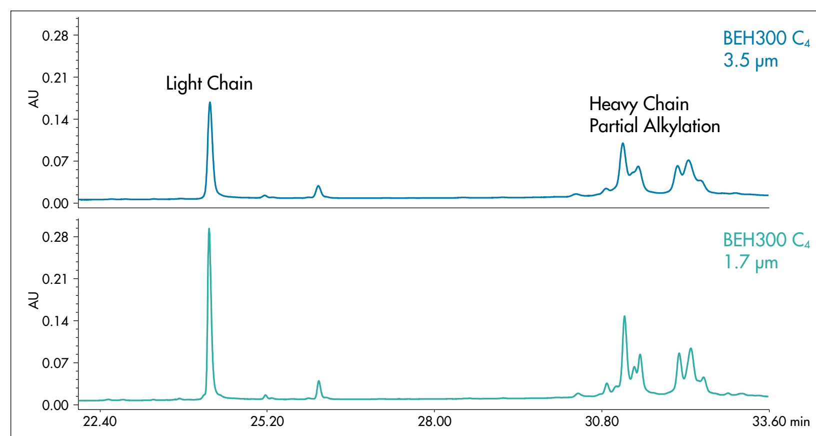

Figure 1. Comparison of a reduced and alkylated monoclonal antibody with 3.5 µm and 1.7 µm particles at 40 °C. While the relative position of the peaks are exactly the same in both separations, all of the peaks are narrower, and the improvement is particularly apparent for the multiple molecular forms of the heavy chain.

Gradient Slope

In gradient separations, chromatographers will often change the gradient slope as a primary tool in method development. Gradient slope, defined by the percent increase in organic per column volume, can be adjusted to optimize a separation for resolution of components or speed of analysis. Typical protein separations use fairly shallow gradients of about 3% or less. Reducing the gradient slope does offer an increase in resolution. Sensitivity, however, is reduced as the gradient is made more shallow. The resolution improvement in protein separations is usually at a slower rate than the loss of sensitivity or increase in peak volume. This phenomenon can be seen readily in the separation of a mixture of IgG, shown in Figure 2. By reducing the gradient slope from 3% to 0.5%, there is only a marginal increase in resolution between the humanized and chimeric IgG peaks, while

there is over a 3-fold loss of sensitivity and a 4-fold increase in run time. While gradient slope is a viable tool in method development, it is preferred to reserve that option until other techniques have been examined.

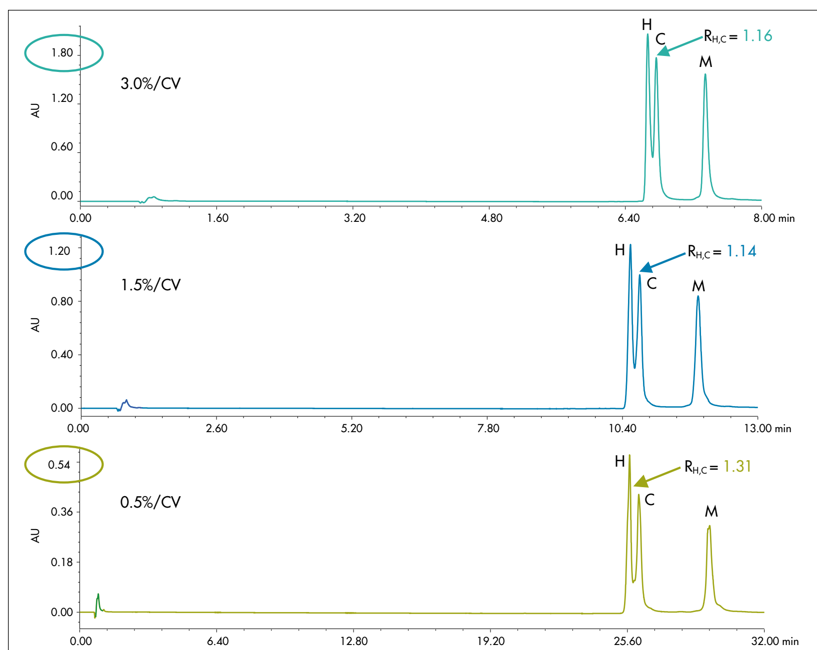

Figure 2. The separation at 80 °C of a mixture of humanized (H), chimeric (C), and murine (M) monoclonal antibodies with varying gradient slopes. There is more than a 3-fold loss in sensitivity, a 4X increase in run time, and a 5X increase in peak volume by decreasing the gradient slope from 3% down to 0.5% with only a marginal improvement in the resolution.

Organic Solvent and Mobile-Phase Modifier Concentration

Alternative organic solvents can alter selectivity of a separation. Historically, acetonitrile has been the solvent of choice in protein separations. The use of isopropanol (IPA) has been common. Gradients of increasing IPA were seldom used because of the high pressure associated with the viscosity of such solvent mixtures. Therefore, an acetonitrile/isopropanol blend (3:7) was a preferred replacement. The higher pressure capability of ACQUITY UPLC instruments allow for use of 100% isopropanol, as shown in Figure 3. All of the proteins elute earlier with IPA, and for this sample, improved resolution of some minor components is observed.

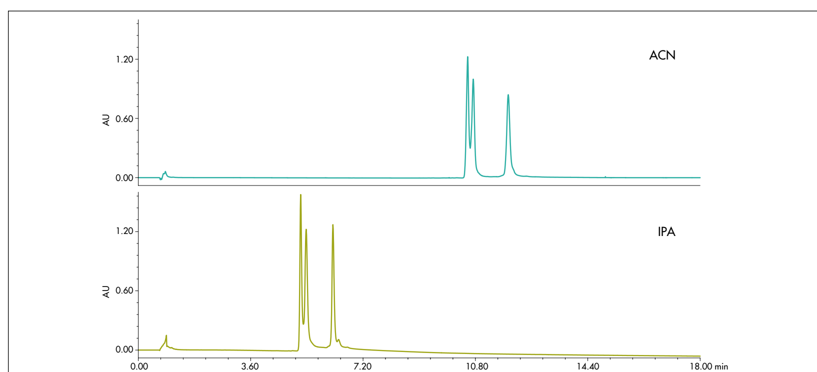

Figure 3. The separation of a mixture of monoclonal antibodies is shown at 80 °C with acetonitrile (ACN) and isopropanol (IPA) as the elution solvent. Reduced retention and improved resolution of trace components is observed with IPA.

Type and concentration of the acid modifier can also influence the separation. Formic acid is the preferred modifier in mass spectrometry applications, and trifluoroacetic acid (TFA) gives better chromatographic peak shape. Altering the acid concentration can change the selectivity of the separation. In general, protein peaks elute earlier with lower trifluroacetic acid (TFA) concentration, reflecting the reduced ion pairing.

There are a large number of organic solvent and acid concentration combinations possible in the development of a separation. This process can be streamlined with the application of Auto•Blend™ Technology, as embodied on the four solvent ACQUITY UPLC H-Class system. Figure 4 shows the preferred configuration of the system for testing the effect of organic solvent and mobile-phase modifier concentration on a protein separation. The conditions to be tested are programmed in the method as percentage flow from each of the four solvent lines. For example, different TFA concentrations are tested by blending a concentrated acid modifier at a series of percentages. This approach was used for the protein separation shown in Figure 5. While all of the peaks elute earlier at lower concentrations of TFA, myoglobin elutes earlier relative to the other proteins. It should also be noted that lower TFA concentration also results in generally wider peaks, which can lead to lower resolution, as can be seen with the different forms of phosphorylase b. Altering modifier concentration can be a very useful tool in method development, particularly where changes in selectivity are needed. The same approach can be used to compare different organic solvents.

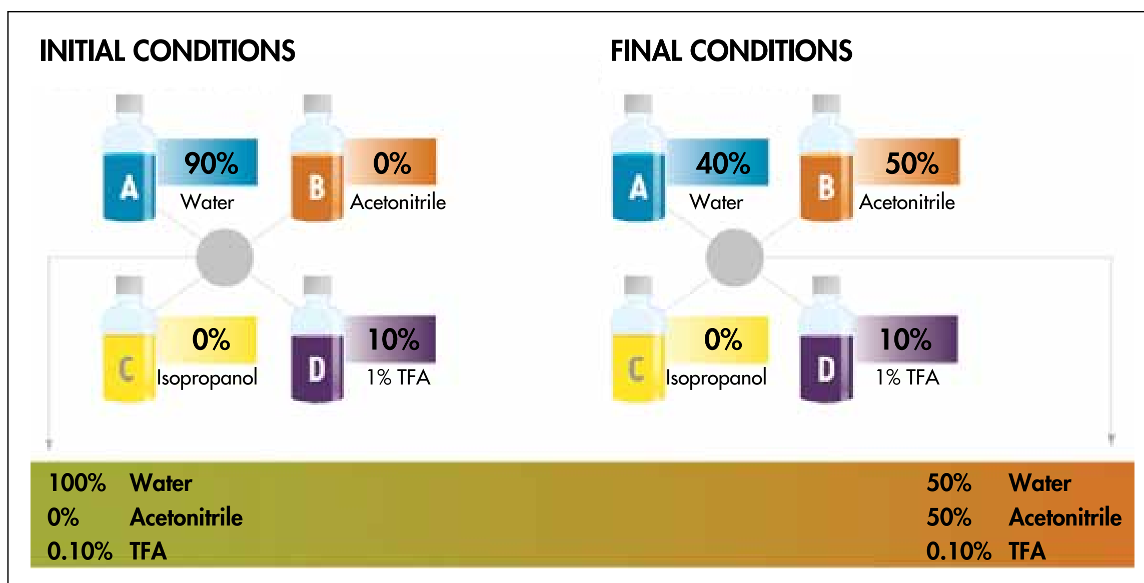

Figure 4. An example showing a possible configuration of four solvent lines of an ACQUITY H-Class system. Auto•Blend Technology enables a user to blend in varying amounts of a concentrated modifier and use different organic solvents in a single set of analyses when developing a method.

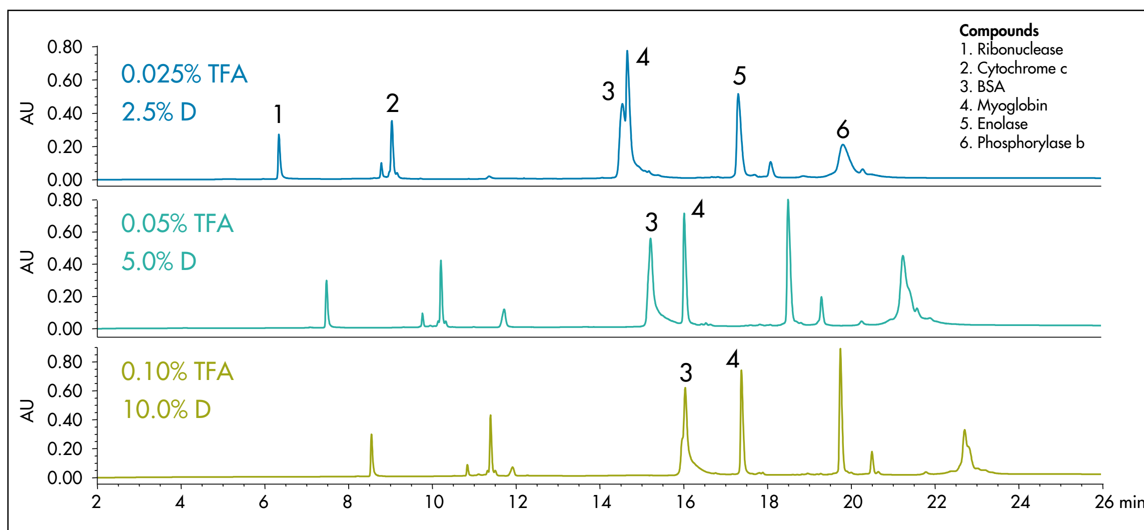

Figure 5. The protein test mixture was separated at 40 °C in the presence of various concentrations of TFA with the other conditions held constant.

Column Temperature

Column temperature has a large effect on reversed phase separation of molecules. Changes in recovery and selectivity are not uncommon with small molecule separations. While increasing the temperature for proteins can significantly improve recovery, particularly for intact monoclonal antibodies (Figure 6), it doesn't generally affect the selectivity of the separation.[2] However, not all proteins require higher temperatures for improved recovery. In fact, some protein separations have more desirable results with lower separation temperatures. Therefore, it is recommended that an evaluation of temperature be included in any method development strategy for new samples.

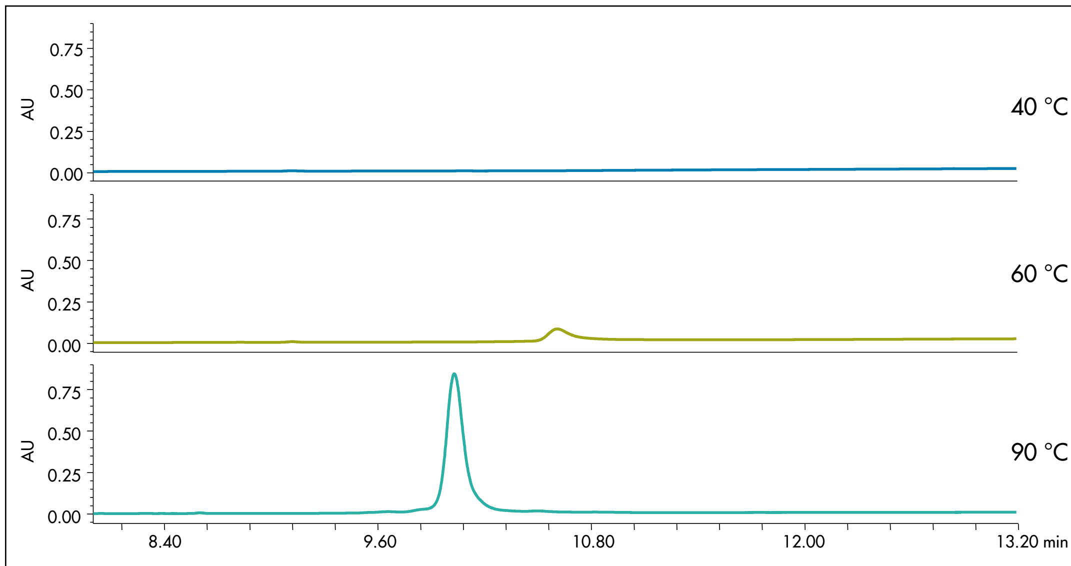

Figure 6. The intact IgG sample gave no observable peak at 40 °C, but recovery for the IgG sample improves with increasing temperature. There is not a measurable increase in recovery or improvement in peak shape above 80 ° C.

Column Length

Increasing the length of the column will increase the resolving power for a separation. This is shown in Figure 7 with the separation of the protein mixture. The additional small peaks surrounding the Phosphorylase b can be seen more readily on the longer column, as seen in the inset, but it comes at the cost of a 3-fold increase in run time and ~40% loss of sensitivity. Depending on the application objective, this may be a useful parameter to improve resolution.

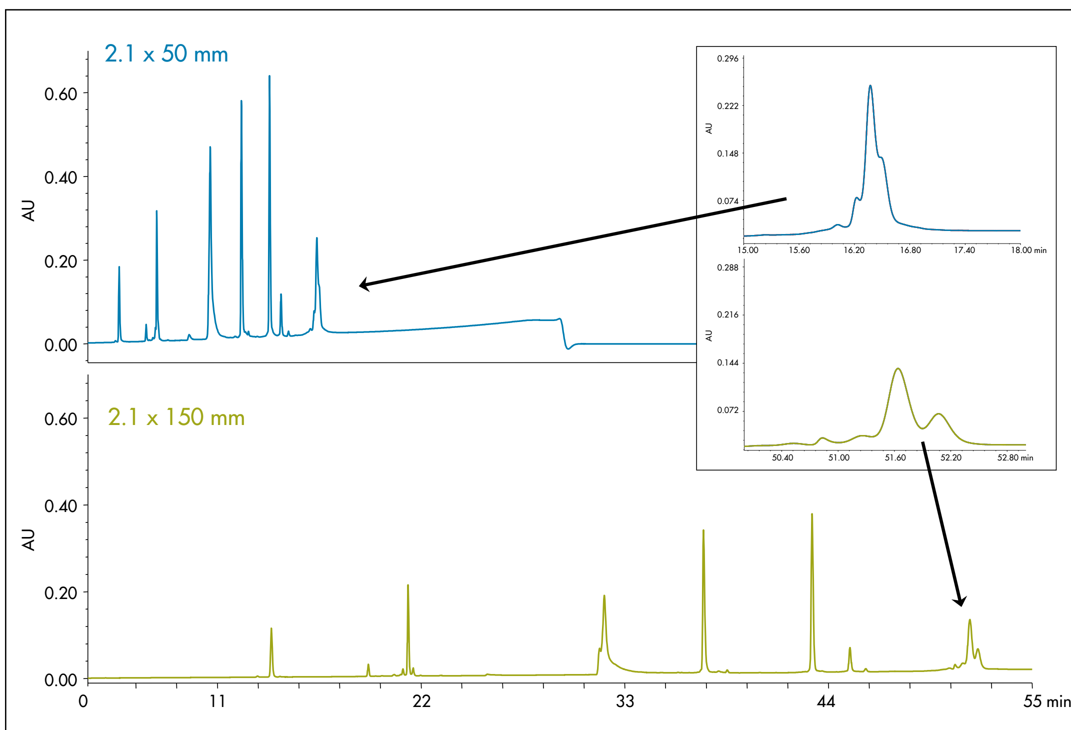

Figure 7. In the separation of the protein mixture at 40 °C, improvement in resolution of the additional small peaks surrounding Phosphorylase b (inset) can be readily seen on the longer column. The gain in resolution comes at the cost of a 3-fold increase in runtime and ~40% loss of sensitivity.

Flow Rate

Flow rate is seldom treated as an important parameter in method development except as an indirect modification of gradient slope. The impact of this variable is, however, more significant for larger molecules. Figure 8 shows the comparison of a protein mix separation at 200 µL/min and 75 µL/min. The inset shows improved resolution with an increase in sensitivity of phosphorylase b at the lower flow rate.

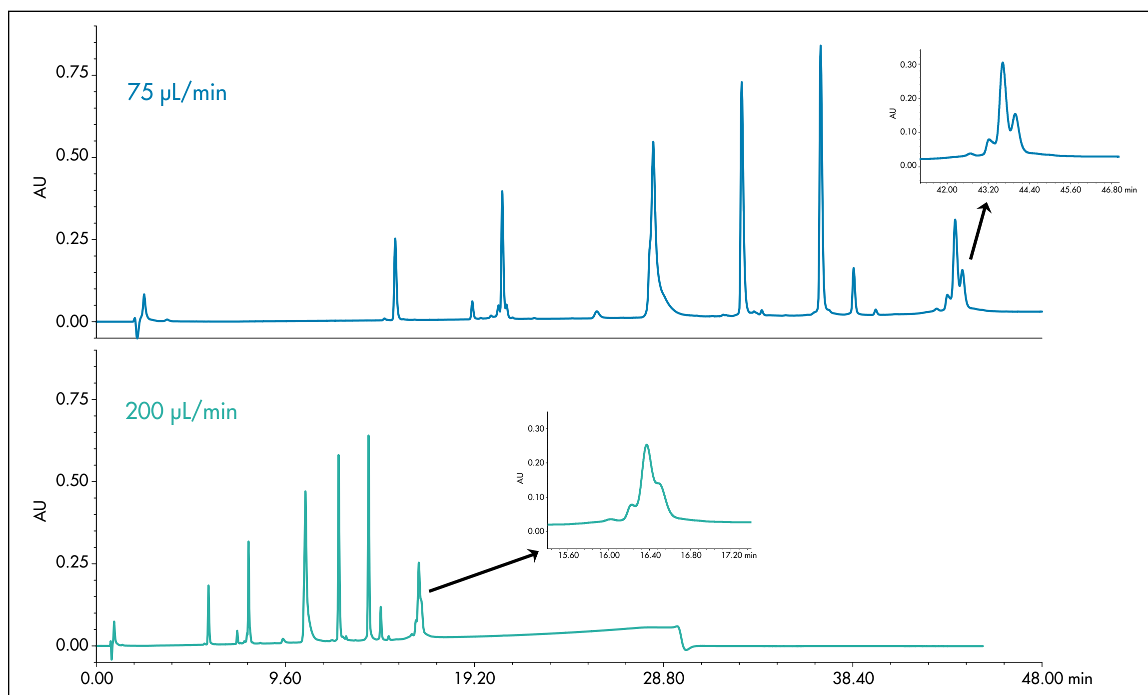

Figure 8. Decreasing the flow rate provides increased resolution without a compromise in the sensitivity, as seen in this separation of the protein mixture at 40 °C. The improved separation of the Phosphorylase b sub-units can be seen (inset) at the lower flow rate. The run time of the analysis is increased proportionally to preserve the same gradient slope in both separations.

CONCLUSIONS

There are many parameters available to the chromatographer in developing methods for separation of proteins. The approach to making adjustments to a method must take into consideration the objective of the analysis.

Protein separations do not tend to show the same dramatic resolution effects seen for small molecules. Therefore, most of the variables discussed here yield small improvements, often at the expense of sensitivity and run time. Smaller particle columns do, however, offer resolution improvements without loss of sensitivity or increased run time. Flow rate, column length, gradient slope, and modifier concentration can then be manipulated to further improve resolution.

Modifier concentration can be a useful tool in developing methods. It can provide resolution improvements by possible selectivity changes. Furthermore, changing the concentration can affect peak shape and detection.

Auto•Blend Technology is a convenient and efficient way to optimize systematically the effects of modifier concentration and organic solvent selection on the separation.

Adjustment in column temperature does not usually provide much selectivity change, but it can have a significant impact on the peak shape and recovery of proteins. It is not always possible to predict the ideal temperature for a protein sample. Therefore, it is good practice to include multiple temperatures in evaluation of appropriate conditions for a protein separation.

Both increased column length and decreased flow rate give increased resolution, both at increased run time. However, decreasing flow rate does not compromise sensitivity, as is the case with the longer column. The longer column, however, permits the injection of a larger sample, which may be valuable in the analysis of trace components.

Benefits in sensitivity, resolution, and run time can be achieved with smaller particles. But these improvements are best realized when applied with the holistic design of the UPLC system.

References

1. Developing Protein Separation Method on a Reversed Phase UPLC Column, Waters poster 720002974EN
2. Protein Separation Technology ACQUITY BEH300 C_4, 1.7 µm, Waters care and use manual 715001870EN

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[APPLICATION NOTE]

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Quantitation of Monoclonal Antibodies with Reversed Phase Liquid Chromatography using the BEH300 C_4 Column Chemistry

Hillary B. Hewitson, Thomas E. Wheat, Paula Hong, Kenneth J. Fountain

APPLICATION BENEFITS

- Reproduciblre reversed phase chromatographic separations of monoclonal antibodies.
- Minimal carry over and memory effects.
- Linear quantitation over four orders of magnitude dynamic ranges.
- Quantitaion of traces of antibody in mixtures of 100-fold ratio.

WATERS SOLUTIONS

ACQUITY UPLC with TUV, fitted with the Peptide Needle (P/N 205000507) and 425 µL Peptide Mixer (P/N 205000403)

BEH300 C_4, 1.7 µm, 2.1 x 50 mm (P/N 186004495)

KEY WORDS

protein quantitation, peptide needle, peptide mixer, trace impurity quantitation, protein variants, monoclonal antibodies

INTRODUCTION

The quantitative analysis of biopharmaceutical proteins is required at various stages in the development process. Depending on the phase and specific focus of a biotherapeutic lifecycle, quantitation can vary from an estimation of the target protein (+/- 10%) to quantitation of impurities at levels < 1% of the target protein. To accommodate these varying requirements, it is not only necessary to determine the best range for the assay, but to assure that the required dynamic range can be readily and reliably achieved. The BEH300 C4 column chemistry was designed specifically so that the high resolution characteristic of UPLC® would be available for protein analyses. Using the BEH300 C4 column chemistry, quantitation of a commercially-available monoclonal antibody is shown in this application note, demonstrating the linear range. Also shown is an example for detection and quantitation of trace-level impurities.

EXPERIMENTAL

SAMPLE DESCRIPTION: Linearity Samples: Fully humanized IgG4 prepared in 0.1% CF_3COOH, for mass load targets of 0.05, 0.1, 0.5, 1, 5, 7.5, 10, 20, 30, 40, 50 µg on column (3.3 µL injection).

Trace impurity quantitation samples: 0.1%, 0.2% 0.5%, 1%, 2.5%, and 5% murine IgG1 in the presence of a constant 50 µg Humanized IgG4 on column (3 µL injection).

LC conditions

LC System:	ACQUITY UPLC® system (fitted with the Peptide needle and 425 µL peptide mixer)
Detector:	ACQUITY® TUV detector
Sanple vial:	Waters Certified Total Recovery vials (P/N 186000384c)
Column:	ACQUITY UPLC BEH300 C_4, 2.1 x 50 mm, 1.7 µm (P/N 186004495)
Column temp:	80 °C
Sample temp:	10 °C
Injection volume:	3 µL or 3.3 µL
Injection mode:	Partial loop
Flow rate:	0.2 mL/min
Mobile Phase A:	0.1% CF_3COOH in water
Mobile Phase B:	0.1% CF_3COOH in IPA
Weak needle wash:	0.1% CF_3COOH in 5% acetonitrile
Strong needle wash:	0.1% CF_3COOH in 75% acetonitrile
Seal wash:	50/50 acetonitrile water
Gradient:	20–37% B in 14.7 min; 1 min regeneration at 90%; 13 min reequilibration at 20% B
	UV detection at 220 and 280 nm

Data management

Software:	Empower™ 2 CDS

RESULTS AND DISCUSSION

UV detection at low wavelengths (e.g. 220 nm) is often used for protein analysis because of its high sensitivity. At low wavelength, low levels of a protein can be detected. However, detector saturation limits the dynamic range to a 50-fold range of concentrations (Figure 1). Saturation of the detector at 220 nm occurs between 5 and 7.5 µg of protein injected on a 2.1 x 50 mm column. The analysis of the same mass load and injection volume of humanized IgG4 showed almost a 10-fold difference in peak height between the chromatograms at 220 nm and 280 nm (Figure 2). The reduced peak height at 280 nm was also accompanied by changes in baseline characteristics. The low end of the linear range, that is the lower limit of quantitation, is dependent upon both drift and short term detector noise. The noise is similar at the two wavelengths, on the order of 0.00002 AU. The drift, however, is more than ten-fold greater at 220 nm. Such anomalies directly affect the accurate integration of peaks. The increased saturation limit and reduced baseline anomalies combine to give a wider usable dynamic range at 280 nm.

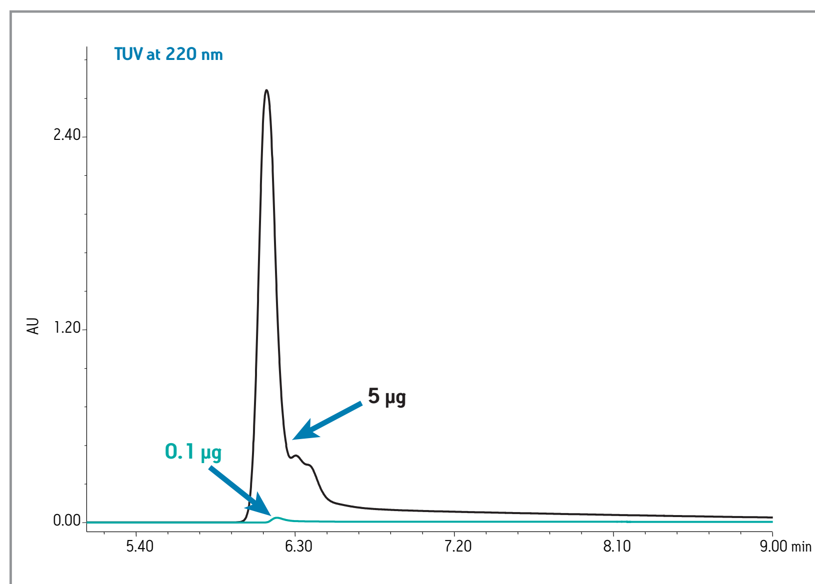

Figure 1. Chromatogram of humanized IgG4 at the low and high end of the linear dynamic range, with detection at 220 nm. Detector saturation is observed at loads above 5 µg on-column.

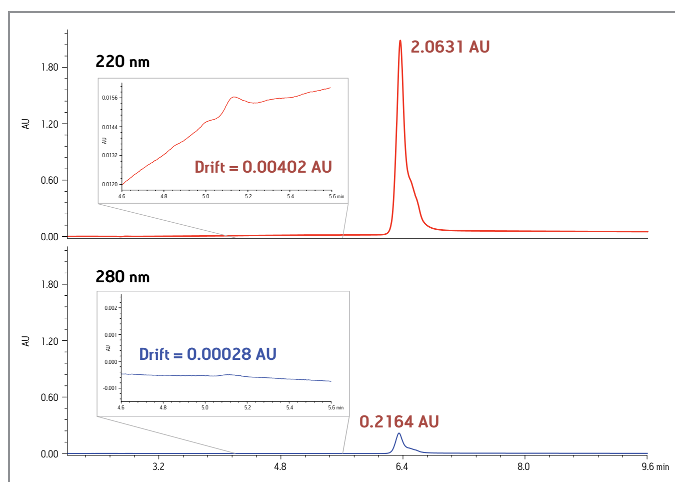

Figure 2. A comparison of the same 5 µg load of a monoclonal antibody is shown at both 220 nm and 280 nm. There is close to a 10-fold difference in peak height. The inset shows a one-vminute segment of baseline prior to the protein peak eluting. There is close to a 14-fold increase in drift observed at 220 nm.

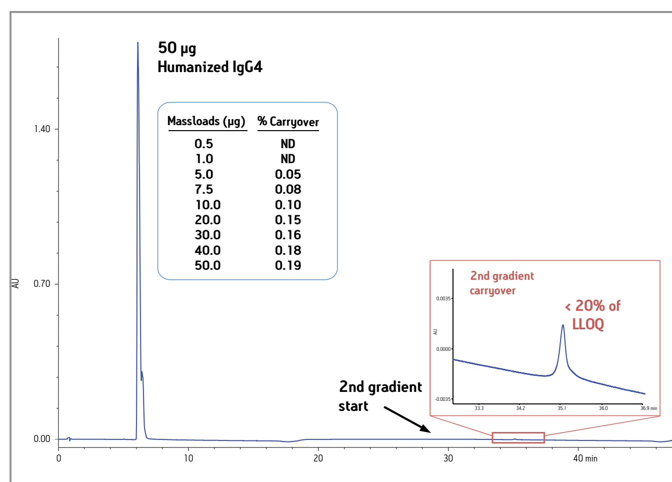

Figure 3. Effect of mass load on carryover. The chromatogram shows a 50 µg load of humanized IgG4 with a second internal gradient. Material eluting near 35 minutes represents a memory effect. The inset table shows the carryover measured for all levels tested, displayed as a percentage of area observed in the first gradient.

The humanized IgG4 was used to determine the linear range with UV detection at 280 nm. Ten different mass loads (0.1 – 50 µg) of protein were analyzed in triplicate at a constant injection volume of 3 µL. At 280 nm, the highest protein load of 50 µg on-column was below the detector saturation limit, and the same variant forms of the protein were still detected. The method was linear across all levels tested, with an R2 value of 0.994. The variability of multiple injections was also less that 2% across the full set (Table 1).

Validation of any quantitative assay must demonstrate the absence of carryover. Observation of carryover has been particularly problematic in protein assays. In developing an application, two sources of carryover are recognized, the fluid path of the instrument and the chromatographic column. Column-related carryover, or memory effect, can be measured by running a second gradient immediately following the sample analysis without making another injection. This internal gradient would show memory effects as a peak at the corresponding retention time in the second gradient.1 This procedure was used for the same set of samples shown in Table 1 (Figure 3). All levels tested showed < 0.2% carryover for the column, which was less than 20% of the lower limit of quantitation. The ability to quantitate proteins from 0.1 – 50 µg with minimal carryover makes this a good method for accurate and consistent quantitation of low level impurities.

The above experiments show the utility of quantitating a single protein peak using reversed phase chromatography. At various stages in the biopharmaceutical development process, it is

Amount on Column (µg)	Average Area	Area % RSD
0.1	39848	1.91
0.5	285091	1.66
1.0	566568	1.47
5.0	2634778	0.52
7.5	3945844	0.08
10.0	5526662	0.03
20.0	9954028	0.21
30.0	14643136	0.88
40.0	22532273	0.79
50.0	27894201	1.10

Table 1. Average values for triplicate injections of humanized IgG4, from 0.1 µg to 50 µg mass load on-column (3 µL constant injection volume). The data set was linear across all levels tested with an R2 value of 0.994. Variability for triplicate injections was <2%.

Impurity			API
Nominal % of API	Measured % of API	Area % RSD	Area % RSD
1.0	0.94	1.61	0.83
2.50	2.48	1.05	0.36
5.00	4.88	2.43	2.78
%RSD values based on 5 replicates			

Table 2. Quantitation of trace amounts of a different protein in an IgG formulation. Five replicate injections were made for each level. The measured % area of the active pharmaceutical ingredient (API) was compared to the nominal values. The area reproducibility for both API and trace impurity is shown for all levels tested.

necessary to quantitate individual proteins in a mixture, most often proteins that are low abundance impurities in the presence of a larger amount of another protein. To demonstrate this, a series of spiked IgG samples was analyzed. These samples contained 50 µg of a humanized IgG with spiked levels of a murine IgG at levels of 1 – 5% of the larger IgG protein peak. As shown in Table 2, the measured impurity amount matched the expected values with good reproducibility.

CONCLUSIONS

The sensitivity and resolution offered by reversed phase chromatography make it a good investigative tool for the characterization of proteins. Consistent analyses are observed over a wide range of protein concentrations without any distortion of the chromatographic retention or peak shape. Reliable and reproducible quantitative results are readily obtained for monoclonal antibodies using the BEH300 C_4 column chemistry. Minimal protein carryover on this column chemistry helps to improve the accuracy of the quantitative results. Through the combination of ACQUITY UPLC and the BEH300 C_4 column chemistry, fast and reliable quantitative results are obtained at any point in the lifecycle of a protein biotherapeutic.

References

1. Protein Separation Technology ACQUITY BEH300 C_4, 1.7 µm, Waters Care & Use Manual, 715001870en.

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[APPLICATION NOTE]

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Two-Dimensional Liquid Chromatography for Quantification and MS Analysis of Monoclonal Antibodies in Complex Samples

Sean M. McCarthy, Thomas E. Wheat, Ying Qing Yu, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Waters® ACQUITY UPLC® H-Class Bio System with 2D technology provides a single system approach for rapid protein titer measurement and mass profiling of proteins. This method can be used during clone selection and process scale-up to determine yield and monitor the mass profile.

WATERS SOLUTIONS

ACQUITY UPLC H-Class Bio System with 2D Technology

Xevo® G2 QTof

BiopharmaLynx™ for MassLynx™ Software

MassPREP™ Micro Desalting Column

KEY WORDS

Affinity chromatography, two-dimensional (2D) chromatography, monoclonal antibodies, Protein A immobilization, affinity purification and quantification, mass profile

INTRODUCTION

Identification, characterization, and quantification of monoclonal antibodies (mAbs) are required at many stages of biopharmaceutical research and development. The primary analytical tools for these assays are liquid chromatography coupled with UV or mass spectrometry (MS). Both techniques can be compromised by interferences in the sample matrix, including high salt concentrations, other proteins, or the components of cell culture media.

A high throughput analytical technique should combine sample preparation and chromatographic techniques to ensure accurate and robust quantification. Affinity chromatography on immobilized Protein A can be used to isolate the antibody from a complex matrix, while reverse phase (RP) LC is useful for introducing a salt-free, concentrated sample into an MS ion source.

To accomplish both affinity purification and MS analysis in a high throughput manner, we utilized the ACQUITY UPLC H-Class Bio System with 2D Technology. The 2D system allows for simultaneous purification and quantification of monoclonal antibodies by Protein A affinity chromatography, and determination of mass profile by MS analysis after desalting on a short RP column. This online 2D UPLC® method requires little to no sample preparation, and an analysis is quickly completed with an instrument duty cycle time of seven minutes.

EXPERIMENTAL

Sample preparation

Samples of trastuzumab were prepared by diluting the sample to a concentration of 1 mg/mL in DMEM cell culture media containing 1 mg/mL of bovine serum albumin (BSA).

UPLC conditions

LC system: Waters ACQUITY UPLC H-Class Bio System with 2D Technology:

- ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (1D)
- ACQUITY UPLC H-Class Bio Sample Manager (FTN)
- ACQUITY UPLC Column Manager A
- ACQUITY UPLC Tunable UV Detector
- ACQUITY UPLC Binary Solvent Manager (2D)

First dimension

Column: Poros A 20, 2.1 x 30 mm (Applied Biosystems)

Column temp.: 20 °C

Flow rate: 1.0 mL/min

Mobile phase A: 50 mM Phosphate Buffer, pH 7.0, 150 mM NaCl

Mobile phase B: 12 mM HCl, pH 1.9, 150 mM NaCl

Detection: UV 280 nm

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	1.0	100	0	0	0	Initial
1.00	1.0	100	0	0	0	1
1.10	1.0	0	100	0	0	1
4.00	1.0	0	100	0	0	1
4.10	1.0	100	0	0	0	1
7.00	1.0	100	0	0	0	1

Second dimension

Column: Waters MassPREP Micro Desalting Column (P/N 186004032)

Column temp.: 80 °C

Flow rate: 0.5 mL/min (desalting and regeneration), 0.2 mL/min (analysis)

Mobile phase A: 0.1% Formic acid

Mobile phase B: 0.1% Formic acid in ACN

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	100	0	Initial
1.80	0.5	100	0	6
3.50	0.5	100	0	6
3.51	0.2	100	0	6
5.00	0.2	5	95	6
5.10	0.5	100	0	6
5.70	0.5	5	95	6
5.80	0.5	100	0	6
6.40	0.5	5	95	6
6.50	0.5	100	0	6

Column Manager

Initial: Left Valve Position 1

1.5 min: Left Valve Position 2

1.8 min: Left Valve Position 1

MS conditions:

MS system: Waters Xevo G2 QTof

Capillary: 3 kV

Sampling cone: 45 V

Extraction cone: 2 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Cone gas flow: 0.0 L/h

Desolvation gas flow: 600.0 L/h

RESULTS AND DISCUSSION

The ACQUITY UPLC H-Class Bio System with 2D Technology can be configured to collect a heart cut (time slice) from a first-dimension separation and divert the fraction for subsequent analysis. Determination of the correct time segment is performed experimentally by measuring the elution time of the peak of interest. The analyst can then enter timed events to control valve positions.

The plumbing diagram shows the system configured for affinity purification followed by RP analysis for intact mass. The sample is introduced into the affinity column in position 1. Following removal of unwanted components (such as BSA and media components), the valve is switched to position 2. The antibody is eluted as a concentrated peak directly onto the head of the desalting column. After collection, the valve is returned to position 1 then the second dimension desalting and gradient are performed.

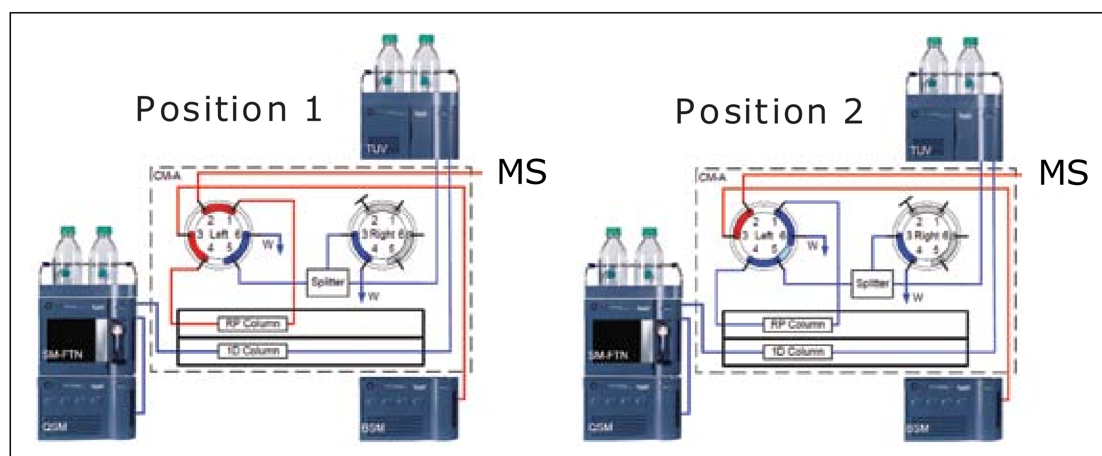

Figure 1. Instrument plumbing configuration. The sample is introduced to the 1D column in position 1. At a specified time, the left valve is switched to position 2 to divert flow to the 2D column. After collection, the valve is returned to position 1 and the second dimension gradient is initiated. A splitter is used between the 1D and 2D to compensate for the differences in mass load needed for each analysis. The splitter can be bypassed by shunting the split flow to waste when the 1D mass load is compatible with the 2D.

In heart cut applications such as this high throughput analysis, it is important that the retention time be reproducible and and carryover be minimal to ensure accurate collection and quantification. As shown in Figure 2, the ACQUITY UPLC H-Class Bio System with 2D technology has both of these attributes. Our data shows reproducible retention time and peak area, allowing for accurate collection and quantification, with no evidence of carryover in blank injections. We also found very good linearity over a range of mass loads, as shown in Figure 3.

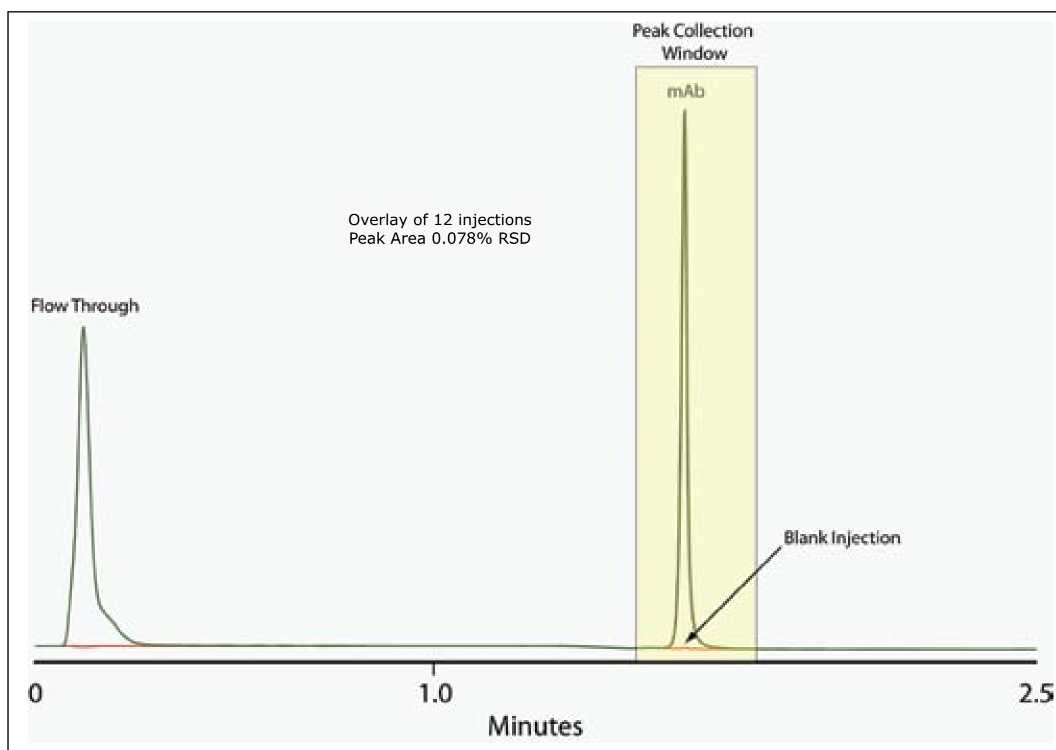

Figure 2. Overlay of 12 1D Protein A affinity chromatograms of trastuzumab, 1 mg/mL in cell culture media (DMEM) with 1 mg/mL BSA. Excellent reproducibility of retention time, peak shape, and peak area were found. Blank injections do not show any carryover.

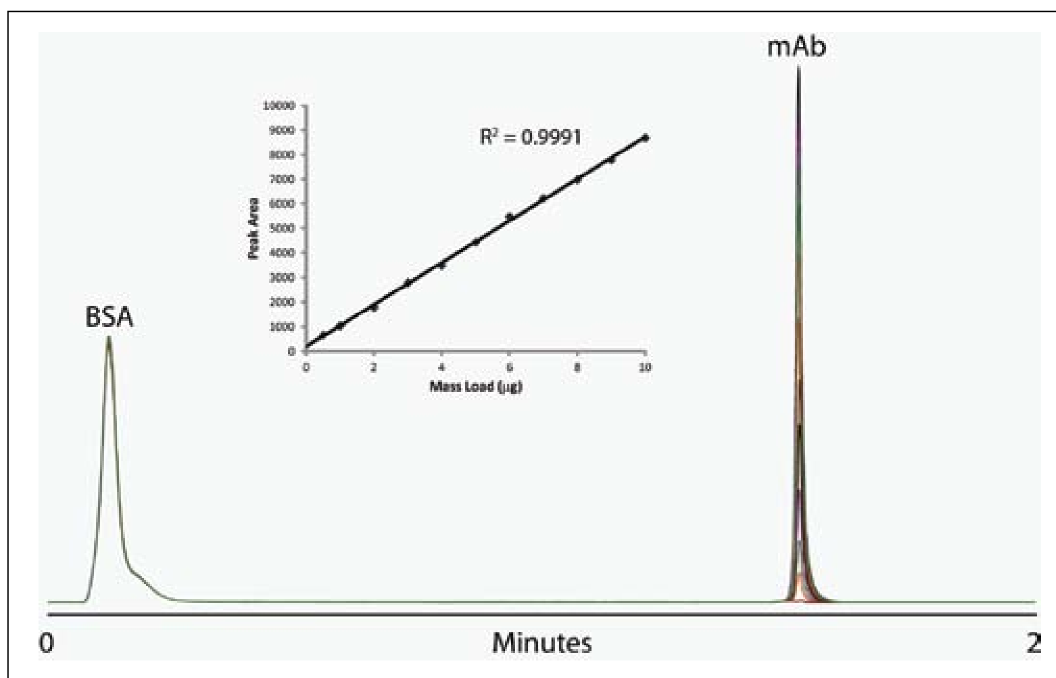

Figure 3. Overlay of serially diluted trastuzumab, from 0.5 µg to 10 µg in cell culture media with 1 mg/mL BSA. Response for mAb is linear over the entire mass load range. Samples run in triplicate with RSD for each mass load <0.1%.

Important aspects of this application include the reproducibility of collection from the first dimension and reproducibility of the MS response. As demonstrated by the overlaid total ion chromatogram (TIC) traces in Figure 4, replicate injections at each mass load overlay very well, and signal intensity increases with mass load. To prevent overloading the second dimension column, we utilized a PEEK tee, shown in Figure 1, as a flow splitter to divert approximately 70% of the first dimension eluent to waste. The split flow going to waste can be shunted; thereby, diverting the entire first dimension flow to the second dimension, if necessary.

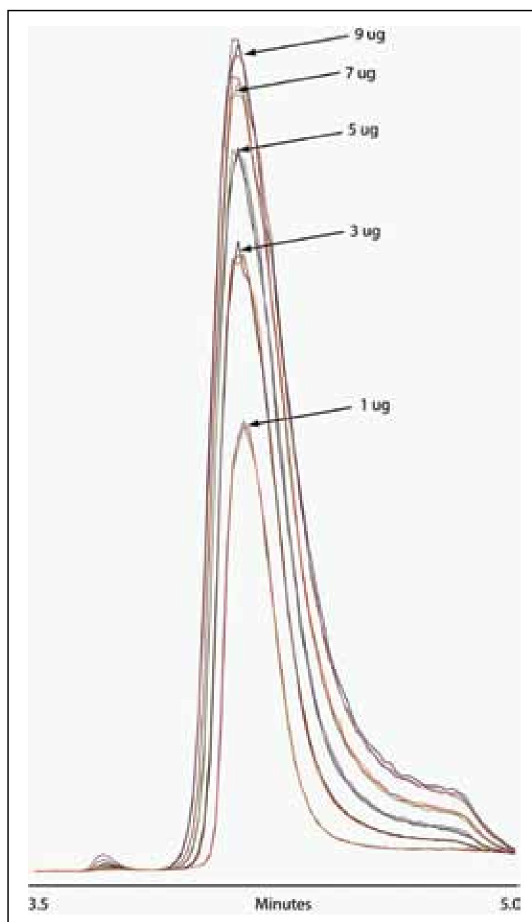

Figure 4. Overlay of total ion chromatograms from desalting column for various mass loads collected from first dimension separation.

For this application, we utilized BiopharmaLynx Software to process the data. The processed spectra for 10-µg and 0.5-µg injections on the affinity column are shown in the top and bottom panels of Figure 5. Since flow was split for each injection, the effective mass loads for each sample were approximately 3 µg and 0.15 µg, respectively. We found excellent agreement between the highest and lowest mass loads used in this application.

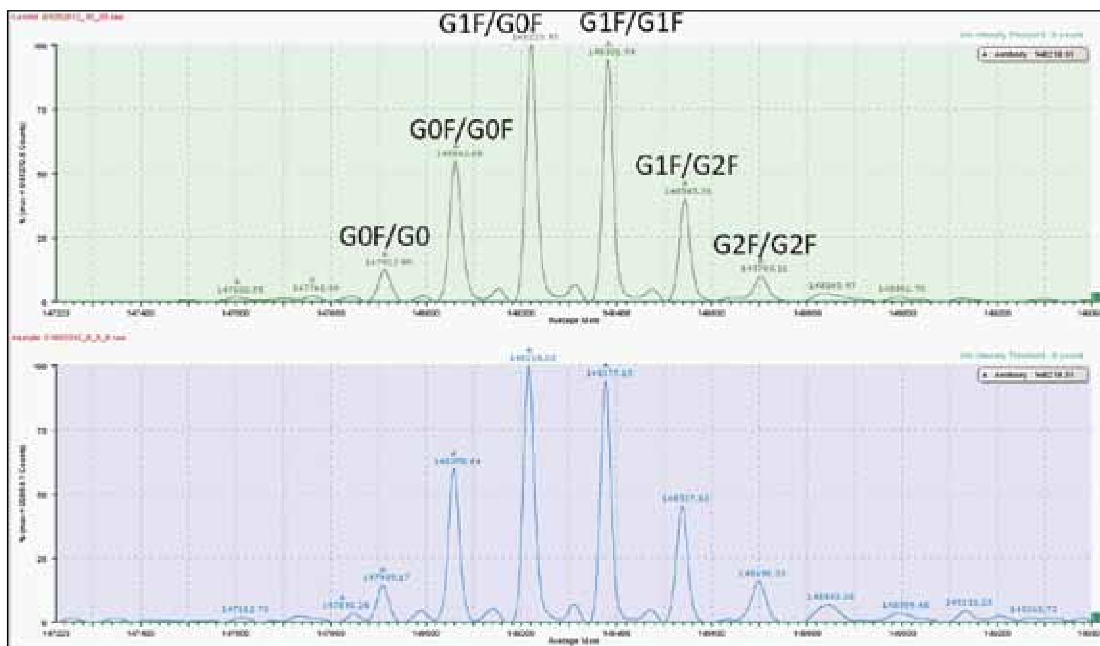

Figure 5. Automated intact protein mass deconvolution using BiopharmaLynx. Data can be batch-processed and quickly screened for mass and glycoprofile. Additionally, data can be compared to a control sample to identify and quickly assess similarities and differences between samples.

CONCLUSION

Using the ACQUITY UPLC H-Class Bio System with 2D Technology, a method was developed to determine monoclonal antibody concentration and mass profile from a single injection. The 2D chromatographic methods were easily defined using an inlet method editor. The system delivered the necessary reproducibility in terms of retention time, recovery, and response for samples from complex matrices using both UV and MS detection. By streamlining sample preparation and enabling high-throughput MS analysis, this online 2D UPLC system can improve a biopharmaceutical organization's productivity in monitoring mass profiles and determining yield during clone selection and production scale-up for monoclonal antibody products.

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Alternative Solvents for the Reversed Phase Separation of Proteins

Hillary B. Hewitson, Thomas E. Wheat, and Diane M. Diehl
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The reversed phase separation of proteins is a useful analytical technique that is used in a variety of applications and at various stages of the biopharmaceutical development process. Acetonitrile has been the solvent of choice because of good peak shape and resolution. It also minimizes viscosity while providing transparency at low UV wavelengths for sensitive detection. Other organic solvents have been used for either improved recovery or different selectivity, but there has been little need for changing the routine use of acetonitrile.

Recently, however, a combination of economic, social, and environmental factors has created an acute shortage of acetonitrile accompanied by a substantial price increase. It is, therefore, necessary to examine more closely the solvent options for reversed phase protein separations. It is especially important to quickly make wise choices that meet the financial requirements without sacrificing the quality of the separations.

Several organic solvents were evaluated. The test mixture of proteins used in the evaluation represents a wide range of properties, including molecular weights from 10 kD to 150 kD, isoelectric points from 4.5 to above 10, and eluting anywhere from 20% to well-above 50% acetonitrile. Three monoclonal antibodies were also used as test samples. The effect of temperature and pressure were observed and summarized for each of these solvents.

EXPERIMENTAL

Samples

Protein Mixture (in 0.1% CF_3COOH in 5% acetonitrile)

Ribonuclease A, bovine pancreas:	0.08 mg/mL
Cytochrome *c*, horse heart:	0.11 mg/mL
Albumin, bovine serum:	0.40 mg/mL
Myoglobin, horse heart:	0.25 mg/mL
Enolase, baker's yeast:	0.43 mg/mL
Phosphorylase *b*, rabbit muscle:	1.18 mg/mL

Mixture of monoclonal antibodies (murine, chimeric, and fully humanized): ~0.5 mg/mL in 0.1% CF_3COOH

Chromatographic conditions

LC system:	Waters ACQUITY UPLC® System PEEK-Sil Needle (PN/205000507) Flow Restrictor (PN/205000547) Peptide Mixer (PN/205000403)
Column:	ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 mm x 50 mm
Column temp.:	40 °C or 80 °C, as indicated
Sample temp.:	4 °C
Flow rate:	200 µL/min.
Mobile phase A:	0.1% CF_3COOH in DI water
Mobile phase B:	0.1% CF_3COOH in indicated solvent
Weak needle wash:	0.1% CF_3COOH in 5% Acetonitrile 600 µl
Strong needle wash:	0.1% CF_3COOH in 75% Acetonitrile 200 µl
Injection volume:	3.3 µL, Partial Loop
Detection:	UV (TUV), 220 nm

Gradient:

Time	% A	% B	Curve
00.00	80.0	20.0	-
29.06	28.6	71.4	6
31.60	28.6	71.4	1
49.60	80.0	20.0	1

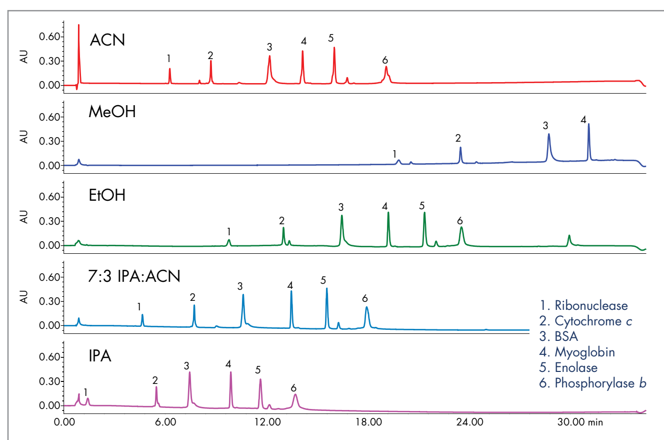

Figure 1. The separation of a mixture of proteins representing a wide range of properties in a variety of solvents at 40 °C.

RESULTS AND DISCUSSION

A standard mixture of proteins was separated using a gradient of increasing acetonitrile. The acetonitrile was replaced with alcohols of progressively longer chain length as shown in Figure 1. A blend of acetonitrile and isopropanol was included in the series, as this mixture has often been used in the past. The blend has lower viscosity than isopropanol while retaining improved resolution and recovery. Methanol gave wide, asymmetrical peaks and did not elute all the proteins within the standard gradient. Ethanol did give a reasonable separation with increased retention compared to acetonitrile. Replacing seventy percent of the acetonitrile with IPA gave good separation with slightly less retention for all of the proteins. Using 100% IPA resulted in even less retention for the proteins, and with reduced resolution for some proteins, such as Phosphorylase *b*.

Increasing the temperature for the analysis resulted sharper peaks and reduced retention for all proteins, as shown in Figure 2. While methanol was not a reasonable choice at 40 °C, it gave acceptable peak shape within the standard gradient at 80 °C. The rest of the standard proteins shifted to lower retention at the higher temperature and generally gave narrower peaks. The shift to lower retention could compromise the analysis of polar proteins, but this gradient uses 20% organic as the initial conditions. The gradient could be modified to lower starting organic concentration as required for the least retained proteins.

The same series of experiments was repeated with a mixture of monoclonal antibodies as show in Figure 3. The same patterns are observed as with the protein standard mixture. Methanol is unacceptable for these proteins, even at the highest temperature. Isopropanol appears to give the best peak shape and resolution.

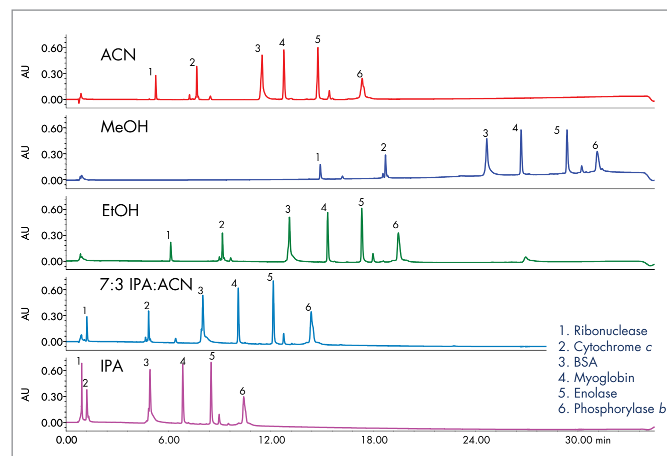

Figure 2. The separation of a mixture of proteins representing a wide range of properties at 80 °C in a variety of solvents.

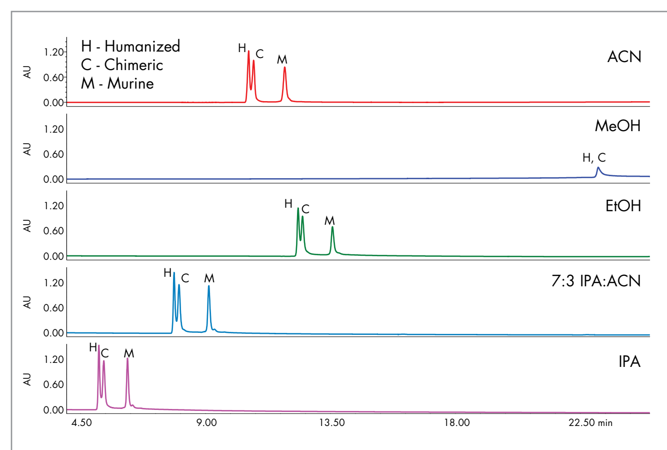

Figure 3. Separation of a mixture of monoclonal antibodies with different solvents.

In the experiments comparing different solvent combinations and temperatures, with both the protein standard mixture and the monoclonal IgG's, there is little or no evidence of selectivity changes. This suggests that the solvent substitutions may be made as convenient without the need for extensive re-development of methods. Re-validation would, of course, be required for regulated methods.

The major challenge in replacing acetonitrile with an alcohol is the relatively high viscosities associated with water-alcohol mixtures. Some combination of increased temperature and reduced flow rate can be used to manage the higher pressures. Table 1 shows the approximate system pressures for an ACQUITY UPLC System, fitted with a pressure restrictor and peptide mixer, for the different solvent and temperature combinations, all tested at 0.2 mL/min. The relative pressures will be the same on all systems so this table is a useful guide for making substitutions. Isopropanol gives almost three times the back pressure of acetonitrile. Raising the temperature from 40 to 80 °C reduces the pressure for all solvents by about 20%. Regardless of the solvents and temperatures tested, the ACQUITY UPLC System has sufficient pressure capacity to handle the separation. While the flow restrictor was installed and used for single variable testing of all solvents, its use above 10k PSI will likely result in leaks at the fittings of the restrictor, and is therefore not recommended for use with isopropanol or ethanol.

Table 1. Estimated pressures with various solvents.

Mobile Phase B	Temp (°C)	Initial PSI	Highest PSI	% B at Highest PSI
100% ACN	40	4800	4800	20
	80	3900	3900	20
100% MeOH	40	5800	6700	45
	80	4900	5500	45
100% EtOH	40	7000	10300	60
	80	5700	8000	60
7:3 IPA:ACN	40	6300	8200	55
	80	5100	6400	55
100% IPA	40	7600	13600	70
	80	6100	11200	70

CONCLUSIONS

Alcohol-based mobile phases can be substituted for acetonitrile in reversed phase chromatography of proteins. Isopropanol is much superior to methanol for this purpose. Elevating the temperature of the separation improves peak shape in all the solvents and for all the samples tested. There is little change in selectivity with the different solvents. These guidelines provide a straightforward approach to mitigating the effects of the acetonitrile shortage on routine reversed phase chromatography of proteins.

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

PEPTIDE MAPPING

Peptide mapping is a gold-standard technique for the comprehensive characterization of biopharmaceuticals, providing detailed structural information for a protein. It is used to comprehensively characterize therapeutic proteins based on the peptide fragments, determine post-translational modifications, confirm genetic stability, and analyze protein sequence when interfaced with mass spectrometry.

Peptide mapping is a challenging application given the number of peaks that must be baseline resolved in LC/UV applications. In addition to the large number of peptides that are generated from enzymatic digestion of a protein, the number of alternative peptide structures (post-translational modification, oxidation, incomplete digestion, etc.) can be equally daunting.

Peptide mapping is a tedious and laborious process if done manually. Good methods often have long run times and may not be as rugged as desired. Typically, UV and/or MS detection is required. When using MS, accurate mass is vital for confidently assigning peptide identifications. All the data acquired needs to be processed, annotated, and compared.

COMPREHENSIVELY DESIGNED FOR PERFORMANCE

Waters delivers comprehensive system solutions for peptide analysis that leverage our extensive expertise in chromatography, mass spectrometry, and informatics.

- UPLC® Technology produces peptide maps of superior resolution and greater sensitivity for more in-depth protein characterization, and offers higher throughput peptide analysis for labs requiring greater efficiency of operation.
- Waters system solutions can be combined with flexible detection options – such as tunable UV, photodiode array, and mass spectrometry – all optimized for UPLC. Waters' portfolio of MS technologies offers versatile configurations that match your peptide mapping application requirements, from single quadrupole LC/MS for confirmation, to hybrid quadrupole and orthogonal acceleration time-of-flight (oa-TOF) tandem mass spectrometers for high mass accuracy for peptide and fragment ions and sensitive detection of low-level variants.
- Automated processing and reporting of mass spectrometry data, with advanced graphical and tabular tools that quickly provide efficient sample-to-sample comparisons, can be performed with BiopharmaLynx™ Software or the UNIFI™ Scientific Information System.

PEPTIDE SEPARATION TECHNOLOGY (PST) COLUMNS

Suitable for a wide range of peptides, including large, small, acid, basic, hydrophobic and hydrophilic, PST columns are QC-tested with a protein digest for assured, consistent peptide mapping performance. BEH particle technology was developed for UPLC applications and is optimized for use with both trifluoroacetic acid (TFA) and formic acid (FA) modifiers. The same separation chemistry is provided with either 3.5-µm particles for HPLC analysis or with 1.7-µm for use with UPLC Systems. PST columns are specifically QC-tested with protein digests to confirm batch-to-batch reproducibility. This column chemistry is, therefore, suitable for the development of methods that are to be used routinely for extended periods.

For a full listing of peptide chromatography columns and products for peptides, go to **www.waters.com/pst**, or, for the Waters bioseparations products catalog, **www.waters.com/biosep**.

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Quantitative Aspects of UPLC Peptide Mapping

Thomas E. Wheat, Ziling Lu, Beth Gillece-Castro, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Throughout the development of a biopharmaceutical protein, peptide mapping is used to demonstrate genetic stability and to confirm the integrity of the protein. Changes in retention time, often in combination with MS or MS/MS detection, reveal changes in the primary structure of the protein.

Modifications such as oxidation, deamidation, deletions, sequence clips, and glycosylation all affect chromatographic behavior. The modified peptides must be separated from the native peptides for a peptide mapping method to be useful. The presence of a modified peptide in the map of a sample reflects the presence of modified protein in the original sample.

In the initial characterization of a protein, it is important to develop a peptide mapping method that resolves modified peptides from native peptides so that all possible modifications may be detected. As development of the biopharmaceutical advances, these peptides must be quantitated. Quantitation is generally expressed as area or height percent of the native peptide.

In this way, the peptide map can provide information on the mixture of protein forms in each sample so that the safety and efficacy of the preparation may be assured. Methods must, therefore, exhibit excellent sensitivity and linearity for quantitative work.

UltraPerformance LC® (UPLC®) has demonstrated significant advantages compared to HPLC for peptide mapping. UPLC gives increased resolution, higher sensitivity, excellent peak shapes for glycopeptides, and the potential to increase throughput.[1-2]

In this application note, we focus on the quantitative aspects of UPLC peptide mapping with UV detection. The technique is evaluated with respect to both chromatographic and detection linearity since the altered and normal peptides occur at extreme molar ratios. Reproducibility of the area measurement at these extreme ratios is also examined. Results from a mixture of peptide standards and from a digest spiked with a known amount of peptide are shown.

MATERIALS AND METHODS

Samples:	Waters MassPREP™ Peptide Standard Mixture Waters MassPREP Hemoglobin tryptic digest
Sample buffer:	95% Buffer A/5% Buffer B
Instrument:	Waters ACQUITY UPLC® System
UPLC column:	Waters Peptide Separation Technology ACQUITY UPLC BEH 130 C_{18}, 1.7 µm, 2.1 x 100 mm
Flow rate:	0.2 mL/minute
Mobile phase:	A: 0.1% TFA in Milli-Q water B: 0.08% TFA in acetonitrile
Gradient:	0 to 50% B in 29 minutes (peptide mixture) 0 to 50% B in 58 minutes (hemoglobin digest)
Temperature:	40 °C
Detection:	UV at 214 nm with 10 mm path length cell at 10 Hz
Quantitation:	Waters MassLynx Software 4.1's QuanLynx™ Application Manager
Peptide ID:	Waters LCT Premier™ oa-TOF Mass Spectrometer
Ionization mode:	Electrospray, positive
Scan:	400 to 1800 m/z at a rate of 2 scans per second

RESULTS AND DISCUSSION

A mixture of peptides, 20 pmoles of each, was analyzed on a Peptide Separation Technology ACQUITY UPLC BEH 130, C_{18}, 1.7 µm, 2.1 x 100 mm column. The separation was monitored at 214 nm. Six replicate injections are overlaid in Figure 1, demonstrating the reproducibility of UPLC for peptide mapping. The same sample was subsequently injected at different levels to test linearity and sensitivity.

Figure 2 shows the analysis of peptide standards from 250 fmol to 100 pmol injected on-column. There is no significant shift in retention or deterioration in peak shape from low to high levels. This confirms that the dynamic range of the chromatographic material and gradient method is sufficient for the analysis of small amounts of one peptide in the presence of much larger amounts of another.

The lower limit is shown in Figure 3, where the six lowest levels are overlaid. The signal-to-noise ratio should be sufficient for quantitation at 250 fmol, and this is consistent with the area reproducibility summarized in Table 1. Peak area response is linear over two-and-a-half orders of magnitude, as shown in Figure 4.

Given a typical injected amount of 100 pmol of digested protein, these quantities would correspond to 0.25% to 10% of a modified peptide. These results on standard peptides indicate that UPLC peptide mapping can be used to quantitate modified peptides in digests over a wide range of concentration.

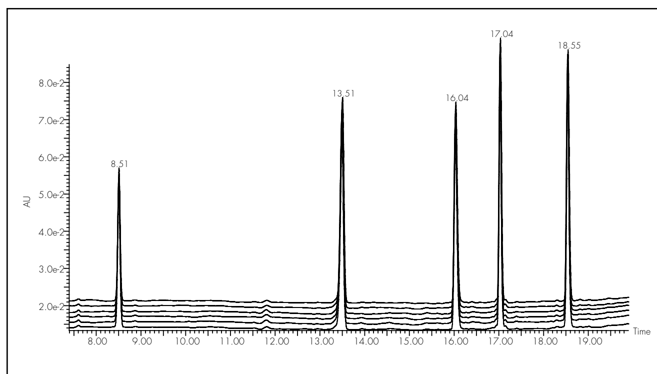

Figure 1. Six replicate 20 pmol injections of peptide mixture standard were made on a Peptide Separation Technology ACQUITY UPLC BEH 130 C_{18} 1.7 µm, 2.1 x 100 mm column. Retention time, peak height, and peak area reproducibility were measured for a peptide mapping gradient.

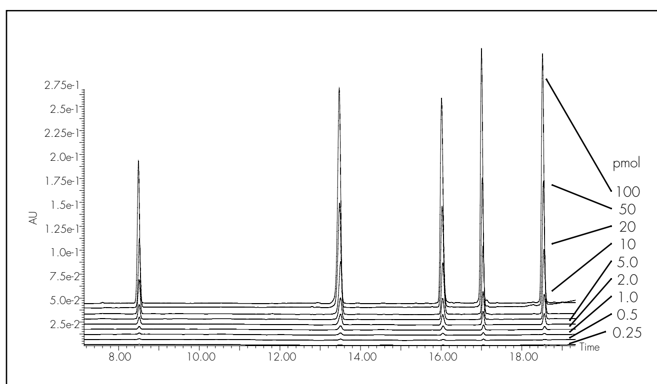

Figure 2. The peptide mixture was analyzed from 250 fmol to 100 pmol on-column.

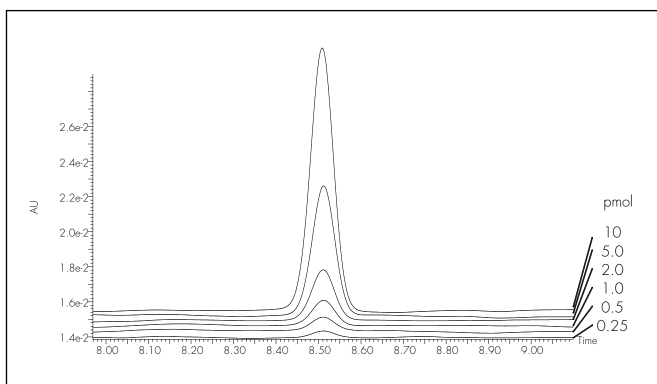

Figure 3. Chromatograms of one peptide from 250 fmol to 10 pmol show the high sensitivity region of the standard curve.

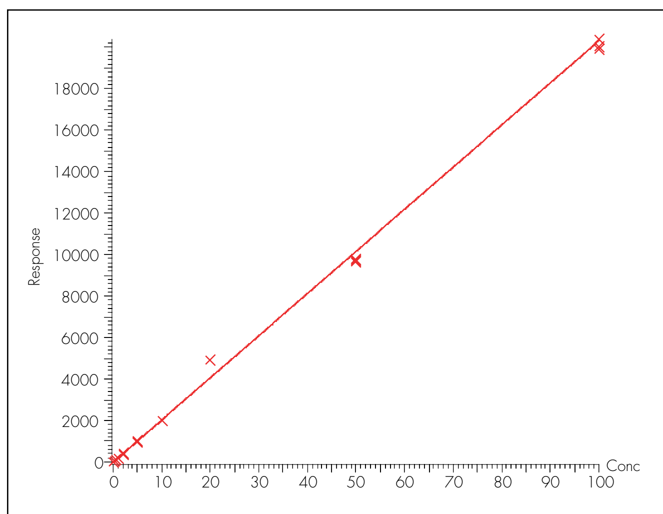

Figure 4. Linearity of UV detector response is shown for triplicate injections from 250 fmol to 100 pmol of the peptide standard mixture. This peptide eluted at 8.5 minutes, as shown in Figures 2 and 3 and Table 1.

A known amount of a specific peptide was added to an actual protein digest to test estimates of quantitation. This peptide serves as a surrogate, illustrating the behavior of modified peptides in the digest. A tryptic digest of hemoglobin (200 pmoles) was spiked with a peptide corresponding to a concentration range of 0.2 to 2% of the native material.

The resulting chromatograms are shown in Figure 5. The whole digest elutes between 7 and 53 minutes. Figure 5 focuses on the segment of quantitative interest and magnifies the elution profile of the surrogate peptide. The surrogate peptide, marked with a * in Figure 5, elutes just before minute 29. The peptide can be detected easily at the 0.2% level. Chromatographic resolution is maintained at this low level relative to the significantly larger peak that elutes 0.25 minutes earlier.

Peptide Load (pmoles)	Mean Area	R.S.D. Area	% R.S.D. Area
0.25	18.98	0.70	3.7%
0.50	38.35	1.13	2.9%
1.00	74.48	1.10	1.5%
2.00	151.39	0.79	0.5%
5.00	392.83	2.50	0.6%
10.00	820.85	12.18	1.5%
20.00	1933.30	6.55	0.3%
50.00	4368.58	26.18	0.5%
100.00	9051.69	117.65	1.3%

Table 1. Reproducibility of peak area for the peptide eluting at 8.5 minutes. Triplicate injections from 250 fmol to 100 pmol were analyzed.

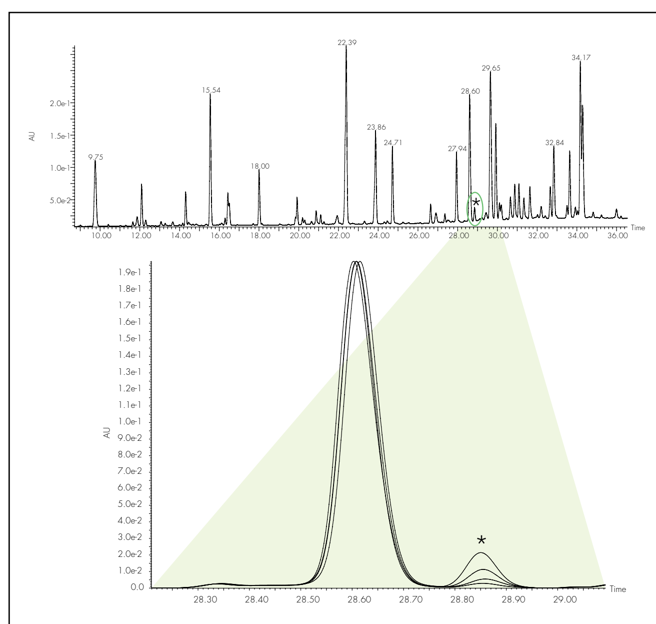

Figure 5. A tryptic digest of bovine hemoglobin was spiked with the peptide mixture as a surrogate to represent modified peptides. The surrogate peptide concentrations were 0.2 to 2% of the digest concentration on a molar basis. Panel A shows the peptide map of bovine hemoglobin with peptide standards spiked at 2% molar basis. A total injection on-column of 200 pmol of hemoglobin digest was made. Panel B focuses on the elution position of the surrogate.

CONCLUSION

UPLC yields qualitatively and quantitatively reproducible peptide maps. Chromatographic resolution and peak shape are constant over nearly three orders of magnitude of sample amount on column. Peak areas may be reliably and reproducibly quantitated to sub-picomole levels, as low as 250 fmoles on column.

This level of sensitivity is obtained with UV detection on 2.1 mm columns without resorting to special detection strategies. The detector response is linear with sample amount. This sensitivity in combination with robust chromatographic behavior enables detection of low level peptides in a complex digest. In turn, the technique can be used to measure the specific variants of protein structure that may be observed in the development of a biopharmaceutical protein.

References

1. Enabling Significant Improvements in Peptide Mapping with UPLC, J. R. Mazzeo, T. E. Wheat, B. L. Gillece-Castro, Z. Lu, Waters Application Note (720001339EN).
2. Next Generation Peptide Mapping with UPLC, J. R. Mazzeo, T. E. Wheat, B. L. Gillece-Castro, Z. Lu, *BioPharm International*, January 1, 2006.

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

[APPLICATION NOTE]

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Enabling Significant Improvements for Peptide Mapping with UPLC

Jeffrey R. Mazzeo, Thomas E. Wheat, Beth L. Gillece-Castro, and Ziling Lu
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Peptide mapping continues to be the preferred technique for the comprehensive characterization of biopharmaceutical products. Its applications include:

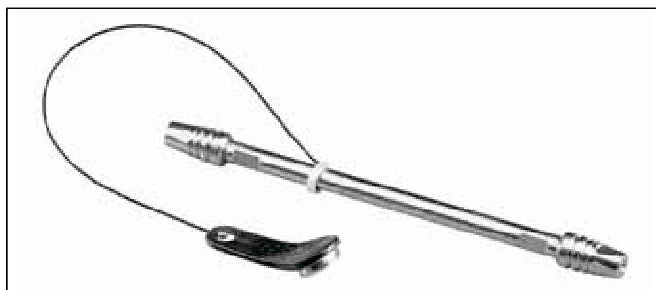
- Identification of proteins based on the elution pattern of peptide fragments
- Determination of post-translational modifications
- Confirmation of genetic stability
- Analysis of protein sequences when interfaced to a mass spectrometer

In a peptide map, it is necessary to resolve each peptide fragment into a single peak. Therefore, peptide mapping represents a significant chromatographic challenge due to the inherent complexity of protein digests. In addition to the large number of peptide fragments that are generated from the enzymatic digestion of a protein, the number of alternative peptide structures (e.g., post-translational modifications, oxidations, etc.) can be significant.

The capabilities of UltraPerformance LC® (UPLC®) Technologies make higher resolution peptide mapping possible. This application note demonstrates the advantages of UPLC for peptide mapping.

EXPERIMENTAL

LC system:	Waters ACQUITY UPLC® System
MS system:	Waters Q-Tof micro™ Mass Spectrometer
Software:	Waters MassLynx™ 4.0 Software
Columns:	Waters Peptide Separation Technology ACQUITY UPLC BEH 130, 1.7 µm, 2.1 x 50 mm and 2.1 x 100 mm BioSuite™ PA-A 3 µm, 2.1 x 100 mm BioSuite 3.5 µm, PA-B 2.1 x 100 mm
Flow rate:	0.1 or 0.3 mL/min
Mobile phase:	A: 0.02% TFA or 0.1% FA in H_2O B: 0.018% TFA or 0.1% FA in ACN
Gradient:	Linear gradients from 5 to 50% B, times as indicated
Temperature:	40 °C
Injection vol.:	20 or 50 µL
Detection:	ESI/MS
Samples:	Waters MassPREP™ Peptide Standard Mixture MassPREP Enolase Digest Tryptic digest of α-1 acid glycoprotein



An ACQUITY UPLC Column.

RESULTS AND DISCUSSION

The chromatographic benefits of UPLC are largely derived from reduced band-broadening that is, in turn, a consequence of reduced diffusion distances in small particles. This process is quantitatively described in the van Deemter equation that relates height equivalent of a theoretical plate to linear velocity.

This relationship is shown graphically in Figure 1 for a peptide of 1500 MW on 3.5 µm and 1.7 µm packings. The minimum in the curve corresponds to the maximum efficiency, and greatest resolving power, for each particle size. At linear velocities, or flow rates, above and below the optimum, resolving power declines.

As expected, the smaller particles have higher resolving power at a higher linear velocity. In quantitative terms, the 3.5 µm particles have a minimum plate height of 8.11 µm at a linear velocity of 0.17 mm/sec. In contrast, a minimum plate height of 3.94 µm is observed at 0.33 mm/sec with the 1.7 µm particles.

In practical terms, these principles suggest that the small particles used in UPLC could double the resolving power in a peptide mapping experiment and could simultaneously reduce the separation time because the optimum is achieved at a higher linear velocity.

For the 3.5 µm particle, the optimum linear velocity corresponds to a flow rate of about 24 µL/min on a 2.1 mm I.D. column. In practice, such a flow rate would never be used for a peptide map because the separation times would be far too long. It is common practice to operate at a higher flow rate, typically about 250 µL/min on 2.1 mm columns. This linear velocity of about 1.7 mm/sec corresponds to a plate height of about 21 µm. This loss of resolution with a 10-fold increase in separation speed has come to be an accepted compromise. For 1.7 µm particles, resolution is much better preserved at the higher flow rate.

These chromatographic principles suggest several ways to approach improving peptide maps using UPLC. First, the smaller particle packing will improve both resolution and sensitivity by reducing diffusion-related band broadening. Second, the reduced plate height is consistent with obtaining the same or better resolution with shorter columns and higher flow rates. Third, the compromise between separation time and resolution will be more favorable with the smaller particles.

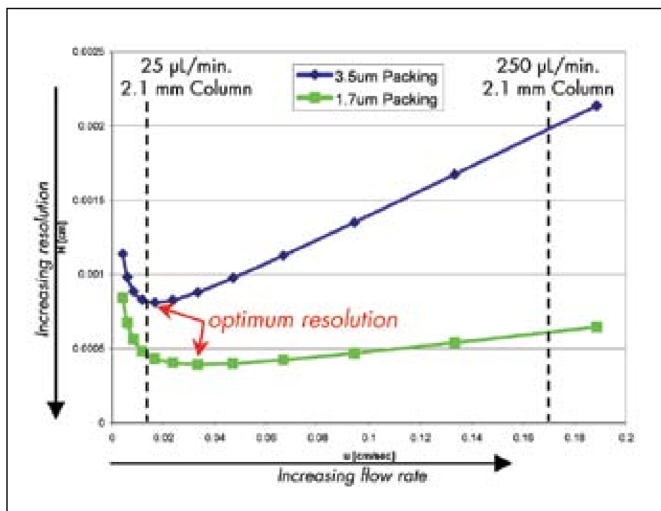
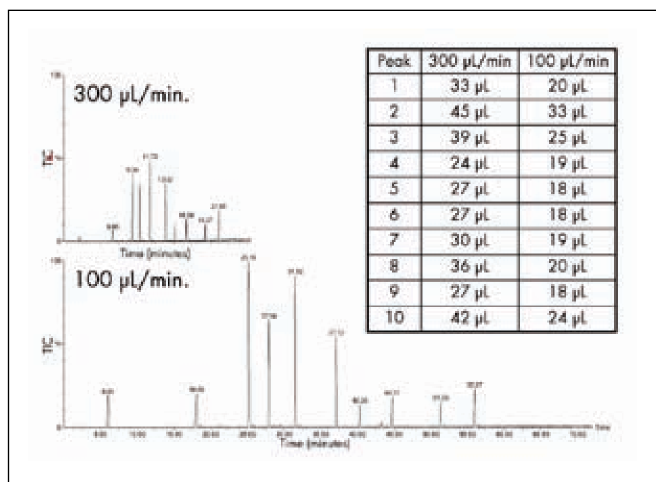


Figure 1. van Deemter plot of a 1500 Da peptide.

The influence of volumetric flow rate on peptide separation performance with 2.1 mm I.D. columns was investigated. A standard peptide mixture was separated on a UPLC column run at 100 µL/min and at 300 µL/min, as shown in Figure 2. Flow rate, or linear velocity, was the only variable because the gradient change/column volume was the same, ensuring that the chromatographic selectivity is constant. In experimental terms, a 75 minute gradient was used at 100 µL/min and a 25 minute gradient at 300 µL/min.

To compare the results, peak volumes, calculated by multiplying the flow rate by peak width at the base, are shown in the inset. Running at 100 µL/min provides, on average, about a 1/3 reduction in peak volume.



Peak	300 µL/min	100 µL/min
1	33 µL	20 µL
2	45 µL	33 µL
3	39 µL	25 µL
4	24 µL	19 µL
5	27 µL	18 µL
6	27 µL	18 µL
7	30 µL	19 µL
8	36 µL	20 µL
9	27 µL	18 µL
10	42 µL	24 µL

Figure 2. Effect of flow rate on the MassPREP peptide standard mixture. Both chromatograms are plotted to the same intensity scale.

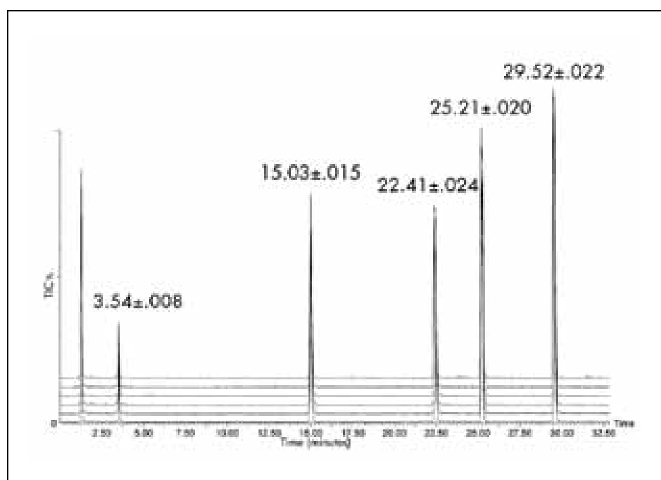


Figure 3. UPLC retention time reproducibility for peptide mapping.

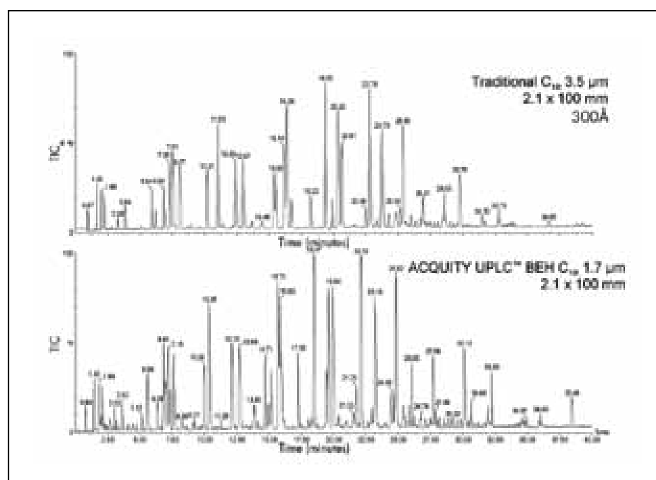


Figure 4. Suitability of UPLC for peptide mapping.

As expected from the chromatographic principles described above, peptide peak volumes, and, therefore, resolution and sensitivity, are optimum at 100 µL/min. The flow rate is low compared to what is traditionally used for peptide mapping with 2.1 mm I.D. HPLC columns. The commonly accepted flow rate represents a compromise between resolution and run time but it also reflects instrumental limits in reproducibly pumping liquid at flow rates less than 150 µL/min with accurate and precise gradients. The ACQUITY UPLC System performs extremely well at a flow rate of 100 µL/min in gradient mode. This performance is demonstrated by the overlay of six gradient runs of a peptide standard, shown in Figure 3. The average and standard deviations of retention times are listed for each peak.

The chromatographic principles also suggest ways to reduce the run time of a peptide map. Peptide maps run by HPLC often require cycle times as long as 3 to 5 hours to separate all the peptides within the digest, especially for large proteins like antibodies. While faster peptide maps would be desirable, it is critically important that resolution not be compromised. The test results must provide the same level of information. The van Deemter equation predicts that plate height will be 2- to 4-fold less with 1.7 µm particles than with 3.5 µm particles. The same resolving power can, therefore, be obtained with a shorter column.

These experiments focus on the physical aspects of the separation as they relate to band-broadening. Successful peptide mapping depends, of course, on the interaction among the peptides, the mobile phase, and the column surface chemistry. In Figure 4, the separation of a tryptic digest of enolase is shown with a 3.5 µm C_{18} HPLC column with 300Å pores, typical of the most common peptide separation columns, and a 1.7 µm UPLC column. Conditions are the same for both columns. In the UPLC separation, more peaks are observed. The overall resolution and sensitivity are higher. In the UPLC map, there are several small peaks that are difficult to discern with the HPLC run.

This result demonstrates that UPLC offers higher resolution and sensitivity when compared to HPLC under the same gradient conditions. As is always observed when comparing two different column chemistries, the separations are not identical in every detail. The overall appearance of the chromatograms is, however, similar. This suggests that the selectivity of the UPLC column is suitable for peptide mapping.

To show how UPLC can resolve the same number of peaks in a peptide map as HPLC but in less time, the separation of an enolase digest was done on a 50 mm length UPLC column with a 20-minute gradient and on a 100 mm length HPLC column with a 40-minute gradient, both with flow rates of 100 µL/minute. These chromatograms are shown in Figure 5. The UPLC separation shows the same number of peaks and a similar overall elution pattern as the HPLC separation, but in half the time. UPLC offers the potential to reduce cycle times for peptide maps.

The higher resolution and sensitivity with UPLC is particularly important when the peptide map is used to detect modified peptides. Higher resolution ensures that modified peptides are resolved from the unmodified form, as well as from other peptides in the digest. Higher sensitivity means that modified peptides can be detected at lower levels. For example, in Figure 6, UPLC is used to separate a deamidated peptide from its unmodified form. UPLC should be the technique of choice for detecting all the peptides in a sample.

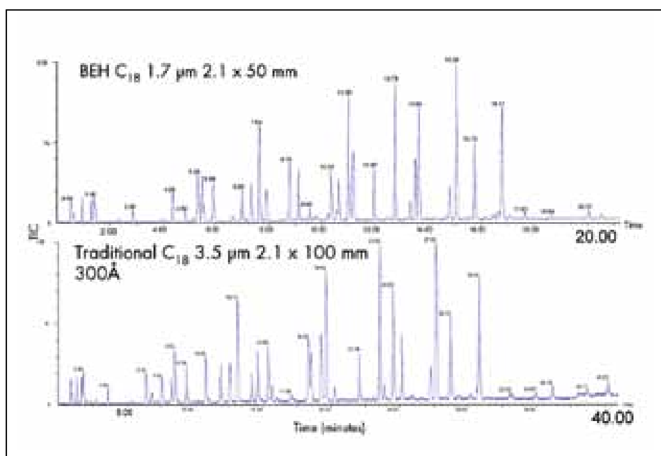


Figure 5. Reduced column length for increased speed.

Peptide mapping is frequently interfaced with electrospray ionization mass spectrometry (ESI/MS) to provide additional information about the eluting peptides, including molecular weight and sequence. MS can also identify modified peptides and glycosylation sites. Therefore, it is important that a peptide mapping technique work well under conditions that are favorable for ESI/MS.

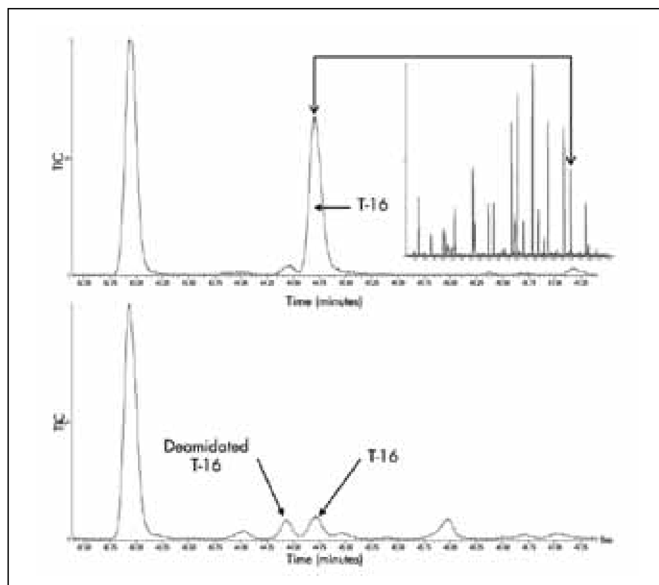


Figure 6. Separation of a deamidated peptide from its native form.

TFA is commonly used as an acidic additive for peptide maps with UV detection, but it can lead to suppression of ionization and reduced sensitivity in ESI/MS. Formic acid is more desirable for LC/MS, as it causes less ion suppression than TFA. However, many reversed phase columns used for peptide mapping show lower retention and broader peaks with formic acid than with TFA.

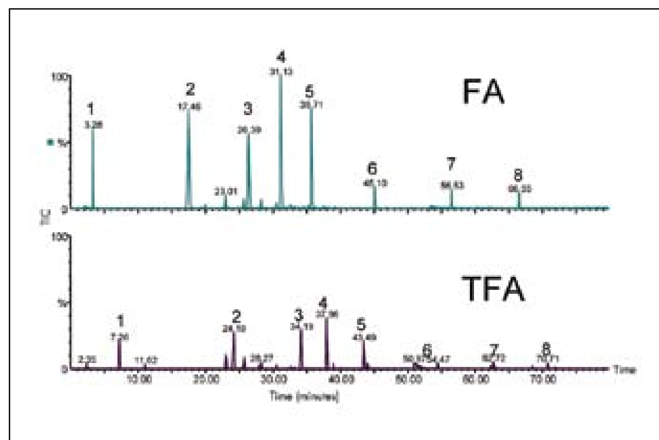


Figure 7. Effect of mobile phase modifier in UPLC Peptide Separation Technology material.

In Figure 7, the separation of several peptides with formic acid is compared with TFA on a UPLC column with MS detection. With formic acid, the peak heights are about 3-fold higher. There is only a slight reduction in retention, corresponding to a difference of a few percent organic at the point of elution, and a slight increase in peak width. This result indicates that the UPLC columns perform extremely well under conditions that are best for ESI/MS.

Glycosylation is an important post-translational modification that plays a critical role in determining the efficacy and safety of a therapeutic protein. Glycosylation can be analyzed on the intact protein by mass spectrometry, as released glycans or as glycopeptides in LC/MS peptide maps. When glycosylation is characterized with LC/MS of the glycopeptides, the site of attachment can be directly determined and structural information can be obtained through MS/MS experiments. This approach is limited, however, by the poor chromatographic peak shape of glycopeptides and incomplete resolution of glycoforms with HPLC peptide mapping. The poor peak shape has been attributed to the large size of the glycopeptides and their heterogeneous structure.

Figure 8 shows the UPLC/MS separation of a tryptic digest of α-1 acid glycoprotein. The MS detection was performed with a Q-Tof micro Mass Spectrometer, which is well-suited for glycopeptides due to its extended mass range. Data is plotted as a selected ion chromatogram for m/z 657, a signature ion for glycopeptides resulting from carbohydrate fragments. The glycopeptides are detected as sharp, symmetrical peaks with UPLC. These characteristics are important for minimizing spectral overlap of different glycoforms of the same peptide. UPLC with ESI/TOF mass spectrometry will be a powerful tool for studying the glycosylation state of proteins.

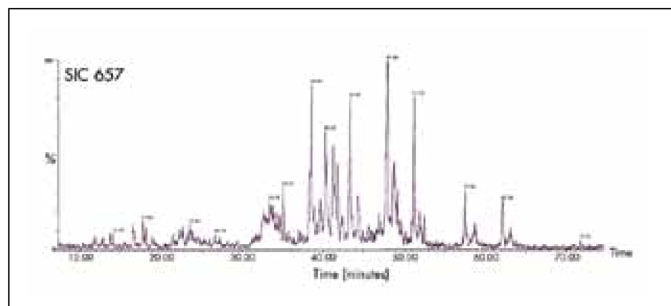


Figure 8. A glycopeptide separation.

CONCLUSION

UPLC facilitates notable improvements in peptide mapping when compared to HPLC. Better resolution is obtained in combination with generally increased sensitivity. Run time can be reduced without compromising resolution by reducing column length and by increasing flow rate. Selectivity is comparable to that of common reversed phase HPLC peptide mapping columns and can be easily transferred to alternative modifiers that give better sensitivity in ESI/MS. UPLC with ESI/TOF is especially suitable for the separation of glycopeptides. The ACQUITY UPLC System is clearly proving to be the next-generation tool for peptide mapping.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Analysis of Deamidation and Oxidation in Monoclonal Antibodies using Peptide Mapping with UPLC/MS^E

Hongwei Xie, Martin Gilar, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Monoclonal antibodies (mAb) are an important class of protein therapeutics. The mAb production in recombinant expression systems is often a source of post-translational modifications (PTM), such as various degrees of glycosilation, oxidation, and deamidation.

Asparagine (N) deamidation and methionine (M) oxidation are two common modifications. Deamidation can diminish the activity and stability of an antibody.

Sulfur-containing methionine is a major target for oxidation. Studies[1-3] on mAb revealed oxidation during storage.

In this application note, we demonstrate the use of data-independent acquisition mass spectrometry, MS^E, with UltraPerformance LC® (UPLC®) for profiling of N-deamidation and M-oxidation sites in mAb after prolonged storage.

Unmodified peptides were resolved from site-specific deamidated isoforms using a Waters® ACQUITY UPLC® System and identified by MS^E. The identities of modified "PENNY" peptides were further confirmed by spiking the sample with synthetic peptide standards. Relative quantitation of the modifications was estimated for both N-deamidation and M-oxidation identified from this antibody.

EXPERIMENTAL

Sample preparation and UPLC/MS^E experimental procedure were identical to a previous description.[4] Briefly, the antibody was digested with enzyme trypsin after reduction with dithiothereitol and alkylation with iodoacetamide. The resulting peptide mixture was separated using an ACQUITY UPLC System and detected by MS^E on a SYNAPT™ MS System. In order to profile substoichiometric modifications in the antibody, 120 pmol of freshly-prepared tryptic digest was injected for the UPLC/MS^E analysis.

The acquired data were processed by Identity^E Software of ProteinLynx Global SERVER™ 2.3. The processed data[4,5] were searched against a database consisting of light and heavy chain sequences of the antibody, with trypsin specificity and one optional miscleavage. Cysteine (C) carbamidome-thylation, asparagine (N) deamidation, glutamine (Q) deamidation, and methionine (M) oxidation were allowed as optional modifications in the search.

UPLC/MS^E experiments for synthetic peptides (as listed in Table 1, purchased from Biomatic, Toronto, Canada) were performed using the same experimental conditions as for the digest. The identities of these peptides were confirmed by MS^E spectra. Their retention time (RT) is recorded in Table 1.

Synthetic Peptides[1]	RT (min)	Elution Order
A)		
GFYPSDIAVEWES NGQPENNYK	59.76	3
GFYPSDIAVEWES isoD GQPENNYK	59.36	1
GFYPSDIAVEWES DGQPENNYK	60.57	7
GFYPSDIAVEWES NGQPEisoD NYK	59.66	2
GFYPSDIAVEWES NGQPEDNYK	60.31	5
GFYPSDIAVEWES NGQPENDYK	60.14	4
GFYPSDIAVEWES NGEPENNYK	60.31	5
GFYPSDIAVEWES DGQPEDNYK	61.06	8
GFYPSDIAVEWES DGQPEDDYK	61.55	9
B)		
DIQMTQSPSSLSASVGDR	42.85	2
DIQMox TQSPSSLSASVGDR	36.07	1

[1] A) N/Q-deamidation, "PENNY" peptide T37 of heavy chain;
B) M-oxidation, T1 of light chain;
isoD - isoaspartic acid; Mox - oxidized M.

Table 1. UPLC elution order of synthetic peptides.

RESULTS AND DISCUSSION

The features and operational aspects of UPLC/MS^E have been described previously.[4,5] Briefly, protein digest is separated by UPLC and on-line detected by MS^E. Two sets of MS data are collected in parallel: low-energy (MS) and elevated-energy (MS^E) chromatograms. The collected data are combined for identification of peptides with the help of sequence database searching analysis. The MS^E acquisition is data independent, which ensures sampling of low-abundance peptides and substoichiometric PTMs. The obtained spectra of such peptides allow for identification of peptide modifications. In this study, we focus on characterization of two major degradation pathways: deamidation and oxidation in mAb the antibody.

In order to profile the modifications, the obtained UPLC/MS^E data were searched against light and heavy chain sequences of the antibdy with N/Q-deamidation (+0.98 Da) and M-oxidation (+16 Da) as optional modifications. The profiling returns 10 modified peptides, including eight deamidated and two oxidized peptides. The modification type, site, relative concentration, and RT of identified modified peptides are listed in Table 2. For comparison, the corresponding unmodified peptides are also included in the Table. All the MS^E spectra of modified peptides were successfully validated.

Protein	Peptide	Start	End	Modification Type	Sequence[1] & Modification Site	MH+	RT (min)	SC (%)[2]
Heavy-Chain	T6	51	59	Deamidation N55	IYPTNGYTR	1085.53	27.69	5.5
	T6	51	59	Deamidation N55	IYPTNGYTR	1085.53	28.8	46.2
	T6	51	59	No Modification	IYPTNGYTR	1084.55	26.96	48.3
	T10	77	87	Deamidation N84	NTAYLQMNSLR	1311.64	42.47	65.1
	T10	77	87	Deamidation N84	NTAYLQMNSLR	1311.64	45.36	13.4
	T10	77	87	No Modification	NTAYLQMNSLR	1310.66	43.7	21.5
	T23	278	291	Deamidation N289	FNWYVDGVEVHNAK	1678.79	51.13	5.1
	T23	278	291	Deamidation N289	FNWYVDGVEVHNAK	1678.79	51.78	9.3
	T23	278	291	No Modification	FNWYVDGVEVHNAK	1677.81	50.07	85.6
	T36	364	373	Deamidation N364	NQVSLTC*LVK	1162.61	42.85	1.6
	T36	364	373	Deamidation N364	NQVSLTC*LVK	1162.61	49.12	2.1
	T36	364	373	No Modification	NQVSLTC*LVK	1161.63	47.06	96.3
	T37	374	395	Deamidation N387	GFYPSDIAVEWESNGQPENNYK	2545.12	59.36	39.2
	T37	374	395	Deamidation N387	GFYPSDIAVEWESNGQPENNYK	2545.12	60.57	9.4
	T37	374	395	Deamidation N392 & Deamidation N389	GFYPSDIAVEWESNGQPENNYK & GFYPSDIAVEWESNGQPENNYK	2545.12	60.31	3.2
	T37	374	395	Deamidation N387 + N392	GFYPSDIAVEWESNGQPENNYK	2546.1	61.06	0.4
	T37	374	395	Succinimide Intermidate N387	GFYPSDIAVEWESNGQPENNYK	2527.1	61.38	1.3
	T37	374	395	No Modification	GFYPSDIAVEWESNGQPENNYK	2544.14	59.76	46.4
	T21	252	258	Oxidation M255	DTLMISR	851.43	28.23	4.7
	T21	252	258	No Modification	DTLMISR	835.43	32.76	95.3
Light-Chain	T3	25	42	Deamidation N30	ASQDVNTAVAWYQQKPGK	1291.98	38.6	1.9
	T3	25	42	Deamidation N30	ASQDVNTAVAWYQQKPGK	1291.98	41.69	32.6
	T3	25	42	No Modification	ASQDVNTAVAWYQQKPGK	1291	40.34	65.5
	T11	127	142	Deamidation N137	SGTASVVC*LLNNFYPR	1798.88	67.01	2.51
	T11	127	142	Deamidation N137	SGTASVVC*LLNNFYPR	1798.88	74.61	3.5
	T11	127	142	No Modification	SGTASVVC*LLNNFYPR	1797.9	72.05	93.99
	T14	150	169	Deamidation N158	VDNALQSGNSQESVTEQDSK	2136.95	26.88	3.8
	T14	150	169	Deamidation N158	VDNALQSGNSQESVTEQDSK	2136.95	28.11	3.2
	T14	150	169	No Modification	VDNALQSGNSQESVTEQDSK	2135.97	27.1	93

[1] C* - carbamidomethyl C
[2] SC - Stoichiometry in percentage, detected in freshly prepared sample

Table 2. Modification type, site, and relative concentration of modified peptides identified from the antibody.

Of the two detected M-oxidations (Table 2), about 5% of M255 in the heavy chain was oxidized. The elution pattern and MS^E spectra of peptide T21 before and after M255 oxidation is shown in Figure 1. Peptide T21 with M255 oxidation elutes about 4 min earlier than the peptide without modification, because M-oxidation increases the hydrophilicity of peptides.

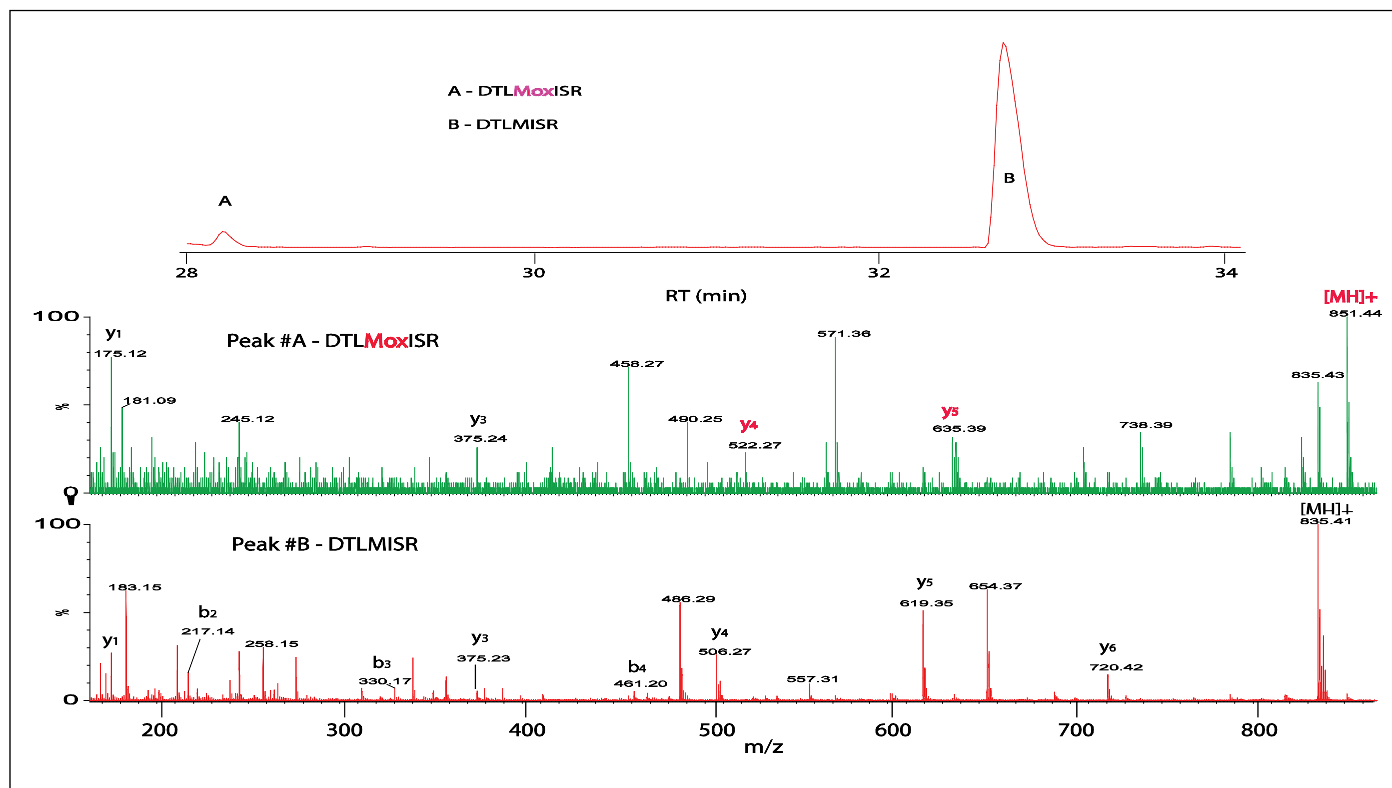

Figure 1. Elution pattern and MS^E spectra of peptide T21 in heavy chain before and after M255-oxidation. Y ions marked in red indicate the M-oxidation.

N-deamidations occur at different levels (Table 2). Each N-deamidation results in two isobaric modified isoforms, isoaspartic acid and aspartic acid, which can't be differentiated by MS. However, N-deamidated peptide with isoaspartic acid elutes earlier than its counterpart with aspartic acid, as confirmed by spiking the sample with synthetic isoaspartic and aspartic isoforms of deamidated peptide standards (see the elution order and RT in Table 1). The elution order is in agreement with literature data.

The peptide T37 of heavy chain with "PENNY" motif has been well studied in literature and suggested as the peptide most susceptible to deamidation in mAb.[6,7] The studies indicate that deamidation occurs on the first two N sites (N387 and N392). In this study, we show separation of six deamidated products (Figure 2). They are identified (see MS^E spectra in Figure 3) to be "PENNY" peptide with isoaspartic acid (isoD) (Peak 2), aspartic acid (D) (Peak 4) and succinimide intermediate (Suc) (Peak 6) of N387 deamidation, as well as newly-found products with deamidation on both N387 and N392 sites (Peak 5), and with deamidation on Q389 (Peak3). N392-deamidated product co-eluted with Q389-deamidated product. This was identified by examining the isotopic patterns of y-series ions in MS^E spectrum of peak 3 (data not shown), and further confirmed by RTs when spiking the corresponding synthetic standards in the sample (Table 1).

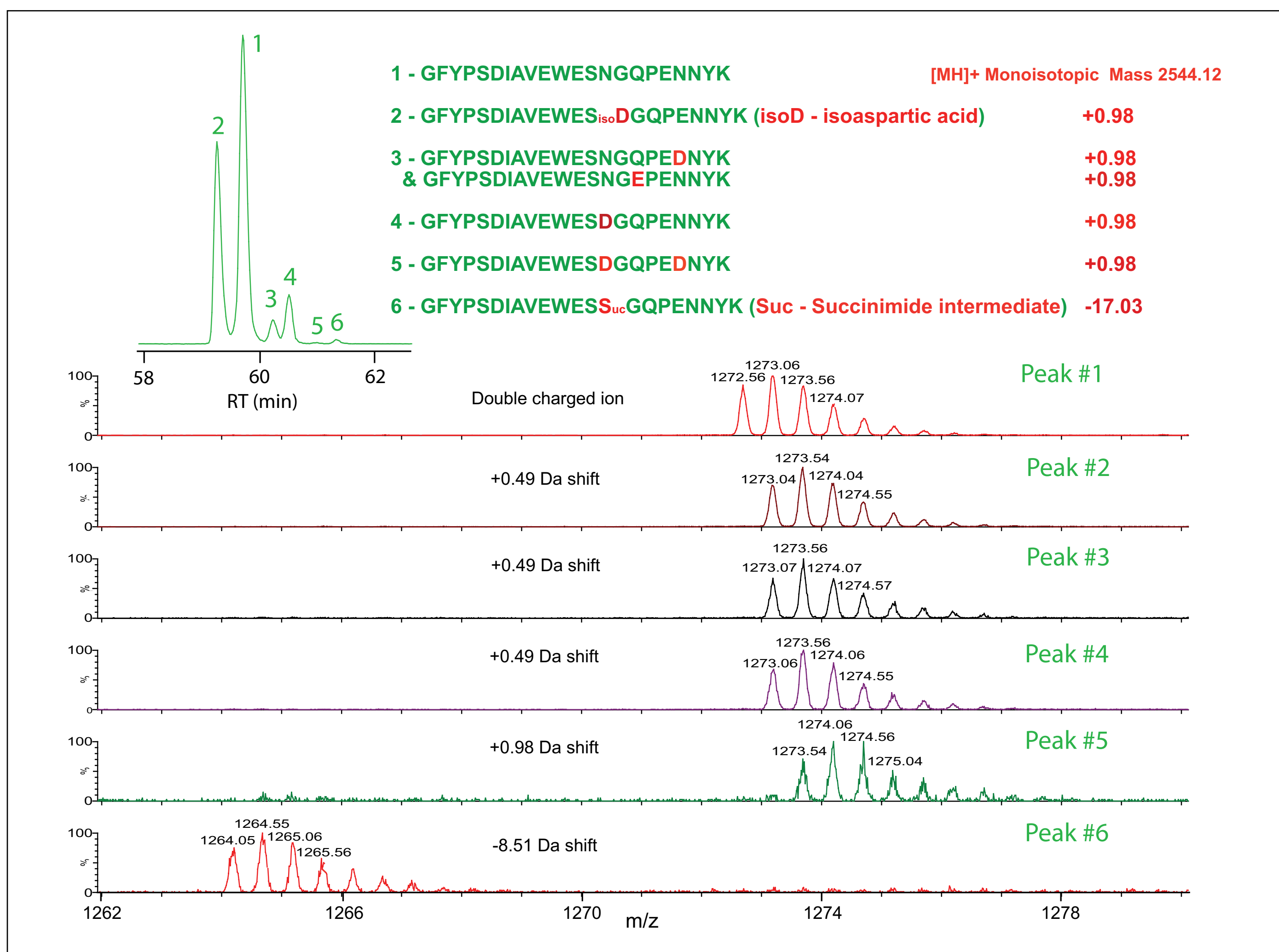

Figure 2. Elution pattern and MS spectra of "PENNY" peptide T37 in Heavy Chain before and after deamidation. Top – Elution pattern; Bottom – MS spectra.

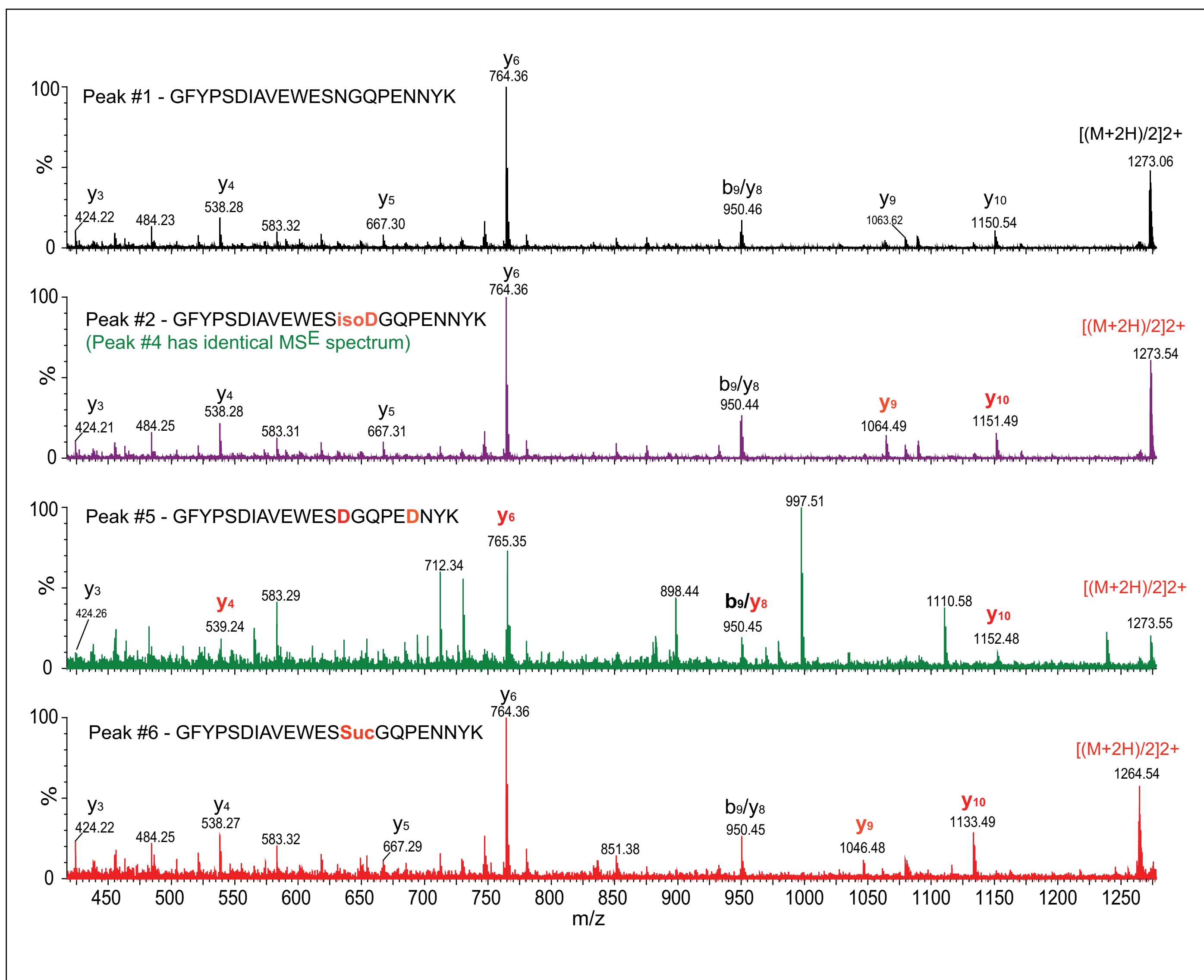

Figure 3. MS^E spectra of Peaks 1, 2 (or 4), 5, and 6 in Figure 2. Y ions marked in red confirm the sequence modifications.

The quantitative results listed in Table 2 show that N387 is the dominant deamidation site of the "PENNY" peptide in this antibody. The total deamidation rate of the "PENNY" peptide was ~ 53.6%.

Comparing the deamidation rate of the "PENNY" peptide with the deamidation of peptides T10 (~ 78.5%) and T6 (~ 51.7%) of heavy chain, we found deamidation on N387, N84, and N55 sites in heavy chain are the major degradation pathways of this antibody.

CONCLUSION

The results demonstrate that UPLC/MS^E is a suitable tool for characterizing PTMs in monoclonal antibodies. MS^E ensures sampling of low-abundance components and acquires indiscriminately MS^E spectra, enabling accurate identification of modified peptides in an unbiased, reproducible manner. The specific conclusions from this study:

- UPLC/MS^E is capable of separating, identifying, and quantifying modified peptides and isoforms
- The high mass resolution and high mass accuracy of the SYNAPT MS System ensures confident identification of modifications with small mass shift (e.g., N-deamidation with 0.98 Da mass difference) and modified isoforms
- Synthetic peptides are helpful for determining modified isoforms and are required for confirmation

In a previous study,[4] we have demonstrated that UPLC/MS^E is able to provide high sequence coverage mapping of mAb tryptic digest, with 97% sequence coverage for both light and heavy chains of the antibody. Therefore, UPLC/MS^E and SYNAPT MS system is an advanced platform for characterization of recombinant proteins, such as monoclonal antibodies.

In current LC-UV/MS peptide mapping methods, the identification of peptide sequences and determination of site-specific modifications typically require multiple tandem mass spectrometry experiments (either DDA MS/MS or targeted MS/MS). The methodology reported here achieves both goals in a single LC run because of available peptide fragmentation information provided by MS^E.

Peptide mapping with UPLC/MS^E improves the analytical efficiency of peptide characterization.

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

[APPLICATION NOTE]

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Identification and Quantification of Protein Modifications by Peptide Mapping with UPLC/MS^E

Hongwei Xie, Martin Gilar, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Protein-based pharmaceuticals are an important class of bio-therapeutic compounds. Although proteins are relatively stable molecules, they are subject to a variety of covalent modifications that occur during manufacturing, formulation, and storage. Modifications have the potential to affect activity and safety of protein drugs. Effective monitoring of them is imperative for ensuring the quality of therapeutic proteins.

Liquid chromatography (LC)-based peptide mapping is suitable for detection of covalent modifications. However, for identification of site-specific modifications, tandem mass spectrometry (MS/MS) sequencing is required. This is especially the case when several possible sites in the peptide can be modified.

In data-dependent acquisition (DDA) LC/MS/MS sequencing, multiple experiments usually need to be performed to elucidate the structure of peptides in protein digests. Detailed DDA LC/MS/MS analysis of peptide maps is a time-consuming task.

Recently, we have demonstrated that peptide mapping with UltraPerformance LC® (UPLC®) and data-independent acquisition mass spectrometry (UPLC/MS^E) is effective for characterization of protein maps,[1-3] providing high sequence coverage and assignment of nearly all LC peaks in the map. The parallel and unbiased data-independent acquisition of precursor and fragmentation data by MS^E [4-5] overcomes the limitations of DDA and also delivers reliable quantitative data.

In this application note, we demonstrate the utility of UPLC/MS^E for identification and quantification of covalent modifications in a yeast alcohol dehydrogenase (ADH) digest. Two common modifications, methionine (M) oxidation and asparagine (N) deamidation, are examined. Modified peptides were clearly distinguished from low-abundant peptides originating from impurity proteins or unexpected peptides resulted from non-specific digestion.

EXPERIMENTAL

Sample preparation and UPLC/MS^E experimental procedure were similar to previous descriptions.[1-2] Briefly, ADH was digested with enzyme trypsin after reduction with dithiothereitol and alkylation with iodoacetamide. The resulting peptide mixture was separated using ACQUITY UPLC® System and detected using MS^E on a SYNAPT™ MS system. Freshly prepared tryptic digest (120 pmol) was injected for the UPLC/MS^E analysis. The analysis was repeated three times.

LC conditions

LC system:	Waters ACQUITY UPLC System
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Column:	ACQUITY UPLC Peptide Separation Technology C_{18}, BEH300Å, 2.1 x 100 mm, 1.7 µm
Column temp.:	40 °C
Gradient:	0-50% B in 90 min
Flow rate:	0.2 mL/min
Detection:	MS^E

The acquired data were processed by Identity^E Software, part of ProteinLynx Global SERVER™ 2.3. The processed data[1-2] were first searched against a yeast database (containing 6139 open reading frames) with trypsin specificity and one potential miscleavage. Then, the data were searched again against the ADH1 sequence with no enzyme specified. N-deamidation, M-oxidation, N-terminal acetylation and C-carbamidomethylation were allowed as variable modifications.

RESULTS AND DISCUSSION

In order to profile sub-stoichiometric modifications in the protein, 120 pmol ADH tryptic digest was injected for the UPLC/MS^E experiments. Figure 1 shows an example UPLC/MS^E chromatogram.

The collected MS^E data were first searched against a yeast database in order to verify the identity of ADH1 and search for potential impurity proteins. Target protein ADH1 and two impurity proteins, ADH5 and Ylr301wp, were found. The concentration of impurity proteins, normalized to ADH1, was 5% and 0.74% for ADH5 and Ylr301wp, respectively.

Subsequently, the MS^E data were searched again against a truncated database containing only ADH1 sequence. The search was purposefully relaxed (no enzyme specificity) to allow for identification of non-specifically cleaved and modified peptides. The ADH1 sequence coverage was 98%.

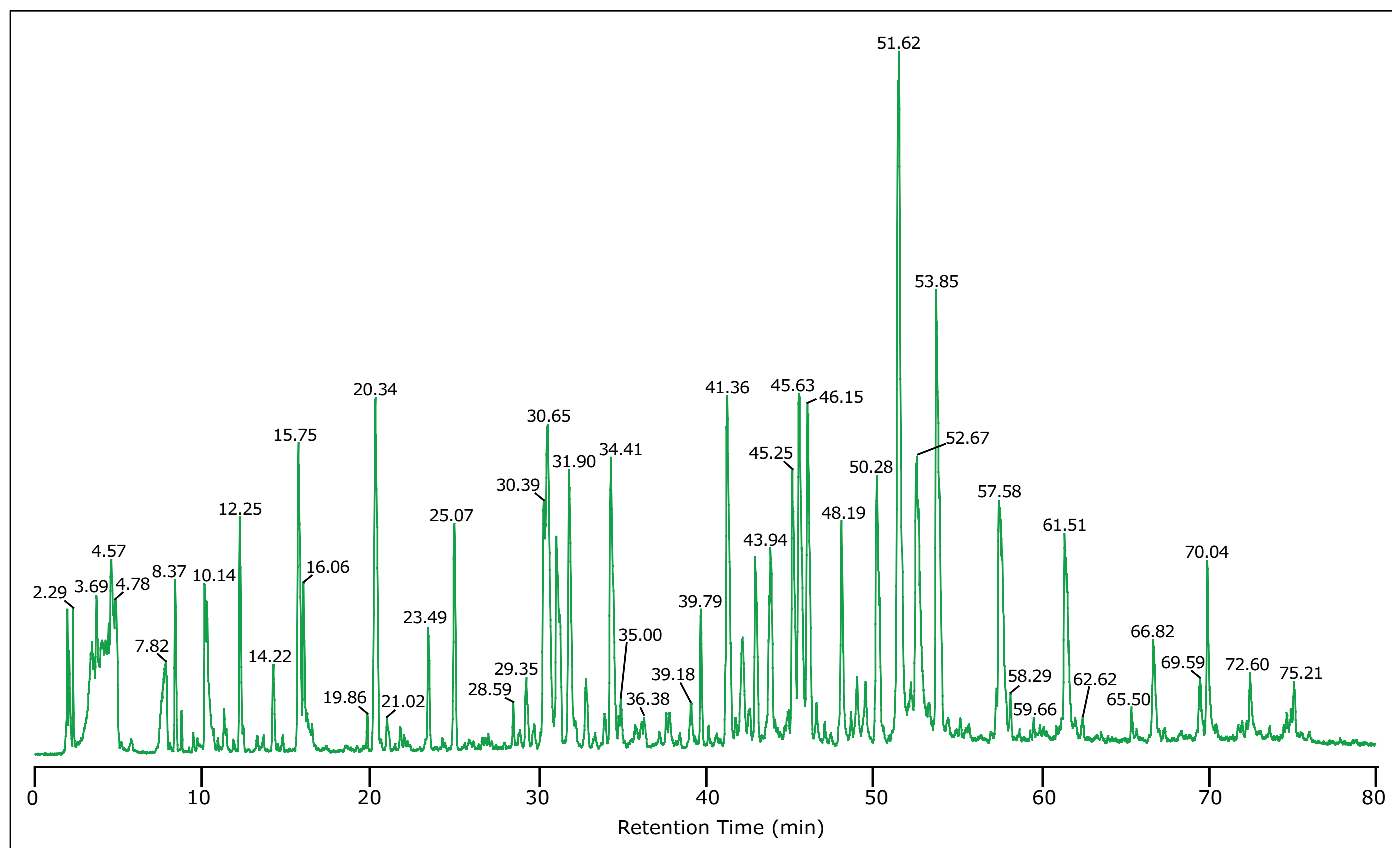

Figure 1. UPLC/MS^E chromatogram of ADH tryptic digest.

Table 1 shows sequences of eight modified amino acid sites located in seven tryptic peptides, including N-terminal acetylation, three oxidized methionines and four deamidated asparagines. The modification type, site, and stoichiometry as well as retention time (RT) of identified modified peptides are presented. The comparisons of MS^E spectra of peptide T12 with and without M-oxidation, and peptide T22 with and without N-deamidation, are shown in Figures 2 and 3, respectively, demonstrating confident identification of the modifications.

The relative quantification of modified peptides calculated from MS signal intensity (Table 1) shows that over 99% of N-terminal of ADH1 were found to be acetylated. Three methionines in the protein sequence were found to be oxidized to a relatively low degree, less than 2% for M75 and M168 sites, and approximately 10% for M270 site. In addition to M-oxidation, the peptide T21 was nearly completely deamidated at N262 site (97%), present in four isoforms as shown in Table 1. Similarly, high percentage of deamidation was observed for N94 site.

Peptide	Modification Type	Sequence[1] & Modification Site	Cleavage[2]	MH+	RT (min)	Stoichiometry ± SD[3] (%)
T1	N-terminal Acetylation	SIPETQK	Fully	844.441	25.07	99.1 ± 0.01
T1	No Modification	SIPETQK	Fully	802.431	16.37	0.9 ± 0.01
T7	Oxidation M75	LPLVGGHEGAGVVVGM GENVK	Fully	2035.064	46.59	1.8 ± 0.30
T7	No Modification	LPLVGGHEGAGVVVGMGENVK	Fully	2019.069	52.70	98.2 ± 0.30
T12	Oxidation M168	SANLM AGHWVAISGAAGGLGSLAVQYAK	Fully	2716.388	65.82	1.7 ± 0.33
T12	No Modification	SANLMAGHWVAISGAAGGLGSLAVQYAK	Fully	2700.393	70.01	98.3 ± 0.33
T21	Deamidation N262 + Oxidation M270	AN GTTVLVGM PAGAK	Fully	1403.720	34.03	1.0 ± 0.01
T21	Deamidation N262 + Oxidation M270	AN GTTVLVGM PAGAK	Fully	1403.720	43.99	8.8 ± 1.35
T21	Deamidation N262	AN GTTVLVGMPAGAK	Fully	1387.725	43.97	84.1 ± 1.72
T21	Deamidation N262	AN GTTVLVGMPAGAK	Fully	1387.725	44.27	3.0 ± 0.39
T21	No Modification	ANGTTVLVGMPAGAK	Fully	1386.741	43.07	3.1 ± 0.01
T5	Deamidation N31	AN ELLINVK	Fully	1014.583	43.54	1.4 ± 0.10
T5	Deamidation N31	AN ELLINVK	Fully	1014.583	47.18	1.4 ± 0.19
T5	No Modification	ANELLINVK	Fully	1013.599	45.66	97.2 ± 0.08
T22	Deamidation N282	C*C*SDVFN QVVK	Fully	1356.592	37.93	4.8 ± 0.01
T22	Deamidation N282	C*C*SDVFN QVVK	Fully	1356.592	44.95	3.3 ± 0.04
T22	No Modification	C*C*SDVFNQVVK	Fully	1355.608	43.07	91.9 ± 0.08
P1	Deamidation N94	WLN GSC*MAC*EYC*ELGNESNC*PHADLSGYTHDGSFQQY	Partially	4358.689	59.67	100[4]
P2	Deamidation N94	WLN GSC*MAC*EYC*ELGNESNC*PHADLSGYTH	Partially	3533.360	55.82	100
P3	Deamidation N94	WLN GSC*MAC*EYC*ELGNESNC*PHADLSGY	Partially	3295.253	58.80	100
P4	Deamidation N94	WLN GSC*MAC*EYC*ELGNESNC*PH	Partially	2688.988	52.51	100
P5	Deamidation N94	WLN GSC*M	Partially	868.333	42.63	100

[1] C* - Carbamidomethyl C; Amino acid in red is with modification; Each deamidated N results in two isofroms, isoaspartic acid and aspartic acid.
[2] Fully - Fully Tryptic; Partially - Partially Tryptic.
[3] In percentage (%) , an average from 3 replicate analyses and calculated by
Intensity of the Modified Peptide / (Intensity of the Modified Peptide + Intensities of Related Unmodified and Other Modified Peptides)
SD - Standard Deviation.
[4] Identified partially tryptic peptides with N94 deamidation from the longest peptide T10 (28 amino acids, M.W. 7601.4); No corresponding peptides without N94 deamination was identified.

Table 1. Modification type, site, and stoichiometry of modified ADH1 peptides.

Although the tryptic peptide T10 (consisting of 69 amino acids, molecular weight 7601.4) was not detected, a series of partially-tryptic N94-deamidated peptides covering part of the missing T10 sequence were found (P1, P2, P3, P4, P5 as shown in Table 1). The non-deamidated versions of these peptides were not detected. Finally, the deamidation of N31 and N282 sites was found to be less than 10%.

Peptide RT shift upon modification can be used as an additional confirmation of sequence modification. In general, N-terminal acetylation increases, while M-oxidation decreases the peptide retention. The RT of N-terminal peptide T1 shifted from 16.37 min to 25.07 min after N-terminal acetylation. In contrast, the RT shifted from 52.7 min to 46.59 min for peptide T7 and from 70.0 min to 65.82 min for peptide T12 after M-oxidation in these peptides.

In the case of N-deamidation, the RT trends are more complex because of the presence of two product isoforms: isoaspartic acid and aspartic acid. Usually, N-deamidated peptide with isoaspartic acid elutes earlier and the other deamidated one with aspartic acid later than the unmodified peptide. There are exceptions to this rule, but the retention order observed here was always the isoform with isoaspartic acid < aspartic acid isoform.

The retention pattern of unmodified peptide T22 and its two N282-deamidated isoforms is presented in Figure 3a. Since the two deamidated isoforms are isobaric (both +0.98 Da mass difference from the unmodified T22), they cannot be distinguished from MS or MS^E data. In such cases, the UPLC separation and RT information of these peptides are important for the identification. However, MS^E can easily differentiate N-deamidated peptide from unmodified peptide, as shown in Figure 3, with a high mass resolution and high mass accuracy platform such as the SYNAPT MS System.

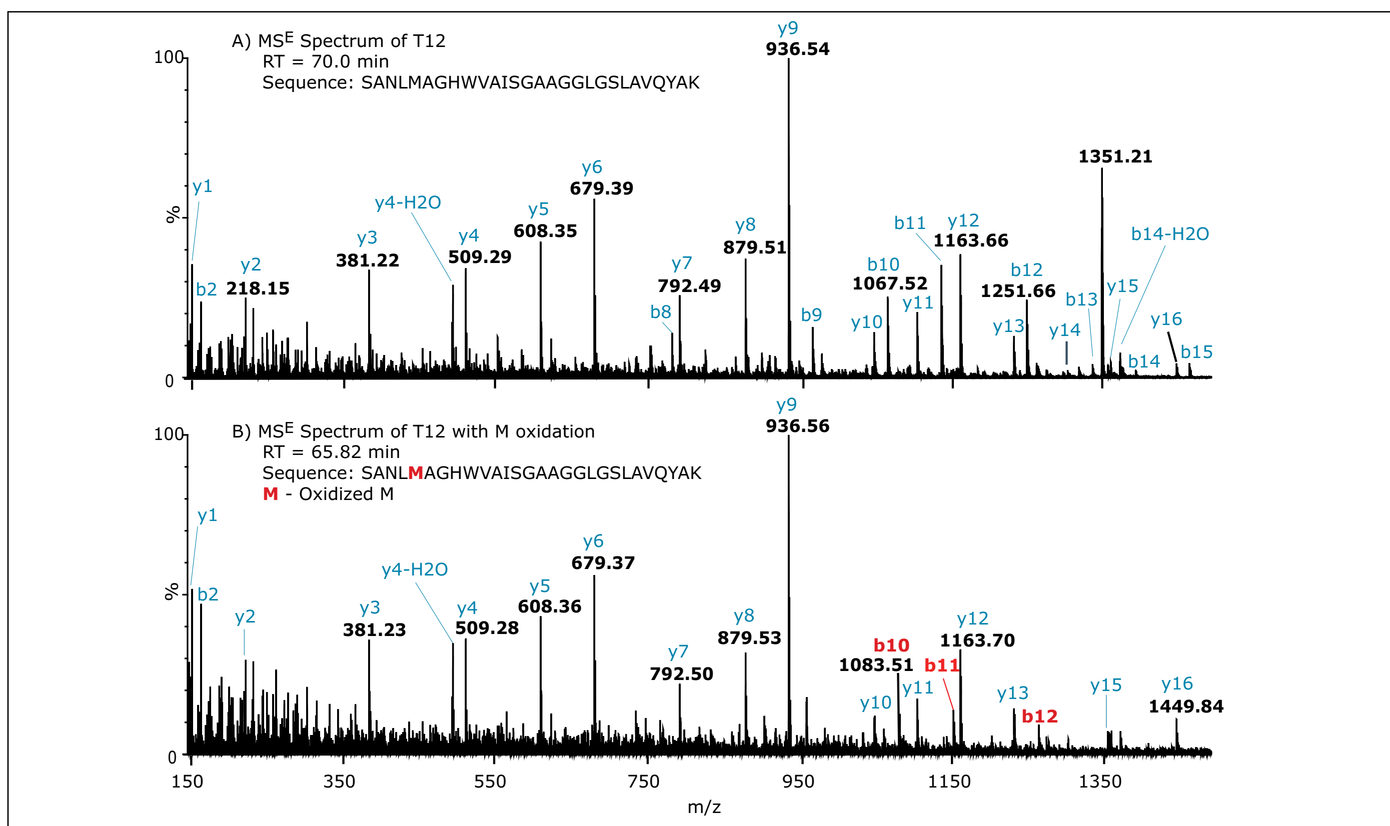

Figure 2. MS^E spectra of peptide T12 (SANLMAGHWVAISGAAGGLGSLAVQYAK) without (top, A) and with (bottom, B) M168 oxidation. b10, b11, and b12 (marked in red) clearly show the oxidation.

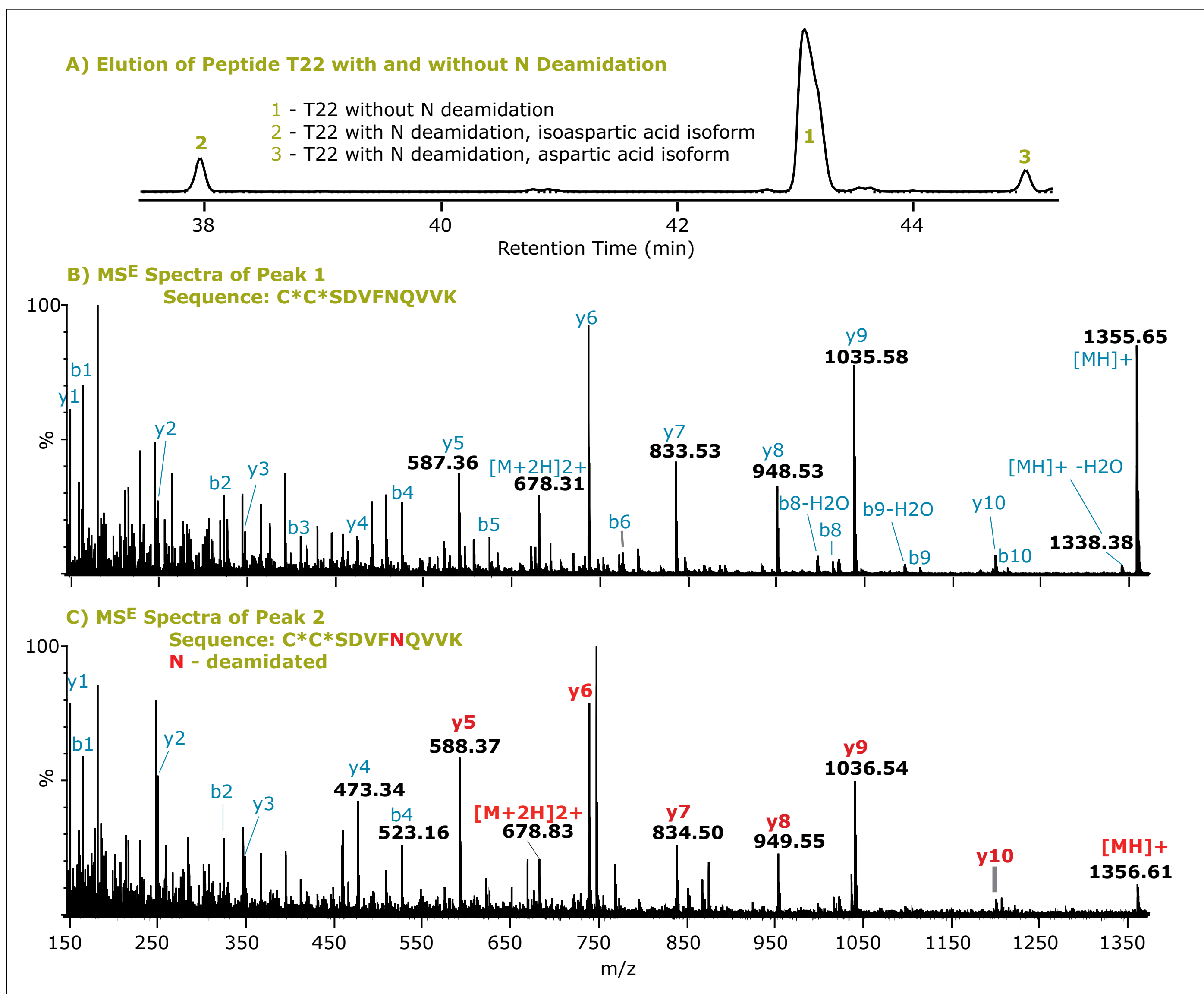

*Figure 3. Elution pattern and MS^E spectra of peptide T22 (C*C*SDVFNQVVK) without and with N282 deamidation. A) Elution pattern; B) MS^E spectrum without N282 deamidation; C) MS^E spectrum with N282 deamidation, isoaspartic acid isoform. y ions (marked in read) starting from y5 clearly show the deamidation. C* is carbamidomethyl C.*

CONCLUSIONS

The results presented here demonstrate application of UPLC/MS^E for characterization of protein peptide maps. UPLC/MS^E was successfully used for identification and quantification of modifications in ADH1. The stoichiometry of modifications ranged from 1% to 99%. In conclusion, peptide mapping with UPLC/MS^E allows for:

- High sequence coverage
- Separation and identification of modified peptides, with modification site and type determined
- Quantification of protein modifications
- Successful determination of sub-stoichiometric modifications at 1% level
- Distinguishing sub-stoichiometric protein modifications (on the peptide level) from peptides originating from impurity proteins contaminating ADH1 at 5% and 0.7% relative levels.

UPLC/MS^E, combining here the ACQUITY UPLC and SYNAPT MS systems, meets requirements for robust and flexible methods that are needed to monitor safety and stability of biopharmaceutical proteins.[1-3] This technology is a fitting choice for biopharmaceutical research and development laboratories. Since it is suitable for establishing protein sequence and characterizing protein covalent modifications and impurities within a single UPLC run, the method will expedite the recombinant protein drug development and manufacturing processes, and reduce their cost.

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[APPLICATION NOTE]

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Effect of Pore Size in UPLC Peptide Mapping

Beth L. Gillece-Castro, Thomas E. Wheat, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The enhanced chromatographic resolution associated with UltraPerformance LC® (UPLC®) has been demonstrated for peptide mapping (Mazzeo et al., *Biopharm.*). The technique improves resolution by a factor of three or more.

In addition, the surface chemistry of the ACQUITY UPLC® BEH Technology™ particles has proven especially advantageous for peptide mapping; good retention and peak shape are observed with either TFA or formic acid as a modifier. The former modifier is preferred for best sensitivity with UV detection, while the latter improves signal-to-noise in electrospray MS experiments. Glycopeptides, which exhibit microheterogeneity, have also been shown to have enhanced resolution and peak shape. These benefits have been demonstrated for tryptic digests of several proteins.

The utility of UPLC peptide mapping for characterizing protein structure can be further extended by expanding the range of column chemistries available. Many peptide chromatographers prefer to use large pore packing materials for peptide separations. The Peptide Separation Technology columns for UPLC include both 130Å and 300Å pore size materials.

The separation of two complex peptide digests on these two pore sizes are compared here. One is a tryptic digest of phosphorylase b, a sample with a large number of smaller peptides; and the other is a LysC digest of phosphorylase b, to give a smaller number of larger peptides. Coupling UPLC chromatography to an oa-TOF mass spectrometer allows high sensitivity identification of peptides and glycopeptides by exact mass measurement.

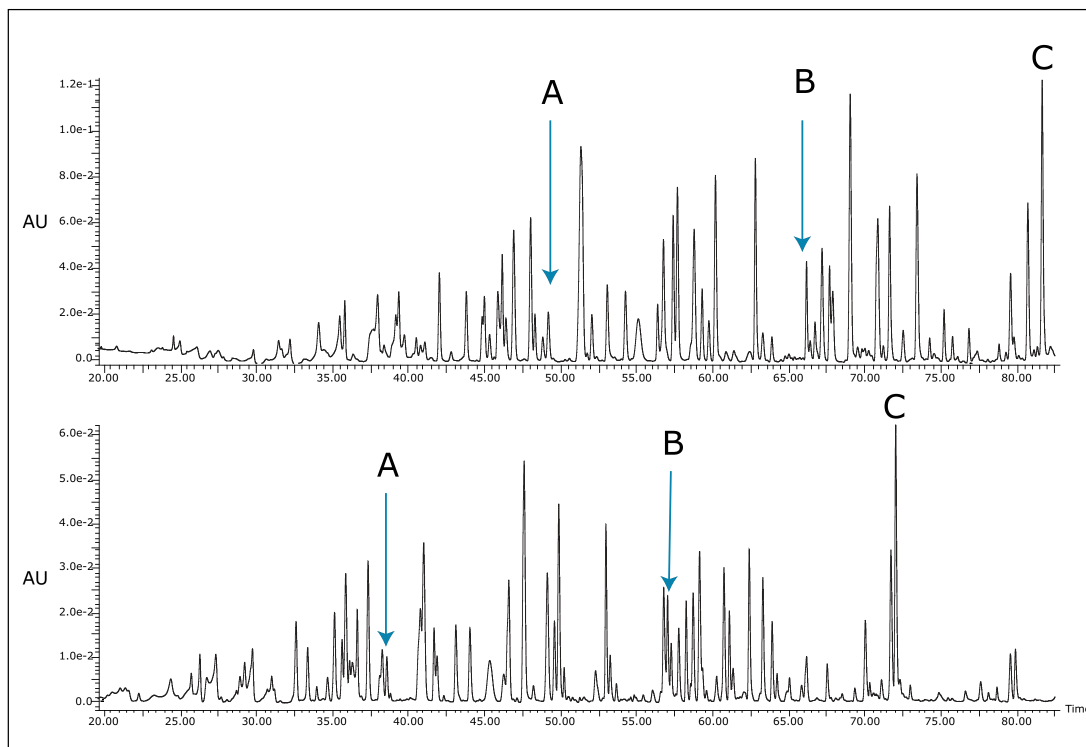

Figure 1. Comparison of the separation of tryptic digest of phosphorylase b on BEH 130, 1.7 μm (top) and on BEH 300, 1.7 μm (bottom) Peptide Separation Technology columns.

RESULTS AND DISCUSSION

Phosphorylase b was chosen to test chromatography due to the large number of peptides that can be derived from this 97 kDa protein. 110 peptides are expected from a complete tryptic digestion, whereas an IgG protein typically forms 50 to 60 tryptic peptides.

Separations of the phosphorylase b tryptic digest are shown in Figure 1. The same sample was analyzed on both the 130Å and the 300Å columns. Three peptides were chosen to monitor specific changes in the chromatogram. The observed peptide maps both have a large number of reasonably well-resolved, sharp, symmetrical peaks. Retention is lower on the larger pore size material.

The change in retention for the specific tracked peaks is equivalent to elution at 4 to 5% lower concentration of acetonitrile. Even with the lower retention on the 300Å column, the four-residue peptide A is easily analyzed.

The selectivity between the two columns is similar, but not identical. For example, a similar pattern of peaks is found between 42 and 46 minutes in the 130Å separation and between 52 and 56 minutes on the 300Å column. Expressed as total resolving power, the calculated peak capacity for the 130Å column is about 1026 and for the 300Å, is 1064. About 230 peaks were recognized on the smaller pore material and 240 on the larger pore.

Digestion of this protein with LysC gives a smaller number of peptides including some larger species. The LysC digests were separated on both columns as shown in Figure 2. Again, peptide retention is generally lower on the larger pore size, and the selectivity is similar, but not identical. The columns, however, still share the same utility described for the separation of the tryptic digests.

The 300Å packing would often be preferred for peptide mapping because the larger molecules are thought to diffuse more freely with larger pores. The chromatographic changes in these experiments do not obviously correlate with the molecular weight of the peptides. For example, the four-residue peptide GRIF is observed as a well-retained and resolved, symmetrical peak in all four maps. A much larger peptide, the LysC peptide representing the

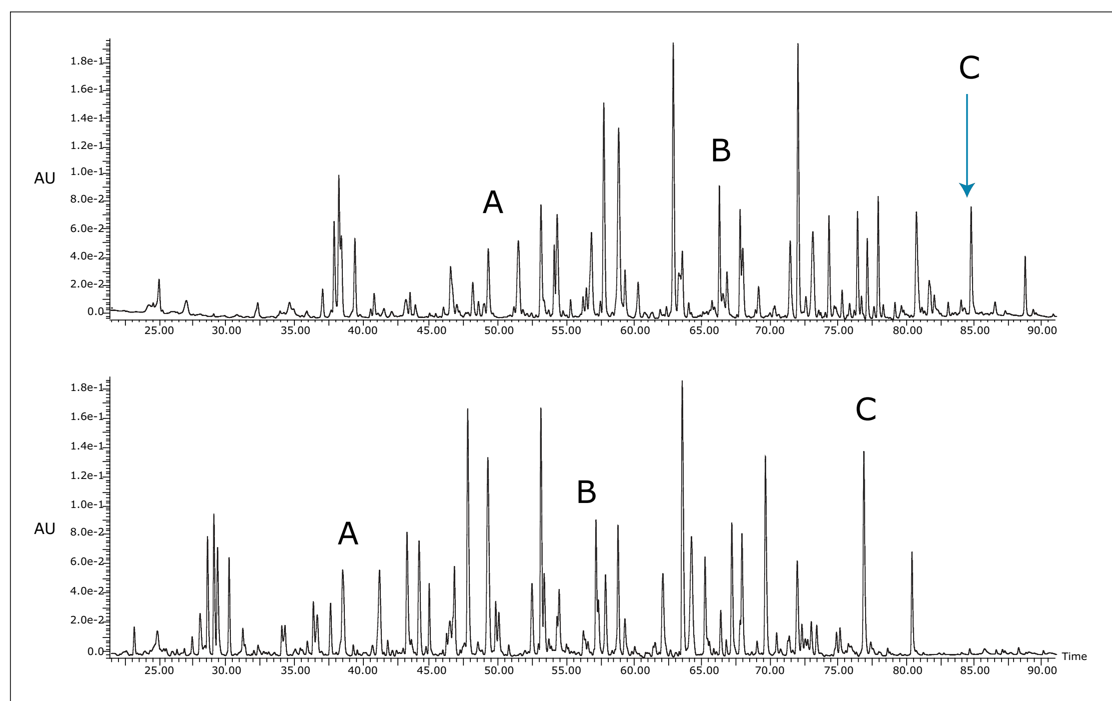

Figure 2. Comparison of the separation of LysC digest of phosphorylase b on BEH 130, 1.7 µm (top) and on BEH 300, 1.7 µm (bottom) Peptide Separation Technology columns.

36 residues with a molecular weight of 4477 Da, also elutes as a symmetrical peak from both columns. We are actively investigating the physical and chemical basis of the differences in chromatography.

Both Peptide Separation Technology columns give useful separations of peptides over a wide range of sizes and chemical properties. The differences in selectivity will prove advantageous in the process of developing an optimized peptide map for a given protein.

MATERIALS AND METHODS

System configuration

- Standard ACQUITY UPLC® System modules, including the Binary Solvent Manager and Sample Manager, were used for the separation.
- A high-sensitivity mixer for peptide analysis (P/N 205000403) was added to the system. Separations were monitored with a TUV detector at 214 nm and with an LCT Premier™ oa-TOF Mass Spectrometer.

Preparation of digests

Phosphorylase b (rabbit) was dissolved in aqueous ammonium bicarbonate (pH 8) to a concentration of 1 mg/mL, and RapiGest™ was added to a concentration of 0.1%. LysC or Trypsin was added to separate aliquots of phosphorylase b solution at an enzyme-to-substrate ratio of 1:50 (w/w), and the samples were incubated overnight at 37 °C. The digestions were terminated by addition of trifluoroacetic acid to a concentration of 0.1%, and stored at <20 °C.

Separation method

The same chromatography method was used for both digests on both columns; that is, for all four experiments.

Injection volume:	10 µL
Columns:	Waters Peptide Separation Technology ACQUITY UPLC BEH 130, 1.7 µm 2.1 x 100 mm ACQUITY UPLC BEH 300, 1.7 µm 2.1 x 100 mm
Temperature:	40° C
Flow rate:	100 µL/min
Solvent A:	0.1% TFA in water
Solvent B:	0.08% TFA in acetonitrile

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Improving LC Peak Shape of Proline-Rich Peptides within an Antibody Peptide Map

Hongwei Xie and Martin Gilar
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Liquid Chromatography (LC) is extensively used in peptide mapping for characterization of therapeutic proteins. Typically, peptide mapping methods employ column temperatures of 40 °C or below. Under such conditions, broad and tailing LC peak shapes are observed for proline-rich peptides. Two tryptic peptides commonly found in the conserved region of monoclonal antibodies (mAb), light chain peptide LT10 (109-TVAAPSVFIFPPSDEQLK-126) and heavy chain peptide HT20 (226-THTCPPCPAPELLGGPSVFLFPPKPK-251), exhibit such undesirable peak profiles.[1]

In this application note, we demonstrate how to improve the LC peak shapes of proline-rich peptides. Improved LC/UV/MS chromatograms of a mAb tryptic digest were achieved by employing elevated column temperature (60 °C) with Waters® Peptide Separation Technology UPLC® C_{18} Columns on an ACQUITY UPLC® System with both TUV and SYNAPT™ MS detection systems.

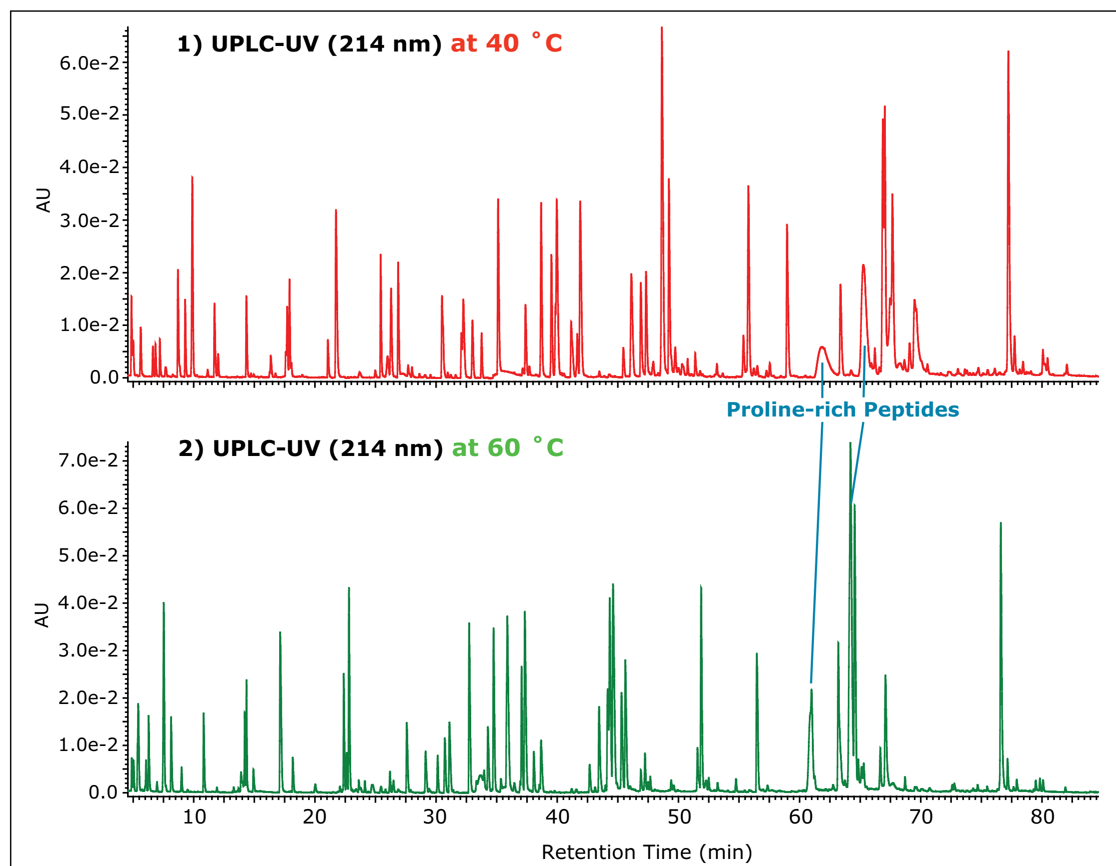

Figure 1. Comparison of UPLC/UV chromatograms of a monoclonal antibody tryptic digest between 1) 40 °C and 2) 60 °C. As expected, peptides elute slightly earlier at 60 °C than at 40 °C and the retention order of some peptides was also changed.

EXPERIMENTAL

Sample preparation procedures were detailed in a previous document.[1] Briefly: the mAb was digested with trypsin, followed by DTT reduction and alkylation with iodoacetamide. The resulting digest was diluted to 1.5 pmol/µL with 95:5 water/acetonitrile containing 0.02% trifluoroacetic acid (TFA) prior to LC analysis.

LC conditions

LC system:	Waters ACQUITY UPLC
Column:	Peptide Separation Technology ACQUITY UPLC® BEH300 C_{18}, 1.7 µm, 2.1 x 150 mm (P/N 186003687)
Temperature:	40 °C or 60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	0.02% TFA in water
Mobile phase B:	0.018 % TFA in acetonitrile
Gradient:	0 to 40% B over 90 min
Detection:	UV (214 nm) and MS^E
Weak needle wash:	100% A, 600 uL
Strong needle wash:	20% A/80% B, 300 µL
Injection volume:	20 µL (30 pmol)

RESULTS AND DISCUSSION

Figure 1 compares the separation (UPLC/UV at 214 nm) of a mAb digest at typical and elevated column temperatures (40 and 60 °C). While excellent resolution of peptides in the digest was achieved at both column temperatures, the 40 °C separation exhibited two broad and tailing peaks between retention times of 60 to 70 min. This is consistent with our previous observations for this sample at 40 °C using 0.1% formic acid as a modifier of mobile phases.[1] By increasing the column temperature from 40 to 60 °C, the shape of these peaks was significantly improved. The components of the two peaks were identified to be proline-rich peptides by MS^E (peptides LT10 and HT20 as shown in Figure 2).

Figure 2 directly compares the LC/MS extracted ion chromatograms (EIC) of peptides LT10 and HT20 under both temperature conditions. As column temperature increased from 40 to 60 °C, the peak widths of these two peptides were reduced by half and peak USP tailing factor reduced from 1.73 to 1.17 for LT10, and 1.45 to 1.2 for HT20, respectively. As expected, the temperature did not affect the peak shapes of other peptides although the retention time of peptides were reduced at higher column temperature

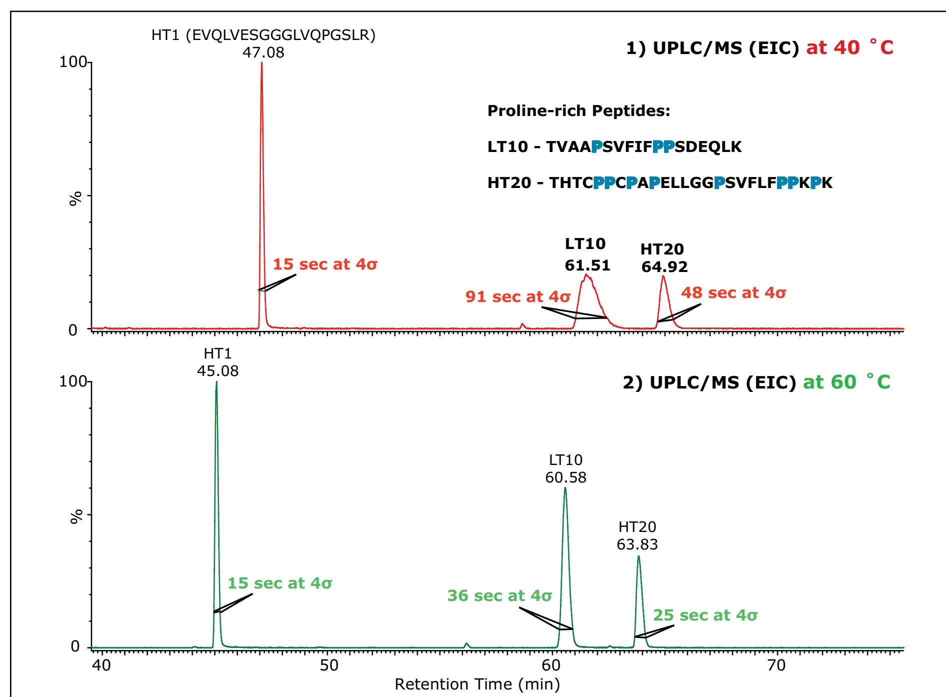

Figure 2. Comparison of LC/MS (EIC) peak shapes of proline-rich peptides between 1) 40 °C and 2) 60 °C. Temperature does not affect peak shapes of not proline-rich peptides, for example, the illustrated peak of heavy chain N-terminal peptide HT1.

(see Figure 2); for example, the illustrated peak of a heavy chain N-terminal peptide HT1 (EVQLVESGGGLVQPGSLR) with same peak width (15 sec) at 4σ and a USP tailing factor of 1.07.

Similar chromatographic behavior in long proline-rich peptides with one or more proline-proline bonds has been reported.[2-5] Griffiths, et al.,[5] reported using high temperature (65 °C) to achieve a symmetrical, quantifiable elution of proline-rich peptide GTEAAGAMFLEAIPMSIPPEVK from a Lys-C digest of recombinant α1-antitrypsin.

At 65 °C, we also obtained sharp and symmetrical peak shape for LT10 and HT20 in the mAb tryptic digest (data not shown). Higher temperature further improves peak shape of these peptides.

CONCLUSIONS

The results presented here demonstrate that elevated temperature (≥ 60 °C) combines with the Waters Peptide Separation Technology UPLC C_{18} Column, used on an ACQUITY UPLC System, to enable sharp, symmetrical peaks for proline-rich peptides from mAb digests. Such improvements to the chromatographic quality of a peptide map will improve the ability to quantify these peaks and interpret these regions of an antibody map.

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Acknowledgements

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ACQUITY UPLC System-to-System Reproducibility for Peptide Mapping

Azita Kaffashan and Thomas E. Wheat
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Recombinant proteins and monoclonal antibodies are developed for therapeutic purposes. Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTMs), and analyze potential impurities. Any difference in structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amounts of the peptide with and without a particular modification are used to measure the fraction of the protein in the particular sample that carries that modification. Changes in area proportions correspond to the fraction of the protein molecules in the sample having a particular modification.

UltraPerformance LC® (UPLC®) shows greater resolution and higher sensitivity for peptide mapping as compared to HPLC. To achieve maximum resolution, all elements of the analysis, including the instrument, column, solvents, and sample must be optimized to work together as a complete system. Using the UPLC® Peptide Analysis Solution, ACQUITY UPLC has been shown to give consistent chromatographic separations and reproducible quantitation for peptide mapping.[1]

When a completely satisfactory peptide map has been developed, it will be used on multiple systems within a department, as well as transferred to another department, laboratory, or CRO. Reproducibility of retention time and relative area must be consistent from one ACQUITY UPLC System to another.

In this application note, we demonstrate reproducibility of the peptide map of a protein digest run on three identical ACQUITY UPLC Systems. Retention time, area, and relative area reproducibility of selected peaks were evaluated.

EXPERIMENTAL

Experimental design

Three identical ACQUITY UPLC Systems (ACQUITY UPLC 1, 2, and 3) were configured according to the instructions found in the UPLC Peptide Analysis Application Solution.[2] Briefly, a core ACQUITY UPLC System, consisting of an ACQUITY UPLC Binary Solvent Manger, ACQUITY UPLC Sample Manager with Column Heater Module, and ACQUITY UPLC TUV Detector, was modified to be compatible with peptide analysis. A high-sensitivity peptide mixer was installed on the pump. The autosampler was equipped with a 20-µL loop, and a 15-µL PEEK/Sil ACQUITY UPLC peptide needle. One Waters® Peptide Separation Technology Column was used on all three systems. A shallow gradient of 0.5%/column volume was selected as typical of peptide mapping gradients. ACQUITY UPLC Systems 1, 2, and 3 were run on days 1, 2, and 3 respectively. Six consecutive runs were completed on each system before moving the column to the next system. The mobile phase was prepared fresh on day 1 and divided among the instruments. Waters MassPREP™ Enolase Digestion Standard was reconstituted with sample buffer to 10 pmol/µL on day 1. Aliquots of 100 µL were frozen in a -80 °C freezer. On day 1, a fresh, unfrozen aliquot was loaded on ACQUITY UPLC System 1. A frozen aliquot was defrosted and loaded on ACQUITY UPLC Systems 2 and 3 just before the start of the injections. Data were processed using Empower™ 2 Software. The peaks in the chromatograms were integrated using the ApexTrack™ integration alogorithm. The first injection of each day was a system blank run.

Materials and methods

Samples:	Waters MassPREP Enolase Digestion Standard (3 vials of 1 nmol tryptic digest of protein, 8 pmol/ µL)
Sample buffer:	0.2% TFA in 95:5 water/acetonitrile (100 µL per vial of digestion standard)
LC system:	Waters ACQUITY UPLC, configured for peptide analysis (Details in experimental design section)

Column:	Waters Peptide Separation Technology ACQUITY UPLC BEH 300 C_{18}, 1.7 µm 2.1 X 100 mm
Flow rate:	200 µL/min
Mobile phase A:	0.020% TFA in water
Mobile phase B:	0.018% TFA in acetonitrile

Gradient:	Time (min)	%A	%B	Curve
	0.0	98	2	NA
	5.0	98	2	6
	206.0	40	60	6
	206.1	10	90	6
	208.1	10	90	6
	208.2	98	2	6
	234.2	98	2	6

Column temp:	40 °C
Injection volume:	8 µL of 10 pmol/µL of reconstituted MassPREP Enolase Digestion Standard
Mode:	Partial Loop
Weak wash:	600 µL of 95:5 H_2O/ACN 0.2% TFA
Strong wash:	200 µL of 20:80 Mobile phase A/mobile phase B
Sample temp:	4 °C
Detection:	Wavelength: 214 nm Sampling rate: 10 pts/sec Filter time constant: Normal

RESULTS AND DISCUSSION

The peptide map of the MassPREP Enolase Digestion Standard is shown in Figure 1. Empower 2 Software using ApexTrack integration was used to integrate all chromatograms. The software-generated integrated chromatogram showed over 300 peaks, of which three were compared in this study. Early-eluting (peak A), middle-eluting (peak B), and later-eluting (peak C) were selected as representative peaks in the chromatogram.

Figure 2 shows the overlay of five consecutive runs of the MassPREP Enolase Digestion Standard for the ACQUITY UPLC System 1 run on day 1. There is no observable shift in retention time that compromises the identification of a peak. For all peaks, retention time reproducibility within a single system is better than 0.3% RSD.

Inter-system reproducibility is shown by the overlay of the chromatograms of the MassPREP Enolase Digestion Standard peptide map from injection 3 on ACQUITY UPLC Systems 1, 2, and 3 run over three days in Figure 3. The peaks detected from the three systems were identified and counted without any manual manipulation. The same number of peaks was found in all chromatograms. Additionally, peaks A, B, and C were correctly identified in all chromatograms.

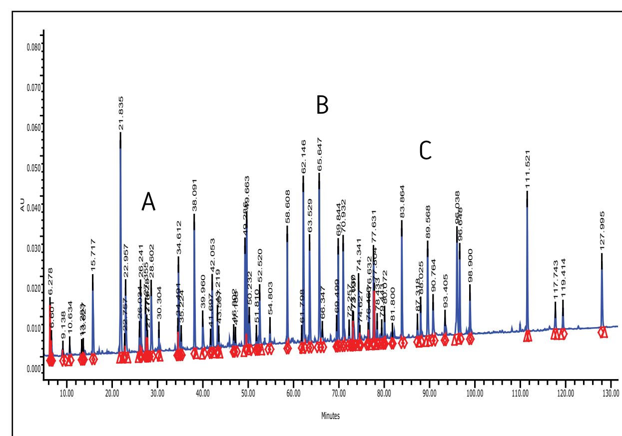

Figure 1. UV chromatogram of the peptide map of MassPREP Enolase Digestion Standard.

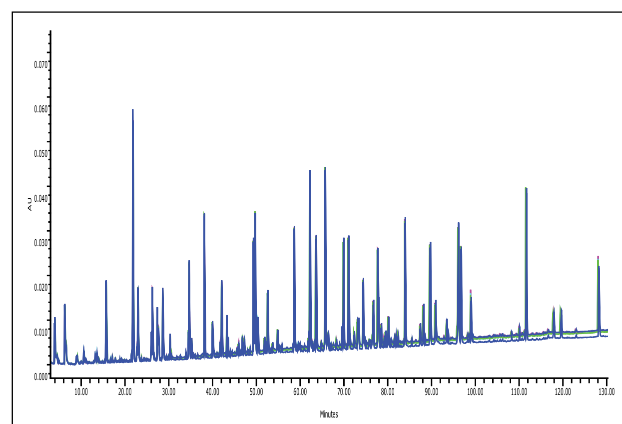

Figure 2. Overlay of five consecutive runs of the MassPREP Enolase Digestion Standard peptide map on ACQUITY UPLC 1.

Table 1 compares the average and standard deviation for retention time for each system, and all of the runs for the three selected representative peaks. As expected, there was more retention time variability in the inter-system runs, compared to runs within a single system. The standard deviation for all of the runs across the three marker peaks was better than 0.20 minutes. As with the runs within a system, there was no shift in retention time for the inter-system runs to compromise the identification of a peak.

The expanded view of a pair of closely resolved peptides that includes peak A is shown in Figure 4. The shape of the peaks and the valley between them is sensitive to all aspects of the separation including flow rate, gradient, and temperature. The consistency of this separation is a measure of the similarity among the three ACQUITY UPLC Systems.

Many factors contribute to judging quantitative behavior. Different peptides have distinctive properties resulting in more or less variability in area. Optimization of the diluents and injection modes will influence the reproducibility of the peak areas. Table 2 compares the average and percent relative standard deviation of the peak area for the three marker peaks using all of the runs from each of the three ACQUITY UPLC Systems. The peak area %RSD for the three peaks within a system is better than 3.3%. The peak area %RSD for all runs is between 5.0% and 6.3%.

For quantitative characterization of a protein sample, the amount of the modified structure is often reported as a percentage of the native structure. Area ratios are a useful measure of the reliability of quantification across three systems. The area ratios shown in Table 3 for the three marker peaks are essentially identical.

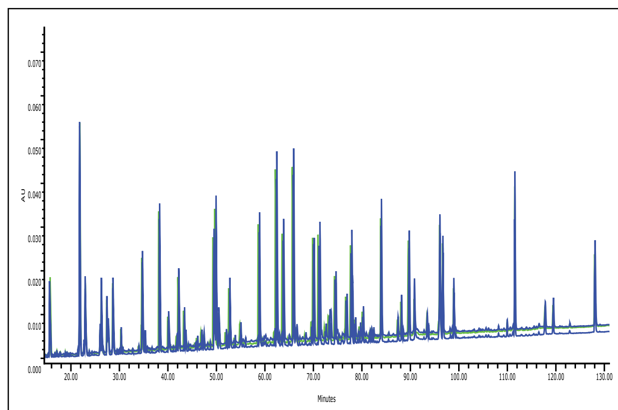

Figure 3. Overlay of injection 3 of the peptide map of the MassPREP Enolase Digestion Standard on ACQUITY UPLC Systems 1, 2, and 3.

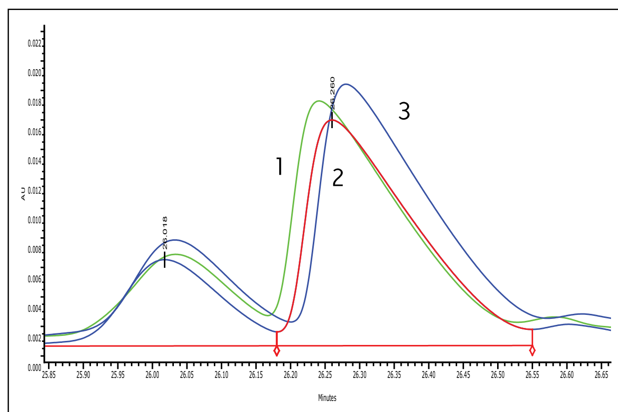

Figure 4. Overlay of injection 3 of peak A on ACQUITY UPLC Systems 1, 2, and 3.

Retention time	Mean	Peak A Standard deviation	Mean	Peak B Standard deviation	Mean	Peak C Standard deviation
ACQUITY UPLC System 1	26.242	0.007	65.686	0.048	87.377	0.068
ACQUITY UPLC System 2	26.284	0.005	66.007	0.120	87.522	0.077
ACQUITY UPLC System 3	26.266	0.006	65.867	0.026	87.474	0.045
All	26.264	0.019	65.900	0.185	87.458	0.088

Table 1. Retention time average and standard deviation of peaks A, B, and C for ACQUITY UPLC 1, 2, 3 and all runs.

Area	Peak A		Peak B		Peak C	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
ACQUITY UPLC System 1	172802	0.799	430452	0.670	40855	2.140
ACQUITY UPLC System 2	187035	0.999	467935	1.284	44650	2.186
ACQUITY UPLC System 3	165696	0.384	415678	0.752	39062	3.267
All	175178	5.294	438021	5.278	41522	6.292

Table 2. Area average and %RSD of peaks A, B, and C for ACQUITY UPLC Systems 1, 2, 3, and all runs.

	ACQUITY UPLC System 1	ACQUITY UPLC System 2	ACQUITY UPLC System 3
Ratio			
A/B	0.401	0.400	0.399
C/A	0.236	0.239	0.236
C/B	0.095	0.095	0.094

Table 3. Area ratio of peaks A, B, and C for ACQUITY UPLC Systems 1, 2, and 3.

CONCLUSION

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTMs), and analyze potential impurities. ACQUITY UPLC peptide mapping provides the high resolution required by these applications. A peptide mapping method can be developed and consistently observed on a single system. With careful attention to detail, the same separation can be transferred to additional ACQUITY UPLC Systems. The quantitative reliability within such a transfer is more than satisfactory for relative quantitation. The total system solution including the instrument, column, and solvents is essential to achieving these results. Protein characterization laboratories can develop fully-defined peptide maps on the ACQUITY UPLC System. The peptide mapping method can be transferred to another department, laboratory, or CRO using the same instrument and column chemistry.

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Xevo G2 QTof: The Flexibility to Treat Large Peptides Like Normal Peptides within an LC/MS Peptide Mapping Experiment

Scott J. Berger, Asish Chakraborty, St. John Skilton, Hilary Major, and Weibin Chen
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

The second-generation Xevo™ G2 QTof is fundamentally designed to extend routine analysis to peptide maps containing peptides of a large mass range.

WATERS SOLUTIONS

Xevo G2 QTof

BiopharmaLynx

KEY WORDS

QuanTof, peptide mapping

INTRODUCTION

The question of when a large peptide is large enough to be considered a small protein is not just philosophical when such peptides are encountered within an LC/MS peptide map. The utility of identifying typical protein digest components by the mass of their ^{12}C (or monoisotopic peak) decreases as the peptide gets larger. At some point, either instrument or physical limitations are encountered, and it becomes more practical to use average peptide mass as an identifier for that peptide. This can complicate data analysis for peptide maps containing digests with wide variations in peptide mass.

If larger peptides can be processed algorithmically like smaller peptides, common data treatment can be applied across peptide mapping data sets, and generate results with uniform standards of component identification and quantification. This uniformity of data processing also facilitates automation of LC/MS data analysis in MassLynx™ Application Managers such as BiopharmaLynx™, which searches peptide mapping data using monoisotopic mass information, and requires at least partial resolution of a peptide's isotopic structure for proper component detection.

It is a fundamental misconception that mass resolution is the only limiting factor for directly obtaining monoisotopic peptide mass results. While sufficient MS resolution is required to detect isotopic features, this capability concurrently requires sufficient dynamic range to detect the monoisotopic peak for the most intense and least intense charge states of that large peptide. This combined resolution and dynamic range challenge grows more acute as peptide size is increased. In particular, three effects dominate:

- The relative abundance of the monoisotopic peak rapidly decreases with increasing peptide mass (see Table 1)
- The dominant charge state(s) (and required MS resolution to detect isotopic features) increases with increasing peptide mass
- The number of charge states (and dynamic range needed to detect them) increases with increasing peptide mass

The second-generation Xevo™ G2 QTof is fundamentally designed to address both aspects of the large peptide detection challenge. Instrument resolution, now routinely greater than 20,000, can clearly resolve isotopic structure for peptides below 10,000 MW, and permits automated processing of higher intensity

data for peptides approaching 15,000 MW. In this secondary mass range, the challenge of dynamic range becomes limiting as the monoisotopic peak decreases from ~1/50th to ~1/700th the relative height of the most abundant isotope in the isotopic envelope for each detected charge state. The QuanTof ™ hybrid ADC technology (enabling greater than 10,000 spectral dynamic range) introduced with the Xevo G2 QTof facilitates superior detection, mass accuracy, and quantitation for lesser abundance charge states than was possible using previous conventional ADC or TDC mass detection technologies.

DISCUSSION

It is the combination of increased MS resolution coupled with the novel hybrid ADC mass detection technology that enables routine LC/MS peptide mapping analysis of digests containing higher MW peptides. This capability enables biotherapeutic characterization studies using enzymes with more limited digest specificities than trypsin (e.g., ArgC, AspN, LysC), intentional under-digestion of proteins (more missed cleavages ~ better coverage of smaller or poorly ionizing digested peptides), and analysis of non-reduced peptide maps (for purposes of disulfide mapping). The extensive bioinformatics capabilities of BiopharmaLynx for automating processing and reporting of such data sets further increases the efficiency and uniformity for processing these more exotic peptide mapping workflows.

The largest peptide encountered in most antibody tryptic peptide maps is the T15 peptide that is derived from the constant region of the antibody heavy chain (DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TQTYICNVNHKPSNTK). In most peptide maps, it is detected in a modified form (containing achemically alkylated cysteine), as one of the later eluting peaks, and a resulting monoisotpic mass of 6712.3071 Da. Figure 1 shows the summed spectrum for the HC T15 peptide from the LCMS peptide map of a commercialized biotherapeutic monoclonal antibody. These data were acquired on a Xevo G2 QTof operating in positive electrospray ionization mode with resolution mode detection settings.

In the positive electrospray ionization mode, the peptide is detected in multiple charge states (4^+ to 7^+). The isotopic distributions for the most intense 5^+ charge state and a minor 4^+ charge state are depicted in an expanded form (Figure 1, left and right inset spectra, respectively). Although the dominant charge state is ~20-fold more intense than the minor charge state (relative peak heights), consistent isotopic resolution and isotopic envelope character are maintained when comparing the two spectra. The quantitative consistency within each isotopic distribution can be simply demonstrated using the ratio of most intense isoptopic peak to monoisotopic peak height. These consistent values, 8.9 (5^+) and 8.8 (4^+), are expected for beam instruments such as a QTof, but are not seen with ion trap based instruments, where large peptide ion populations are biased by the Automatic Gain Control (or AGC) functionality used to control space charge effects. This quantitative isotopic fidelity across dynamic range permits the quality of an isotopic distribution to be used in addition to the raw accurate mass information during processing of peptide mapping data.

The larger issues of dynamic range can be explored by first noting that the peak height for the 4^+ monoisopic peak is ~175-fold less intense than the most abundant isotope of the 5^+ peak. Furthermore, the baseline noise level in the 4^+ spectrum indirectly demonstrates that the peptide itself does not ionize particularly well compared to others in the map. In fact, the best ionizing peptide in the map had an instrument response ~8-fold higher than this T15 peptide. So even in this relatively simple case of a 6700 MW peptide that ionizes reasonably well, detection of isotopic features over a dynamic range greater than 1400 was required for effective identification and quantitation of the unmodified HC T15 peptide.

Peptide Mass	Monoisotopic peak (% of total)[1]	Relative Monosiotopic Peak Height[2]	Isotopic Envelope Dynamic Range[3]
2,000	31.400	93.870	1.1
5,000	5.700	26.230	3.8
10,000	0.300	2.100	47.6
15,000	0.018	0.140	714.2
20,000	0.001	0.010	10,000

Table 1. The relative abundance of the monoisotopic peak rapidly decreases with increasing peptide mass.

[1] Percentage of peak area for the monoisotopic peak relative to the entire isotopic envelope of a given charge state.

[2] Relative peak height of monoisotopic peak to most abundant isotopic peak in the isotopic envelope for a given charge state.

[3] Ratio of peak heights for monoisotopic peak to most abundant isotopic peak in the isotopic envelope for a given charge state.

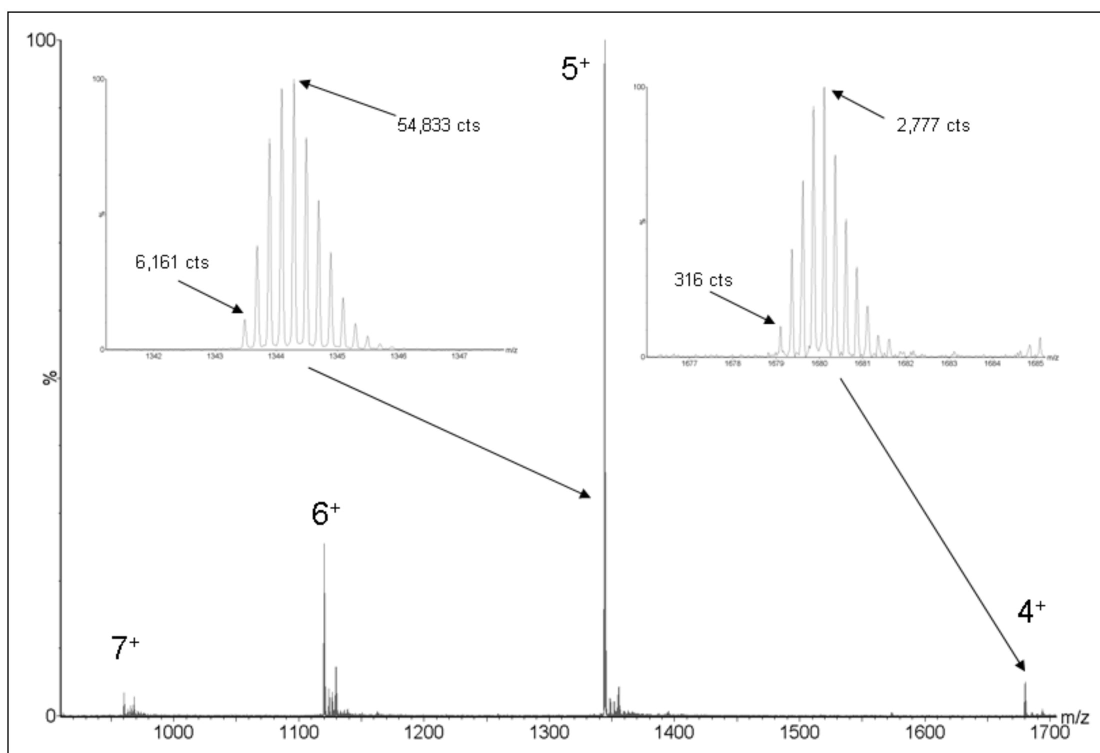

Figure 1. Ratio of monoisotopic to most abundant isotopic peak is ~ 1:9 for both charge states.

CONCLUSION

The detection of poorer ionizing peptides, and lower level modified variants of the T15 peptide demonstrate the true dynamic range challenges encountered during routine LC/MS analysis of digests containing larger peptides. While this typical antibody peptide mapping example is shown to be well within the capabilities of the Xevo G2 QTof, the data contained within Table 1 clearly show the utility and necessity of this latest generation Xevo instrument for extending routine analysis to peptide maps containing peptides of even greater mass range.

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

[APPLICATION NOTE]

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Maximizing Chromatographic Resolution of Peptide Maps using UPLC with Tandem Columns

Hongwei Xie, Martin Gilar, and Jeff Mazzeo
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

The ACQUITY UPLC System, configured with a tandem of 2.1 x 150 mm columns, improves resolution of peptide maps and reduces coelution. This improved resolution is particularly useful for identification and quantification of protein heterogeneities with UPLC-UV.

WATERS SOLUTIONS

ACQUITY UPLC System

Peptide Separation Technology Columns

SYNAPT MS System

KEY WORDS

Peptide mapping, peptide coelution, mapping of mAb tryptic digests, reproducibility

INTRODUCTION

LC-based peptide mapping is extensively employed for protein characterization, from early biotherapeutics development, to quality control of production and monitoring stability and activity after long-term storage. The use of this technology to identify and quantify low levels of impurity proteins, sequence variants, and post-translational modifications is critical for the assessment of protein drugs.

However, due to the complexity of protein enzymatic (often tryptic) digests, peptide coelution and poorly resolved chromatographic peaks are quite common in peptide mapping. In addition to recent advances in MS and particularly MS^E,[1] a good LC assay removes issues of ion suppression and isobaric interferences that may affect sensitive identification and accurate quantification of heterogeneities. Furthermore, a high-resolution LC assay can be more readily transferred for quality control purposes when utilizing UV detectors.

The use of sub-2-µm particles allows UPLC® to push the limits of both peak capacity (higher efficiency) and sensitivity (sharper peaks). With UPLC, speed of analysis is also improved due to higher linear velocities.[2] These features make UPLC-based
peptide mapping powerful and attractive. Currently, the dimensions of 1.7-µm Peptide Separation Technology BEH C_{18} columns used with an ACQUITY UPLC® System include 2.1 x 50 mm, 2.1 x 100 mm, and 2.1 x 150 mm. The pressure generated by these columns is far below the pressure limit (15,000 PSI) of the ACQUITY UPLC System at typical operating conditions (flow rate 0.2 mL/min, column temperature 20 to 65 °C), which leaves room for achieving further separation if longer columns are properly configured.

In this application note, we demonstrate how to maximize ACQUITY UPLC separation power by using a tandem of two 2.1 x 150 mm columns. The resolution of an IgG1 tryptic digest was compared between the tandem column configuration and single columns, with online detection by both a TUV detector and a SYNAPT™ MS system with MS^E detection mode. The UPLC-UV/MS^E reproducibility of the new configuration was evaluated. BiopharmaLynx™ 1.2 Software was used for identification of the eluted peptides.

EXPERIMENTAL

LC conditions

LC System:	ACQUITY UPLC with a standard peptide mapping mixer (425 µL)
Column:	Peptide Separation Technology (PST) BEH300 C_{18}, 1.7 µm, 2.1 x 100 mm, 2.1 x 150 mm, or a tandem of two 2.1 x 150 mm columns
Column temp.:	65 °C
Flow rate:	200 µL/min
Sample Injected:	10 µL (100 pmole)
Buffer A:	0.02% TFA in water
Buffer B:	0.018% TFA in ACN
Gradient:	A linear gradient of 1-40% B was scaled with column lengths (100 mm, 150 mm, and 300 mm by coupling two 150 mm columns) for run times of 60, 90 and 180 min, respectively.
Detection:	TUV (214 nm) and MS^E

MS conditions

The SYNAPT MS System and MS^E methods setup and operating conditions were the same as in previous descriptions.[5-6]

Data management

BiopharmaLynx 1.2 MassLynx Application Manager[3,7]

Materials and reagents

Waters MassPREP™ Enolase tryptic digestion standard was used. The IgG1 Antibody digest was prepared from a commercially available monoclonal antibody (mAb) by a RapiGest-assisted 4-h trypsin digestion protocol.[3-4]

RESULTS AND DISCUSSION

A tandem of two 2.1 x 150 mm columns were coupled by capillary metal tubing and installed in the ACQUITY UPLC Column Manager. We first tested the system pressure generated by the configuration and the chromatographic reproducibility. At flow rate 200 µL/min and column temperature 65 °C, the system pressure was ≤7500 psi, far below the pressure limits of both the ACQUITY UPLC System (15,000 psi) and the 425 µL standard peptide mapping mixer (10,000 psi). We also tested the system pressure at different column temperatures, and demonstrated that the configuration with a tandem of two columns was feasible as a working system if the column temperature set above 40 °C.

Excellent chromatographic repeatability was observed (Figure 1) in six continuous runs of MassPREP Enolase tryptic digestion standard using a gradient of 1-40% buffer B in 180 minutes. The retention time (RT) fluctuation from injection to injection is within 4 seconds for each eluted peak in the chromatograms. For example, the RT fluctuation observed for the lately eluted enolase tryptic peptide T37 (YPIVSIEDPFAEDDWEAWSHFFK, MW 2827.3 Da, RT around 150.19 min) is 3.3 seconds. The average relative standard deviation (RSD, calculated by standard deviation SD / average RT * 100%) is 0.017%. This proved the configuration did not affect the good reproducibility of the ACCQUITY UPLC System, as has been demonstrated in previous work studying ACCQUITY UPLC system-to-system reproducibility for peptide mapping.[8]

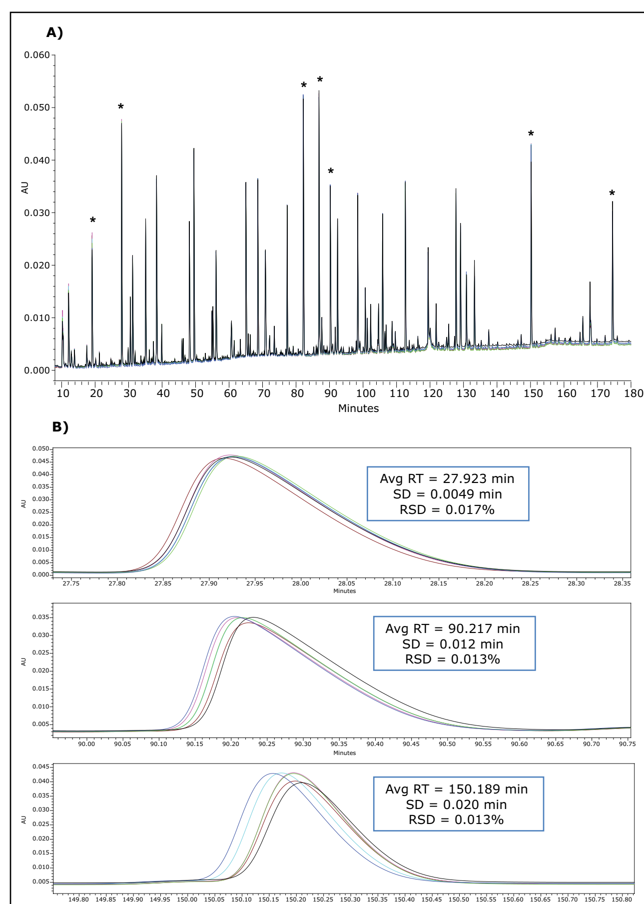

*Figure 1. Reproducibility of six continuous injections of Waters MassPREP Enolase tryptic digest. A) UPLC-UV chromatography overlay. The retention time fluctuation measured by average RSD is 0.017% (calculated by the seven peaks marked in *); B) A zoom view of retention time fluctuation of 3 selected peaks, which represent an elution at early, middle, and late gradients, respectively.*

In order to evaluate the performance of the new configuration, we next compared the peptide maps of the IgG1 tryptic digest obtained between the tandem of two 2.1 x 150 mm columns and a single 2.1 x 100 or 2.1 x 150 mm column. The comparison was performed at same gradient slope by using a linear gradient of 1-40% B in run times (60, 90, and 180 min, respectively) scaled with column lengths (100 mm, 150 mm, and 300 mm of the tandem of two 150 mm columns). A good scalability and an apparently better separation were observed for the tandem columns (Figure 2). It clearly shows that poor or non-chromatographically-resolved peaks by single columns are separated by the tandem columns, e.g., those peaks in the region marked in shadow, due to significantly improved peak capacity. The calculated peak capacities for the 100 mm, 150 mm, and the tandem of two 150 mm columns in this peptide mapping experiment are about 420, 500, and 700, respectively.

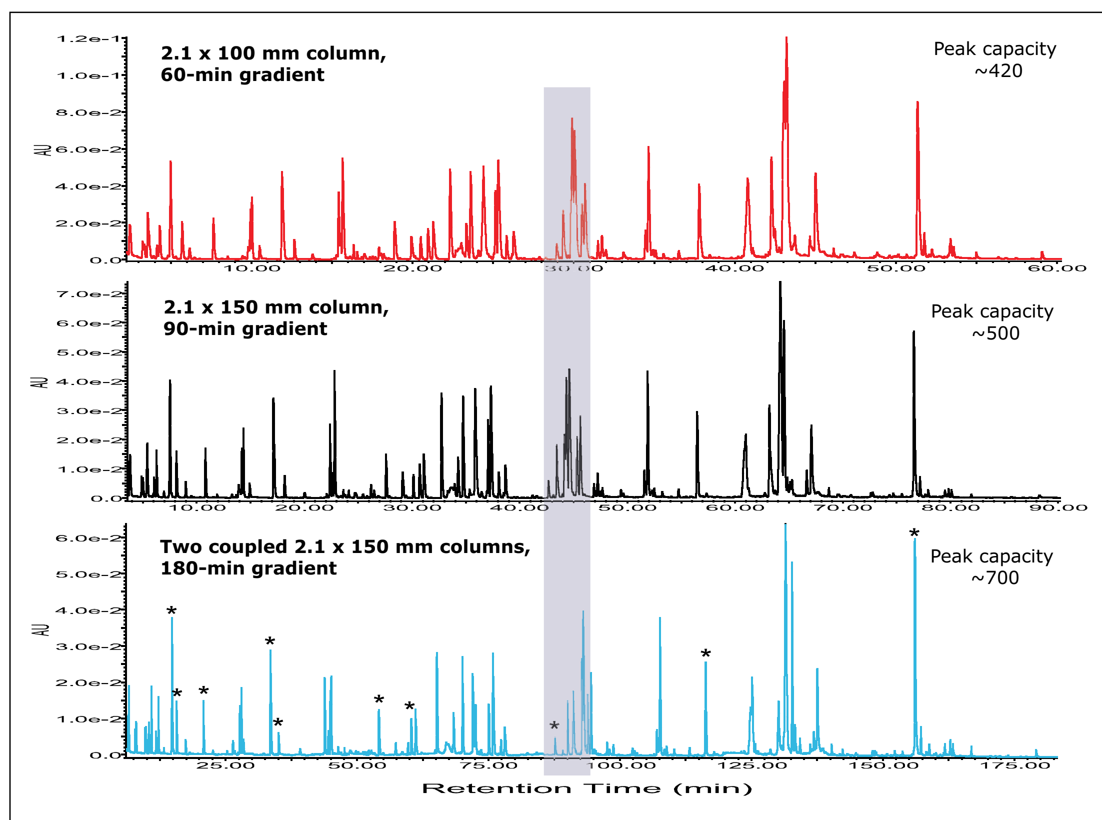

*Figure 2. Comparison of UPLC-UV (214 nm) maps of the IgG1 tryptic digest obtained at different column configurations with same gradient slope (see text). From top to bottom: 2.1 x 100 mm, 2.1 x 150 mm and two coupled 2.1 x 150 mm columns. The calculated peak capacity using 10 selected peaks marked with * is illustrated in each configuration.*

Figures 3 and 4 further demonstrate the improved resolution of the tandem column configuration compared to the single columns by a zoom view of two detailed separation examples. The heavy chain tryptic peptides HT6 (IYDTNGYTR) and HT11* (AEDTAVYYC*SR, C* - carbamidomethylated C) were completely coeluted in both maps obtained by the single 2.1 x 100 mm or 2.1 x 150 mm column as shown in Figure 3, but they were partially (in half-height) separated by the tandem columns. Meanwhile, Figure 3 also shows that N-deamidated HT6* and light chain tryptic peptide LT14 (VDNALQSGNSDESVTEQDSK) achieved baseline resolution by the tandem columns, which was not the case for the single columns, particularly, for the single 2.1 x 100 mm column. Similarly, as shown in Figure 4, the unmodified heavy chain tryptic peptide HT37 (GFYPSDIAVEWESNGQPENNYK, peak 1 in Figure 4) and its deamidated products (peaks 2, 4, 5, and 6, see Figure 5 for details), which were only partially resolved by single columns (particularly for the 2.1 x 100 mm column), were baseline resolved by the tandem columns.

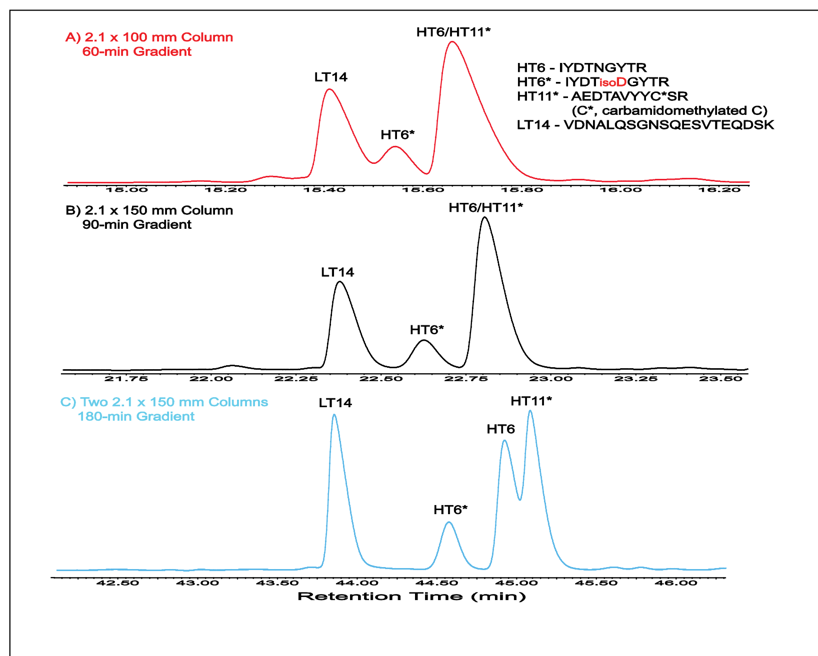

Figure 3. A zoom view for the separation of 4 peptides (LT14, HT6, HT6 and HT11*, see details in the figure illustration) achieved at different column configurations. A) 2.1 x 100 mm; B) 2.1 x 150 mm; C) two coupled 2.1 x 150 mm columns.*

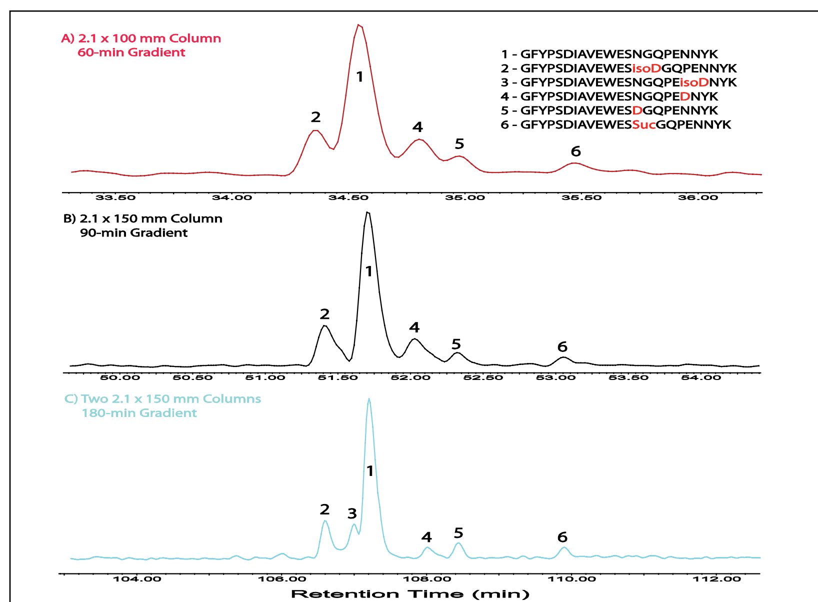

Figure 4. A zoom view for the separation of HT37 and its N-deamidated products (see details in the figure illustration) achieved at different column configurations. A) 2.1 x 100 mm; B) 2.1 x 150 mm; C) two coupled 2.1 x 150 mm columns.

More importantly, the tandem columns separated the deamidated product of peak 3, which was completely coeluted with peak 2 or peak 1 in the maps obtained by single columns. HT37 is also called "PENNY" peptide. It receives particular attention because it tends to be easily deamidated during production and storage, and potentially affects drug quality. The complexity of its deamidation products makes it an analytical challenge. Here, we demonstrated the ACQUITY UPLC System with tandem columns is capable of completely separating modified and unmodified components of "PENNY" peptide.

Mapping of mAb tryptic digests is a typical biopharmaceutical application. By comparing the maps of the mAb tryptic digest obtained at different column lengths, the difficulty of characterizing the structure and modifications of mAbs is shown. Due to the complexity of the resulting digests and the chromatographic challenge of peptide mapping, good chromatographic is paramount. For such applications, the ACQUITY UPLC System configured with a tandem of two 2.1 x 150 columns shows advantages over single (2.1 x 100 mm or 2.1 x 150 mm) column configurations due to the improved resolution and capability to separate unmodified and modified peptides such as N-deamidated peptides.

Meanwhile, using this tandem column configuration, the sequence coverage of the mAb monitored by BiopharmaLynx 1.2 was 97.2% for the light chain and 98.2% for the heavy chain for each of six continuous injections, demonstrating high sequence coverage and excellent reproducibility.

CONCLUSIONS

Coelution is a serious challenge for peptide mapping applications. In this work, we demonstrated that the ACQUITY UPLC System configured with a tandem of 2.1 x 150 mm columns is capable of improving resolution of peptide maps and reducing coelution. This improved resolution is particularly useful for identification and quantification of protein heterogeneities with UPLC-UV. This tandem column configuration was implemented on the ACQUITY UPLC System (binary solvent management) with a column temperature ≥ 40 °C and did not require any additional equipments or parts. Furthermore, this instrumental configuration is reproducible and easy to use.

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Maximizing Resolution in Peptide Mapping Analysis using UPLC with a 300-mm Column

Hongwei Xie and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

This work demonstrates that ACQUITY UPLC® separation power can be greatly enhanced by using columns with an increased column length of 2.1 x 300 mm.

WATERS SOLUTIONS

ACQUITY UPLC System

Peptide Separation Technology UPLC Columns

KEY WORDS

BEH chemistry, pressure limits, peptides, long columns

INTRODUCTION

Reverse-phase liquid chromatography is extensively used for the characterization and quality control of protein products. However, peptide coelution and incompletely resolved chromatographic peaks are frequently observed in peptide mapping analysis due to the complex nature of protein digests. Poor resolution can hinder the detection and accurate quantification of critical components that are important to the quality attributes of protein therapeutics, especially when only ultra violet (UV) detection is employed.

Although improved separation resolution can be achieved by optimizing LC operating parameters, this approach is time consuming, and targeted resolution is not necessarily guaranteed for complex peptide digests such as monoclonal antibody (mAb) tryptic digests. Thus, a column with higher peak capacity can be a more efficient alternative under those circumstances.

The use of sub-2-µm particles allows an ACQUITY UPLC System to produce higher peak capacity (higher efficiency) and better sensitivity (sharper peaks). With UPLC,® the speed of analysis can also be improved due to the higher linear velocity. These features make a UPLC-based peptide mapping approach powerful and attractive.

Currently, the dimensions of Waters Peptide Separation Technology BEH-based 1.7-µm column offerings include 2.1 x 50 mm, 2.1 x 100 mm, and 2.1 x 150 mm. The pressure generated by these column is far below the pressure limit (15,000 PSI) of the ACQUITY UPLC System at a typical operating conditions (flow rate 0.2 mL/min, column temperature 20 to 65 °C), which leaves room for achieving further separation if longer columns are employed.

RESULTS AND DISCUSSION

Here we demonstrate the ability to enhance UPLC separation power with an ACQUITY UPLC BEH130 C_{18} 1.7-µm, 2.1 x 300 mm column (P /N 186005792). The resolution of a protein digest was compared based on the separation achieved using columns with the same column packing materials but different column lengths, 2.1 x 150 mm vs 2.1 x 300 mm. All chromatography was performed with the same sample, eluents, and method on an ACQUITY UPLC System with online detection by both a TUV detector and a SYNAPT® MS System with MS^E detection mode.

The performance of the newly developed 300-mm column was evaluated using Waters MassPREP™ enolase tryptic digest (P/N 186002325) based on the peak capacity achieved in a typical condition for peptide mapping analysis.

The ACQUITY UPLC System pressure generated by the 300-mm column was first investigated. At a flow rate of 200 µL/min and column temperature 40 °C, the maximum system back pressure generated by the tandem columns was ~10,000 PSI, well below the system's pressure limit (15,000 PSI). We also tested the system pressure at different column temperatures, and demonstrated that the configuration with a 300-mm column was feasible as a working system if the column temperature set ≥ 40 °C.

Examples of selected peptide maps were examined to illustrate the resolution obtained with the two columns at the same gradient slope. Improved peptide component resolution with a reduction in coelution was observed with use of the 300-mm column. This improved resolution is particularly useful for identification and quantification of protein heterogeneities with UPLC-UV.

The data were acquired at the same gradient slope using a linear gradient of 5 to 45% B in run times of 90 and 180 min respectively to match with column volumes. A good scalability and an apparently better separation were observed for the 300-mm column (Figure 1). The calculated peak capacities at 4σ for the 300-mm and the 150-mm column in this peptide mapping experiment are about 700 and 500, respectively. Peak capacity (P) was calculated and averaged from six repeated analyses.

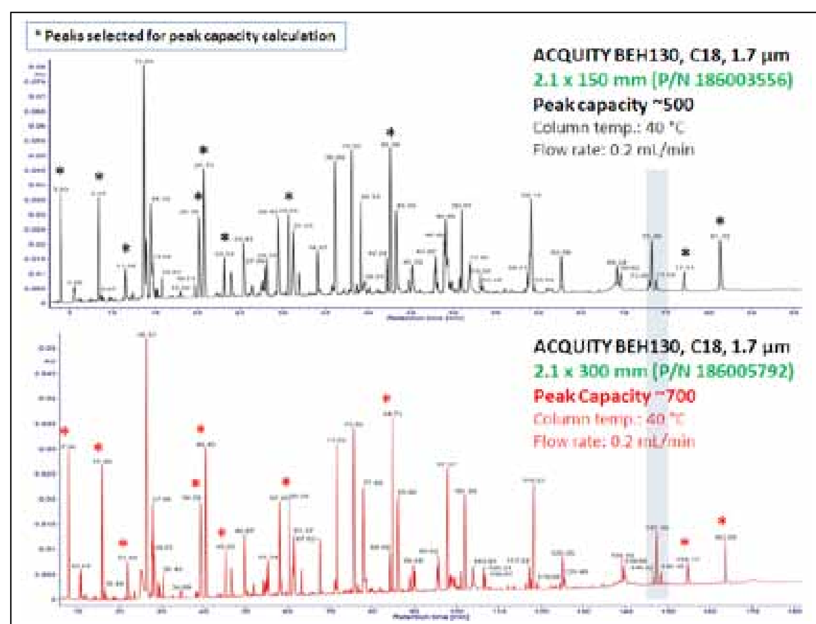

Figure 1. Comparing peptide maps of tryptic digest using 2.1 x 150 mm (top) and 2.1 x 300 mm (bottom) columns. Good scalability and an apparently better separation were observed with the 300-mm column. Ten peaks, marked with an asterisk, were chosen for the peak capacity calculation.

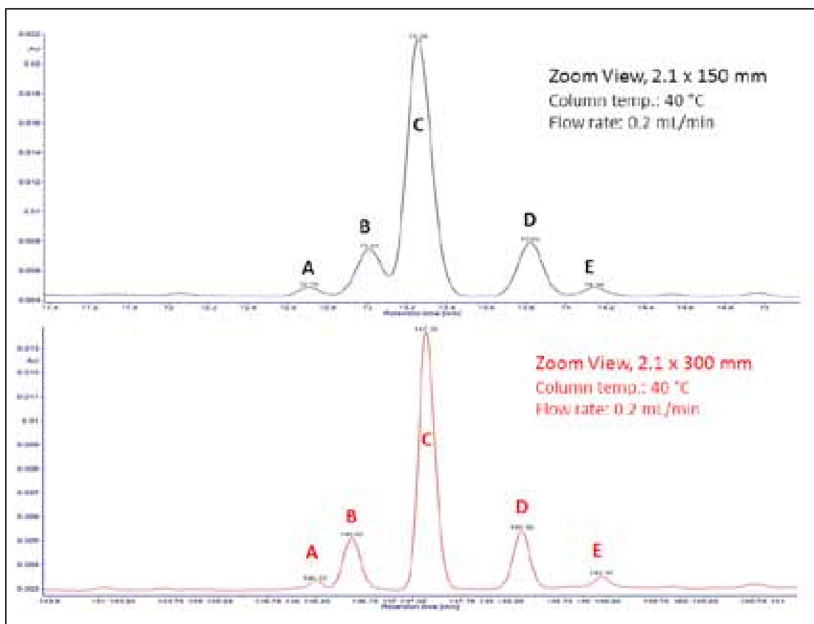

Figure 2. Zooming in on the highlighted area of Figure 1 shows a clear and direct comparison of the peak shape and peak width for the two columns. Peaks that were not chromatographically resolved by the 150-mm column are now completely separated by the 300-mm column due to significantly improved peak capacity.

Ten peaks (marked with asterisk in Figure 1) were chosen for the peak capacity calculation. To make a clear and direct comparison for the peak shape and peak width for the two columns, peaks in the region highlighted in shadow is shown in Figure 2 from the two columns. It clearly shows that non-chromatographically resolved peaks by the 150-mm column are now completely separated by the 300-mm column, due to significantly improved peak capacity.

CONCLUSION

In a comparison of the peptide mapping performance of an ACQUITY UPLC BEH130 C_{18} 1.7-µm column in lengths of 2.1 x 300 mm and 2.1 x 150 mm, we found that the use of a 300-mm column results in improved chromatographic resolution and greater peak capacity. The enhanced performance is particularly useful for the identification and quantification of protein heterogeneities when a peptide mapping experiment is based on an LC-UV method.

The longer column configuration maintains the excellent gradient separation reproducibility that an ACQUITY UPLC System can achieve and operates well below the system's maximum 15,000 pressure limit under the detailed conditions. Use of the 300-mm column can reduce method development time for some challenging separation problems and increase the productivity and efficiency of the workflow.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
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[TECHNOLOGY BRIEF]

Efficient Development of Peptide Maps using the Multi-Solvent Blending Capability of the ACQUITY UPLC H-Class System

GOAL

The ACQUITY UPLC® H-Class System is used for the systematic optimization of peptide mapping conditions in a fully automated protocol using four-solvent blending.

BACKGROUND

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTM), and analyze impurities. Any difference in structure of a protein is reflected in a change in retention time for the peptide containing the modification.

Complete structural characterization of a protein requires resolution of 100 or more peptides that span a wide range of size and molecular properties. The experiments to develop the required separation conditions include changes in the mobile phase modifier and organic solvent composition. Each condition tested requires the preparation of a pair of mixed solvents involving two or three exact measurements.

With the four-solvent blending of the ACQUITY UPLC H-Class System, these solvent mixtures are generated by the instrument from bottles of pure solvent. In this way the labor of preparing many solvent mixtures is reduced, and the possibility of error is minimized. Intermediate compositions can also be easily tested.

The ACQUITY UPLC H-Class System simplifies the development of reliable peptide maps that can be used to characterize the structure of a protein.

The ACQUITY UPLC H-Class System and its four-solvent blending capability enables the user to focus on the quality of the analysis so that reliable results can be obtained efficiently.

THE SOLUTION

MassPREP™ Enolase Digestion Standards were separated on a Peptide Separation Technology ACQUITY UPLC BEH 300 C18 Column with various mobile phase combinations. The ACQUITY UPLC H-Class System was configured with water as Solvent A, acetonitrile as Solvent B, isopropanol as Solvent C, and 1% TFA in water as Solvent D. To assess the effect of acid concentration, three identical gradients of increasing acetonitrile were run with 2.5%, 5%, and 10% D, respectively, corresponding to 0.025%, 0.05%, and 0.1% TFA.

The chromatograms in Figure 1 show significant changes in selectivity as a function of acid concentration in the mobile phase. The experiment can be extended by repeating the same experiments but using a gradient of increasing isopropanol. This is conveniently performed by running the gradient from Solvent A (water) to Solvent C (isopropanol) with the same constant percentage of D as in the previous experiments. The change from acetonitrile to isopropanol reduces retention while also giving substantial changes in selectivity.

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This set of experiments evaluates six different mobile phase formulations to identify the optimum peptide map. The changes in selectivity can be used to obtain the best resolution of the critical peptides in the mixture. To perform these experiments with a binary system or with a switching valve, the scientist would need to prepare 12 bottles of mobile phase with at least 24 volume measurements. With the ACQUITY UPLC H-Class System, only one mixture was used, with two measured volumes, in conjunction with three bottles of pure solvents (Figure 2). The ACQUITY UPLC H-Class System makes it possible to reduce the amount of work, time, and potential for error in reaching the optimum separation conditions.

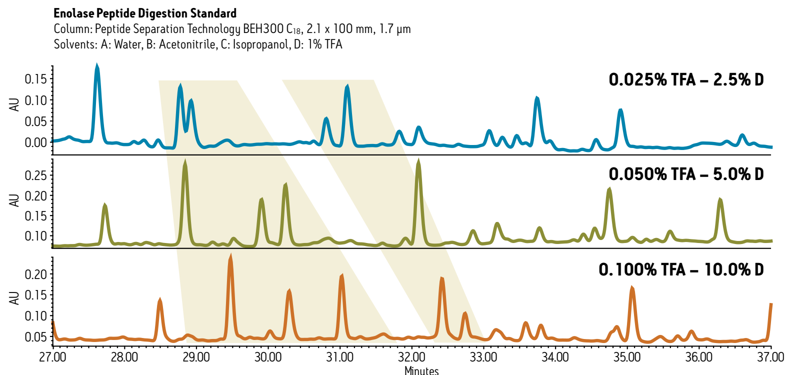

Figure 1. Solvent composition is easily adjusted using the Auto•Blend™ functions of the ACQUITY UPLC H-Class System. In this example, the optimum concentration of TFA for a peptide map is identified simply by varying the percentage of flow taken from the D line. There is no need to make extra bottles of solvent, and intermediate values can be tested with minimal effort.

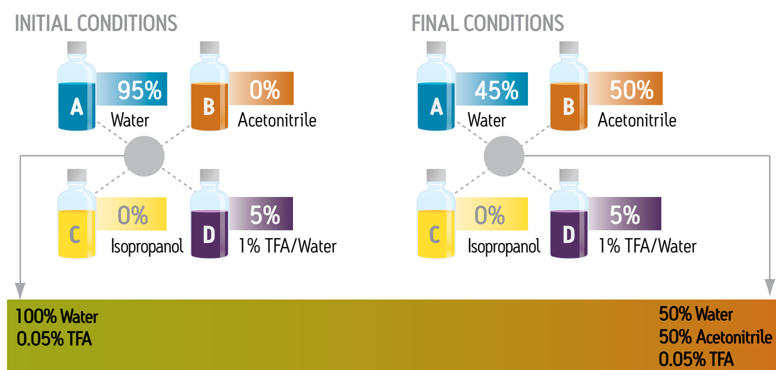

Figure 2. Solvent mixtures are generated from bottles of pure solvent by the ACQUITY UPLC H-Class System's Auto•Blend functionality.

SUMMARY

Biochemists can use the ACQUITY UPLC H-Class System to efficiently develop peptide maps for protein characterization. The system's easy-to-use four-solvent blending capability enables the user to focus on the quality of the analysis so that complete, reliable separations can be developed efficiently. The ACQUITY UPLC H-Class System combines UPLC separation principles with flexible instrument operation to provide the best possible results for bioseparations.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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[TECHNOLOGY BRIEF]

Reliability of Peptide Mapping using the ACQUITY UPLC H-Class System

GOAL

To test the analytical reproducibility of the ACQUITY UPLC® H-Class System in applications that require long shallow gradients to resolve complex mixtures, such as peptide maps.

The ACQUITY UPLC H-Class System provides reproducible and accurate control of peptide mapping separations over extended series of runs.

BACKGROUND

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTM), and analyze potential impurities. Any difference in the structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amount of the peptide with and without a particular modification is used to measure the fraction of the protein in the particular sample that carries that modification. Changes in area proportions correspond to the fraction of the protein molecules in the sample having a particular modification.

To meet these application requirements, long, shallow gradients are required. In the past, such separation conditions have been regarded as challenging for single-pump gradient systems. The ACQUITY UPLC H-Class System was tested with a typical peptide mapping protocol.

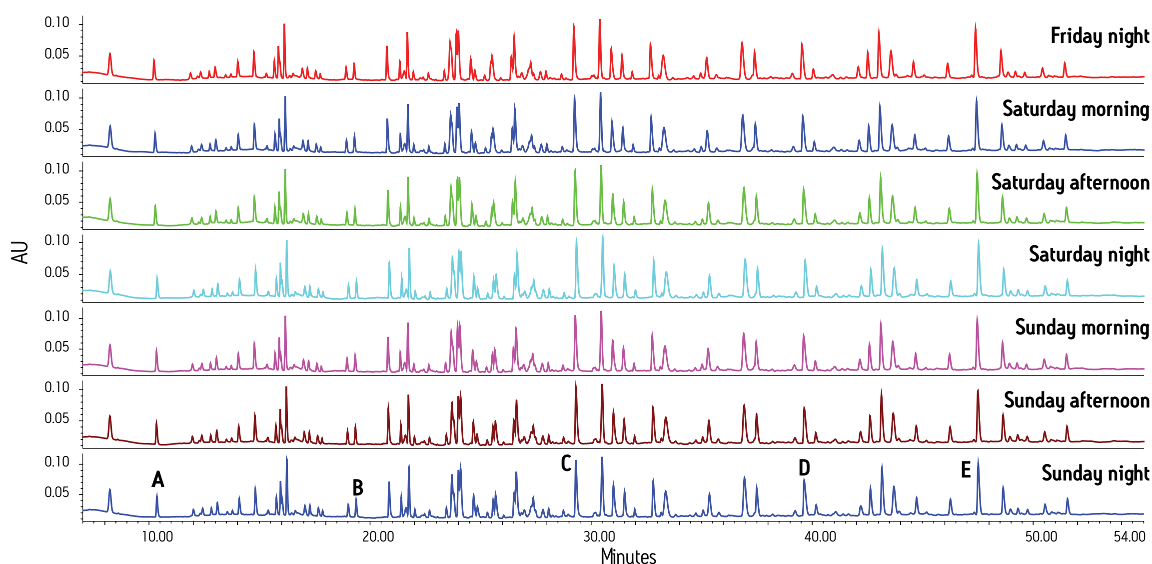

Figure 1. The peptide mapping sample list started on Friday and ran automatically through the weekend. The data was ready to review upon return to work on Monday. Every third separation is shown, demonstrating both excellent reproducibility, resolution, and retention. The five labeled peaks, A to E, were chosen as representative for quantitative analysis, as summarized in Table 1.

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THE SOLUTION

The ACQUITY UPLC H-Class System consisted of the Quaternary Solvent Manager (QSM), Flow-Through-Needle Sample Manager (SM-FTN), Column Heater, and Photodiode Array (PDA) Detector. The optional 250 µL mixer was installed. The MassPREP™ BSA Digestion Standard was separated on a Peptide Separation Technology ACQUITY UPLC BEH 300 C_{18} Column. A shallow gradient of 1.0% per column volume, about 0.6% per minute, was selected as typical of peptide mapping gradients.

The protocol takes advantage of the Auto•Blend capabilities of the instrument. Reservoirs of pure solvents and stocks of concentrated modifiers are used in place of binary, pre-formatted solvent. In this example, a gradient is formed between pure water and pure acetonitrile in lines A and B, while a fraction of the flow is drawn from reservoir D that contains 1% TFA in water. During the gradient, the percentage from line D decreases from 5% to 4%, corresponding to 0.05% to 0.04% TFA, to minimize baseline drift.

The peptide maps are shown in Figure 1 with both retention time and peak area statistics summarized in Table 1. Relative retention and resolution remain constant over this long series of runs. The retention times are sufficiently reproducible as to ensure that peaks will always be correctly identified. Peak area ratios were calculated comparing peaks B, C, D, and E to peak A. The consistency of these area ratios (Table 1) meets the requirements for estimating proportion of modified protein in a set of samples.

	Retention Reproducibility	Relative Quantitation
A	10.311 ± 0.093	1
B	19.331 ± 0.064	0.727 ± 0.008
C	29.284 ± 0.064	3.621 ± 0.014
D	39.614 ± 0.073	2.801 ± 0.009
E	47.470 ± 0.086	3.504 ± 0.020

Table 1. A summary of peak retention times and area ratios. Representative peaks, evenly spaced throughout the separation at 10-minute intervals, were selected for quantitative comparison.

SUMMARY

For meaningful peptide mapping, both quantitative and qualitative reproducibility are required. The ACQUITY UPLC H-Class System provides precise control of peptide mapping separations over large sample sets, so that the analyst can be confident that any deviation in retention time indicates a change in sample composition rather than instrument variability. The observed separations meet this objective while taking advantage of the multi-solvent blending capability. The system has been designed to ensure that both qualitative and quantitative results meet the requirements of modern analytical biochemistry.

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[TECHNOLOGY BRIEF]

Enhanced In-Spectrum Dynamic Range of the Xevo G2 QTof: Improving Peptide Quantitation and Identification in LC/MS Peptide Map Analysis

GOAL

Demonstrate enhanced in-spectrum dynamic range and accurate mass measurements that are achievable using the Xevo™ G2 QTof for peptides spanning four orders of magnitude in concentration during routine UPLC®/MS peptide mapping analysis.

BACKGROUND

Peptide mapping using LC/MS is a powerful methodology that is routinely applied by the biopharmaceutical industry to detect and identify minute variations in biotherapeutic proteins. Information acquired from peptide mapping is useful to demonstrate product quality and consistency, and can further be applied to identify new product-related impurities that arise during development.

The ability to identify and quantify these product impurities relies on proper mass spectrometric detection and data processing for peptides and modified peptides that vary widely in concentration and ionization efficiency. Such analyses require simultaneous quantitative and qualitative analysis of high-abundance product peptides and the low-level impurity peptide variants arising from imperfections in the biology of product production or post-translational chemical modifications of the biotherapeutic.

The complexity and dynamic range of peptide variants are further coupled with challenges introduced by the ESI-MS process. In-spectrum dynamic range

Using automated data processing and informatics to recognize the low-level and coeluting labeled peptide with high mass accuracy demonstrates the power of the Xevo G2 QTof for routine characterization of protein therapeutics.

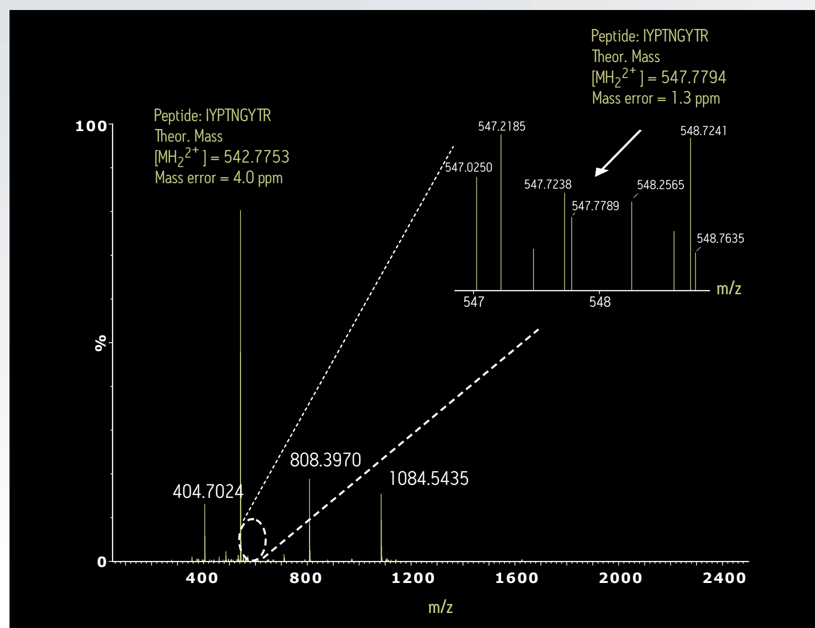

Figure 1. Summed spectrum for the UPLC/MS peak corresponding to the peptide, IYPTNGYTR, highlighting the 2+ charge state of the unlabeled and the spiked-in stable isotope labeled synthetic analog.

becomes critically important to provide accurate mass measurements not only for peptides with highest MS response, but also for peptides that ionize with 1.0% or even 0.1% efficiency of the best ionizing peptides. Without sufficient in-spectrum dynamic range, the instrument will fail to routinely detect these critical quality-indicating or stability-indicating peptides.

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	Protein	Peptide	Fragm...	Start	End	Modifiers	▲1 ...	Control Inte...	Control Mass Error (ppm)
1	Trastuzumab Heavy Chain	IYPTNGYTR	1:T6*	51	59	Isotopically Labeled Arg(1)	14.3	353.0	-2.4
2	Trastuzumab Heavy Chain	IYPTNGYTR	1:T6	51	59		14.3	1101177.0	-0.2
3	Trastuzumab Heavy Chain	IYPTNGYTR	1:T6*	51	59	Deamidation N(1)	15.2	613769.0	1.8
4	Trastuzumab Heavy Chain	IYPTNGYTR	1:T6*	51	59	Deamidation N(1)	16.5	160632.0	1.6

Figure 2. BiopharmaLynx processes and assigns identity to lowest level map components.

THE SOLUTION

The Xevo G2 QTof is capable of accurately measuring the mass of a peptide, within a monoclonal antibody LC/MS peptide map, spanning four orders of magnitude in concentration.

An isotopically-labeled peptide analog that bears an exact sequence match to a tryptic peptide (IYPTNGYTR) within a therapeutic antibody was synthesized and serially spiked in to the digested antibody (50 pmol/µL) at levels spanning four orders of magnitude lower than the unlabeled peak (5 fmol/µL). The spiked digest was separated on an ACQUITY UPLC® BEH 300 C_{18} Column with a 90-min gradient, and LC/MS^E peptide mass and fragmentation data were acquired on a Xevo G2 QTof in the ESI positive resolution mode of the instrument, using GFP as a lockmass reference compound. Due to the identical sequences, these two peptides coelute during chromatographic separation but differ by about 10 Da (5 m/z for the 2+ charge state) from the stable isoptope-labeled Arg on the spiked-in peptide. Data processing was accomplished using manual analysis in MassLynx™ Software or automated analysis using BiopharmaLynx,™ a MassLynx Application Manager supporting automated peptide map and intact protein LC/MS analysis workflows.

Figure 1 shows the MassLynx summed spectrum for the UPLC/MS peak corresponding to the peptide, IYPTNGYTR, highlighting the 2^+ charge state of the unlabeled and the spiked-in stable isotope labeled synthetic analog. The results clearly indicate that high mass accuracy was maintained despite the vast range of peptide intensities. More importantly, both peptides were properly identified with a common retention time and superior mass accuracies for both versions of the peptide (Figure 2) using the advanced processing capabilities of BiopharmaLynx.

The ability to use an automated data processing and informatics workflow to recognize the low-level and coeluting labeled peptide with such high mass accuracy demonstrates the enhanced utility of the Xevo G2 QTof and its QuanTof™ hybrid ADC mass detector for routine characterization of protein therapeutics. This enables organizations to maximize their investment in innovative technology and reduces the average time scientists spend analyzing peptide maps for biotherapeutic proteins by days to weeks.

SUMMARY

The enhanced in-spectrum dynamic range of Xevo G2 QTof mass spectrometer enables the system to analyze a digested protein sample that contains components across a wide dynamic range of concentrations. When coupled with the appropriate informatics tools, such as BiopharmaLynx, the Xevo G2 QTof offers a powerful solution for comprehensive biotherapeutic protein characterization.

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F: 1 508 872 1990
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INFORMATICS FOR BIOPHARMACEUTICALS

GENERATING PRODUCT INFORMATION, NOT DATA, IS KEY TO LABORATORY PRODUCTIVITY

The goal of biotherapeutic characterization studies is not data collection, but rather information generation to guide drug development decisions. Unfortunately, data processing and report generation often become productivity-limiting tasks for organizations tasked with biotherapeutic protein characterization and analysis. The challenge to produce comprehensive biotherapeutic analysis has multiplied as analytical technologies have become more information-rich. The application of proper automated data processing and informatics tools will reduce such burdens, allowing laboratories to apply their scientific resources with greater efficiency and effect.

COMPREHENSIVELY DESIGNED FOR PERFORMANCE

Mass spectrometry plays a critical role in biotherapeutic analysis throughout the product lifecycle. In early development, intact protein and peptide mapping LC/MS studies enable selection of the highest quality expression clones and provide the key characterization data needed to define overall product heterogeneity. In later development, as proper reference standards become available, comparative analyses become more common, and product stability and formulation questions come to the forefront. Software that automates data processing and facilitates these comparisons has been long desired to improve productivity of analytical groups in biopharmaceutical organizations. To address these needs, Waters has created two industry leading biopharmaceutical informatics tools: the BiopharmaLynx™ Application Manager for MassLynx™ Software, and the UNIFI™ Scientific Information System.

BIOPHARMALYNX APPLICATION MANAGER

BiopharmaLynx automates the processing, annotation, and reporting of intact protein MS and peptide map (LC/MS and LC/MS^E) data for maximum laboratory productivity.

- Advanced processing algorithms facilitate identification and quantification of all components within an LC/MS peptide map.
- Targeted bioinformatic search tools assign these components against selected proteins, peptides, and modifications, while accounting for related species that arise as artifacts of sample preparation or the analytical process (e.g., missed cleavages, over-digestion products, adducts, and source fragmentation ions).
- Comparative tabular and graphical displays promote rapid identification of new species and determination of quantitative structural differences between samples.
- Automated batch processing of MaxEnt1 deconvoluted spectra to efficiently extract both the qualitative and quantitative variant profiles for biotherapeutic proteins.

UNIFI SCIENTIFIC INFORMATION SYSTEM

The Waters Biopharmaceutical System Solution with UNIFI is an application-focused platform that achieves what has previously been impossible: Capturing complex high-resolution mass spectrometry and chromatography data on one platform architecture, where next-generation bioinformatics and GxP data management capabilities enable broad organizational deployment.

UNIFI enables efficient collection, analysis, and reporting of biotherapeutic MS and optical detector data within both regulated and non-regulated environments. The UNIFI platform's intrinsic design facilitates data standardization so that information may be exchanged and transferred among departments or with third-party partners. It enables all laboratory functions to work with a common backbone of analytical information comprised of successful methods, processed results, and historical data and reports.

THE BEST OF UPLC AND MS PROCESSING CAPABILITIES, UNITED IN ONE SOFTWARE PACKAGE

UNIFI brings together for the first time the advanced features and robust functionality of software that supports LC and MS techniques simultaneously. It enables scientists to acquire both chromatography and mass spectrometry data within a single, streamlined software environment. Data and results are easy to access and share at all times.

UNIFI includes sophisticated biopharmaceutical workflows. Advanced bioinformatic processing, visualization, and reporting capabilities are all managed within an intuitive user interface. Raw and processed data are innately connected within the relational database architecture: Detailed analysis of a result, linking sample components to chromatographic peaks and mass spectra, is accomplished within a single window interface. This level of informatics integration and automation reduces days of manual intact protein, peptide map, and released glycan data processing to minutes of routine, automated processing and report generation.

The compliance capabilities of UNIFI and the Waters Biopharmaceutical System Solution now enable laboratories in late development, manufacturing, or QC to take full advantage of high resolution mass spectrometry on qualifiable systems capable of meeting regulatory expectations.

At its core, UNIFI enables labs across a company to collaboratively work with a common backbone of analytical results independent of lab function. This accelerates the drug development process and strengthens organizational partnerships, helping to ensure the quality of biopharmaceuticals, while protecting valuable intellectual property.

Biopharmaceutical System Solution with UNIFI: Intact Protein Characterization

GOAL

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

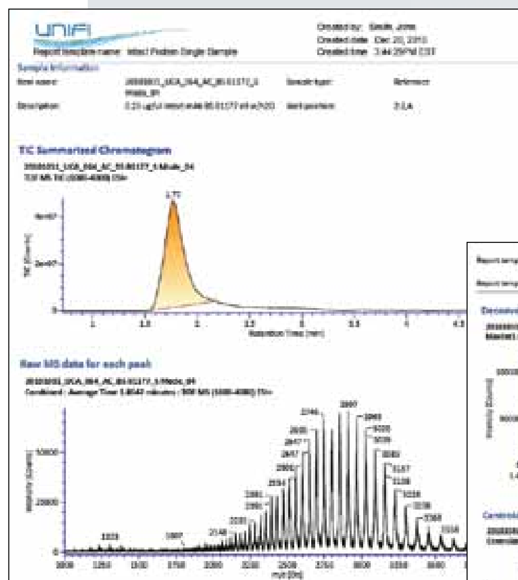
This UNIFI-based platform addresses previous limitations with a comprehensively integrated platform for data acquisition by chromatography and mass spectrometry, with automated reporting.

BACKGROUND

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

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

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



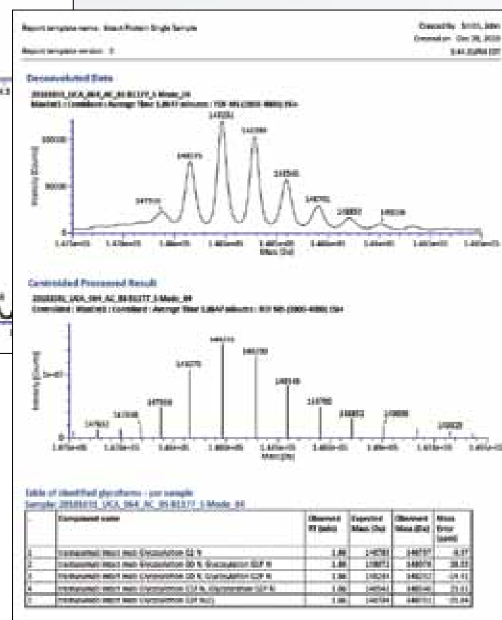


Figure 1. UNIFI's intact protein analysis report of an intact mAb, Trastuzumab. The report shows, from top to bottom, the integrated chromatographic peak, the charge state distribution, the continuum deconvoluted data, the integrated and processed data, and finally the summary table assigning major glycoforms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

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

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

THE SOLUTION

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

UPLC/MS analysis of the mAb Trastuzumab was performed automatically. Aqueous solutions of 0.1% FA and 0.1% FA solution in acetonitrile were used as eluents A and B, respectively. Column temperature set to 80 °C is critical for successful chromatographic separation. The system included an ACQUITY UPLC H-Class Bio, an ACQUITY UPLC BEH300 C4 Column, and a Xevo G2 Tof. The UNIFI Scientific Information System for acquisition, data processing, and reporting completes this comprehensive Biopharmaceutical System Solution.

Table of identified glycoforms - per sample
Sample: 20101031_UCA_064_AC_BS B1177_5Mode_04

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

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

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

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

SUMMARY

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

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

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

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F: 1 508 872 1990
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[TECHNOLOGY BRIEF]

Biopharmaceutical System Solution with UNIFI: Simplifying Verification of Peptide Mapping Results

GOAL

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

BACKGROUND

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

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

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

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

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

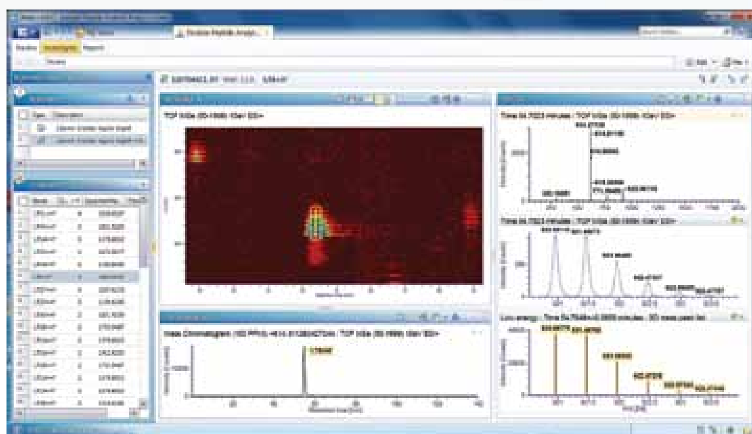

Figure 2. The Investigate tab in UNIFI permits both component-driven and open-ended analysis of raw UPLC/MS^E data.

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THE SOLUTION

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

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

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

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

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

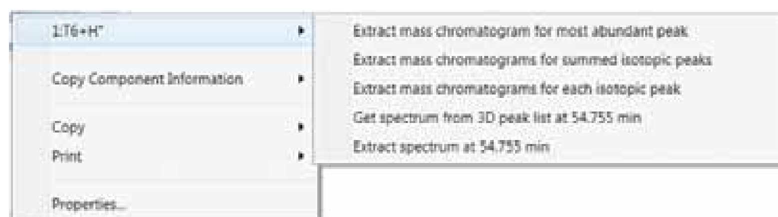

Figure 3. For each component detected, the extracted mass chromatograms, summed MS spectra, and associated processed spectral features can be automatically generated.

SUMMARY

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

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[APPLICATION NOTE]

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

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

APPLICATION BENEFITS

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

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

Xevo® G2 Tof Mass Spectrometer

Biopharmaceutical System Solution with UNIFI

KEY WORDS

Biosimilars, mAb, biopharmaceutical, rituximab

INTRODUCTION

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

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

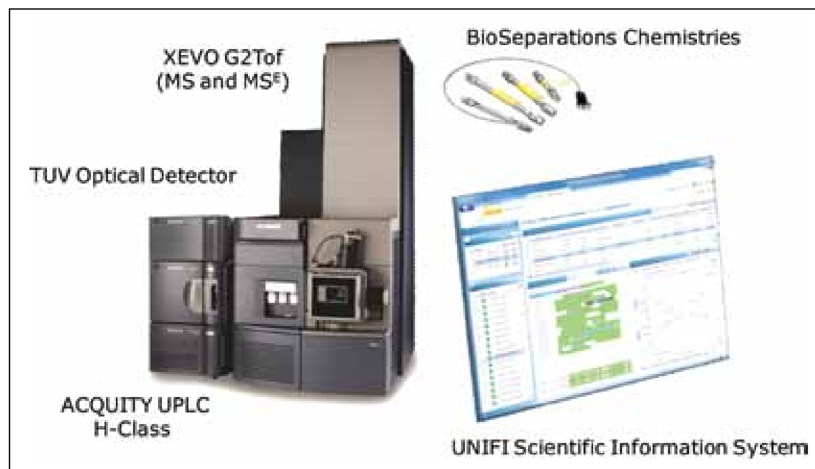

Figure 1. Biopharmaceutical System Solution with UNIFI.

EXPERIMENTAL

Sample Description

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

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

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

Method Conditions

Biopharmaceutical System Solution with UNIFI: ACQUITY UPLC H-Class with Peptide Separation Technology (PST) and Protein Separation Technology (PrST) UPLC® Chemistries

Xevo G2 Tof, ACQUITY UPLC TUV Optical Detector

UNIFI Scientific Information System

Intact Protein LC/MS Conditions

Column: ACQUITY UPLC BEH300 C4, 2.1 x 50 mm

Column temp.: 80 °C

Mobile phase A: water

Mobile phase B: acetonitrile

Mobile phase C: 1% formic acid (aqueous)

Detection: UV 280 nm

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

MS Conditions

Capillary: 2.5 kV

Sampling cone: 50 V

Extraction cone: 4 V

Source temp.: 150 °C

Desolvation temp.: 350 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 800 L/Hr

Partially Reduced Protein LC/MS Conditions

Column: ACQUITY UPLC BEH300 C4, 2.1 x 50 mm

Column temp.: 80 °C

Mobile phase A: water

Mobile phase B: acetonitrile

Mobile phase C: 1% formic acid (aqueous)

Detection: UV 280 nm

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

MS Conditions

Capillary: 3.0 kV

Sampling cone: 30 V

Extraction cone: 4 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 700 L/Hr

Tryptic Digest LC/MS Conditions

Column: ACQUITY UPLC BEH300 C_{18}, 2.1 x 150 mm

Column temp.: 65 °C

Flow rate: 0.2 mL/min

Mobile phase A: water

Mobile phase B: acetonitrile

Mobile phase C: 1% formic acid (aqueous)

Detection: UV 214 nm

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

MS Conditions

Capillary: 3 kV

Sampling cone: 30 V

Extraction cone: 4 V

Source temp.: 100 °C

Desolvation temp.: 250 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 500 L/Hr

Chymotryptic Digest LC/MS Conditions

Column: ACQUITY UPLC BEH300 C_{18}, 2.1 x 150 mm

Column temp.: 60 °C

Mobile phase A: 0.1% formic acid (aqueous)

Mobile phase B: 0.1% formic acid in acetonitrile

Detection: UV 214 nm

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

MS Conditions

Capillary: 3 kV

Sampling cone: 30 V

Extraction cone: 4 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Cone gas flow: 0 L/Hr

Desolvation gas flow: 600 L/Hr

RESULTS AND DISCUSSION

The therapeutic protein comparability workflow started with mAb sample analysis at the intact protein level, followed by the analysis of heavy and light chains after protein reduction, and finally addressed local post-translational modifications (PTMs) and mutations with LC/MS^E peptide map methodology. Such comprehensive workflow is managed by UNIFI for a regulatory environment by integrating data acquisition, data processing, and reporting in a highly automated fashion. The analysis method is completely defined prior to acquisition with the instrument settings, data processing parameters, and a reference to a reporting template included. Each analysis type focuses on a particular application need, such as intact protein analysis or peptide mapping experiment, facilitating the design of a method workflow, as shown in Figure 2. The report templates are composed of the objects that can be entirely configured by the user. The standard report templates include total ion chromatogram (TIC), mass spectra for all or selected ions in a form of either raw, deconvoluted, or centroid data format, and a tabulated summary of the interpreted LC/MS(MS^E) data.

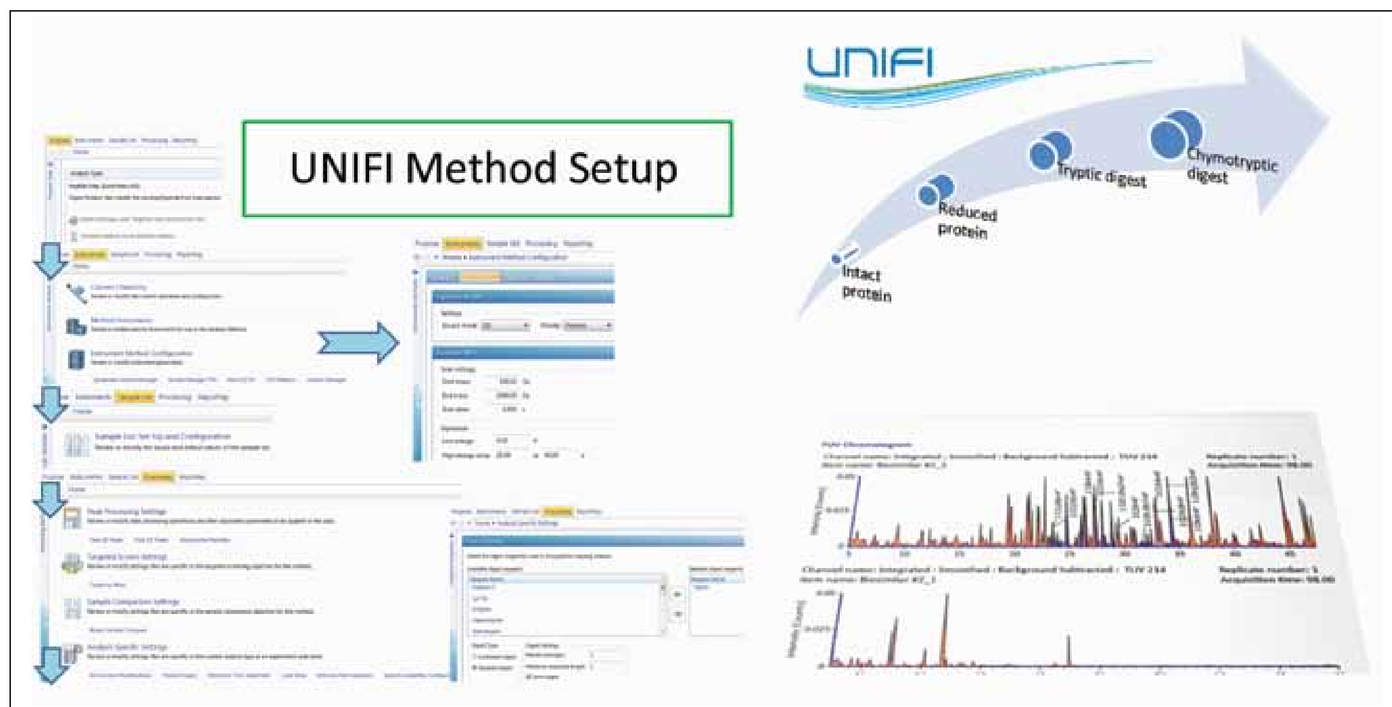

Figure 2. Integrated UPLC/MS analysis of monoclonal antibody with a comprehensive platform for mass accuracy, data processing, and reporting with UNIFI Scientific Information System. All LC/MS parameters, data processing settings, and reporting options are defined in the method prior to acquisition, enabling high-throughput analysis.

Workflow 1: Intact Protein MW Determination and Composition

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

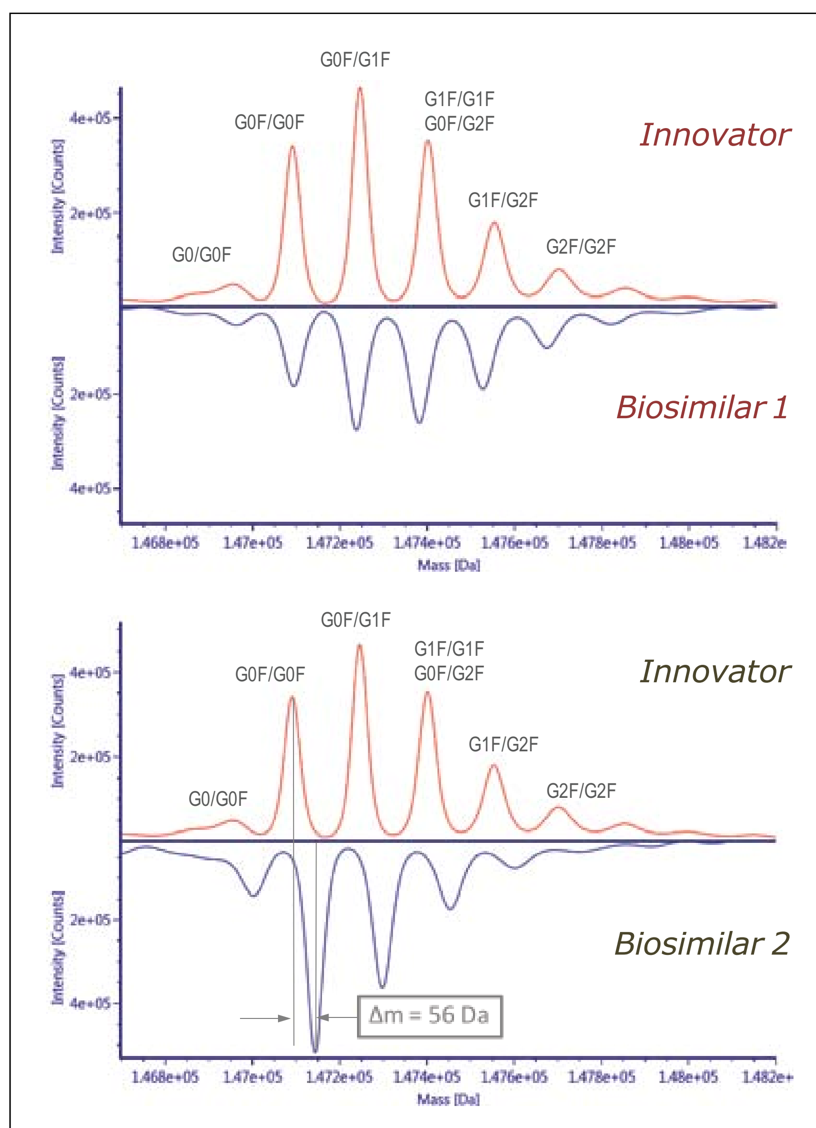

Figure 3. Intact protein mass analysis: MaxEnt1 deconvoluted mass spectra in compare mode. Biosimilar 2 glycoforms have a systematic mass shift of +56 Da with respect to the innovator mAb; whereas, Biosimilar 1 components do not display a systematic mass difference.

Workflow 2: Reduction of mAb

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

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

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

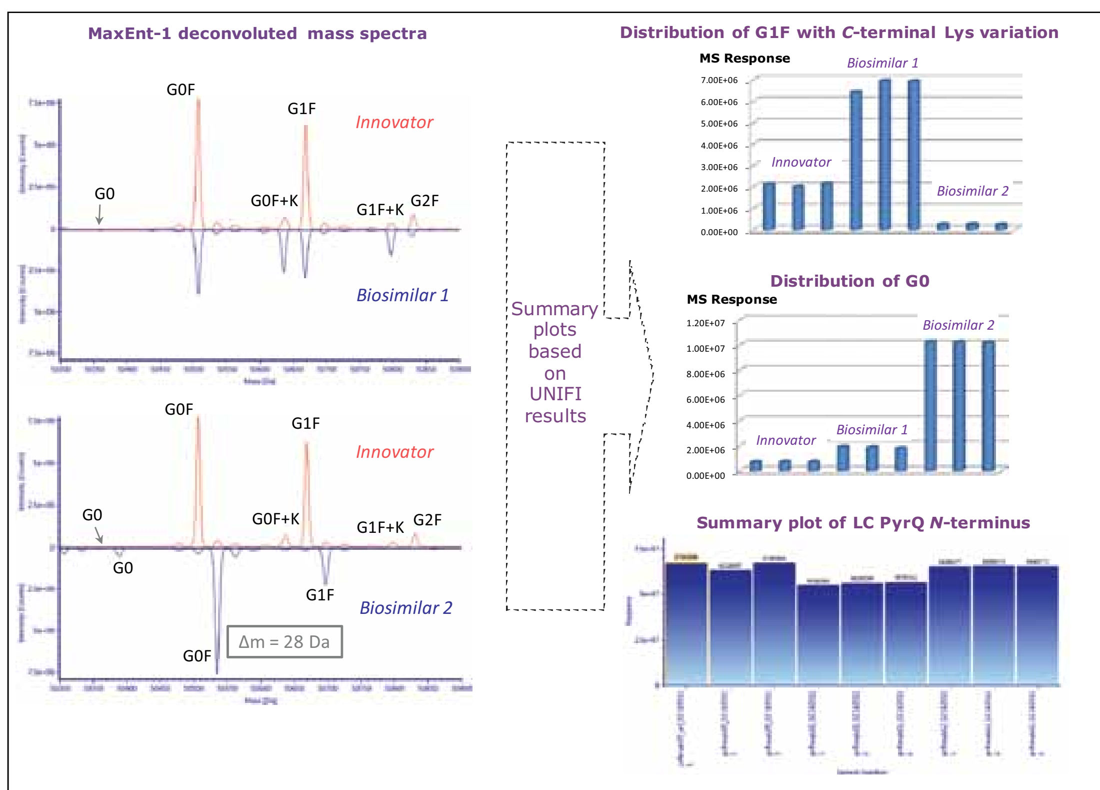

Figure 4. Partially reduced protein analysis. The following examples were measured and compared among three replicate injections of innovator and two biosimilar mAbs: C-terminal Lys variants on the HC, glycoform variant (G0) between biosimilars, amino acid sequence variant (+28 Da) on the heavy chain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

Workflow 3: Peptide Mapping

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

The ultimate inquiry was localizing the amino acid mutation contributing to 28 Da mass shift of the Biosimilar 2. Based on published information,[2] an additional targeted sequence with $Lys_{218} \rightarrow Arg_{218}$ mutation was submitted to the method search. Compare mode view of the tryptic digest chromatogram, or component summary did not show a significant difference between the innovator and Biosimilar 2 mAb; therefore, no conclusion could be drawn about the primary sequence difference. The answer came with use of an alternative, non-specific enzyme, chymotrypsin. The chymotryptic map clearly showed the mass shift in a component view, as seen in Figure 6, and the peptide with a mutation site was automatically highlighted in the chromatogram, peptide map, and the component summary in the Review panel. Filtering the results in the Review by "showing unknown unique components" makes it easy to display the differences between the innovator ("reference") and the biosimilar samples ("unknown").

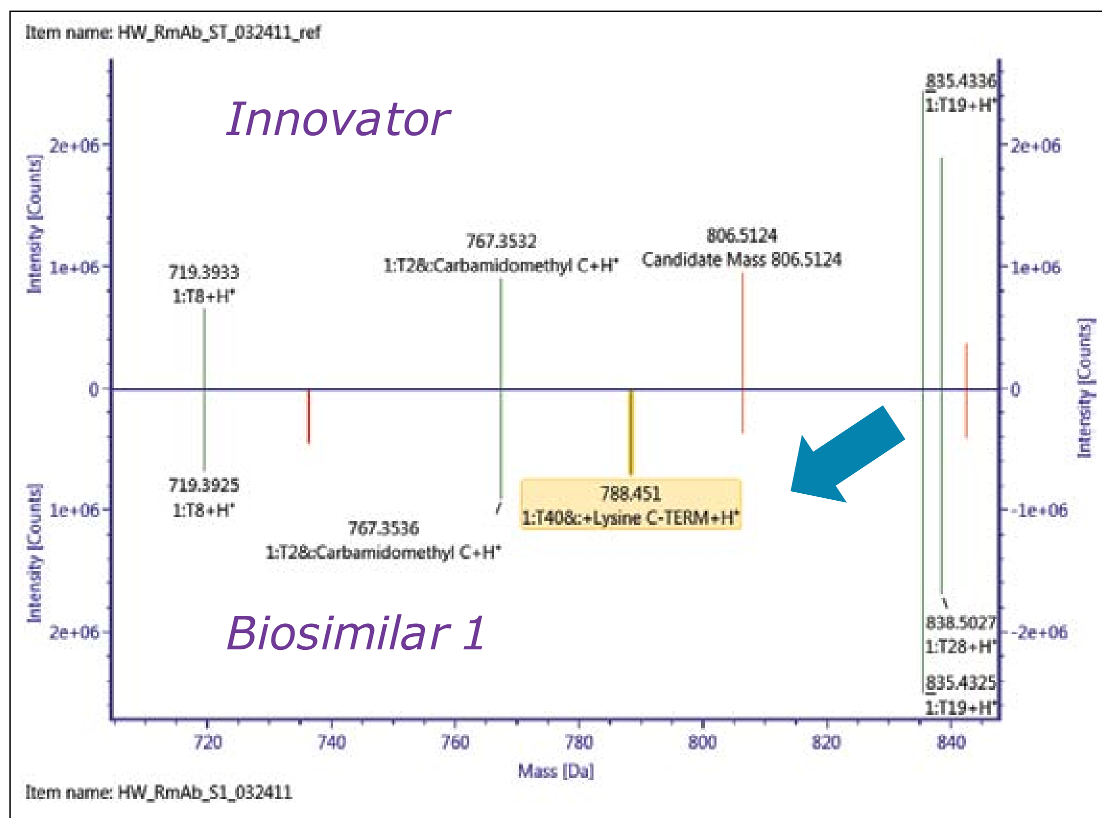

Figure 5. Tryptic digest comparison where components plot in compare mode. C-terminal Lys variant was observed only in the Biosimilar 1 peptide map.

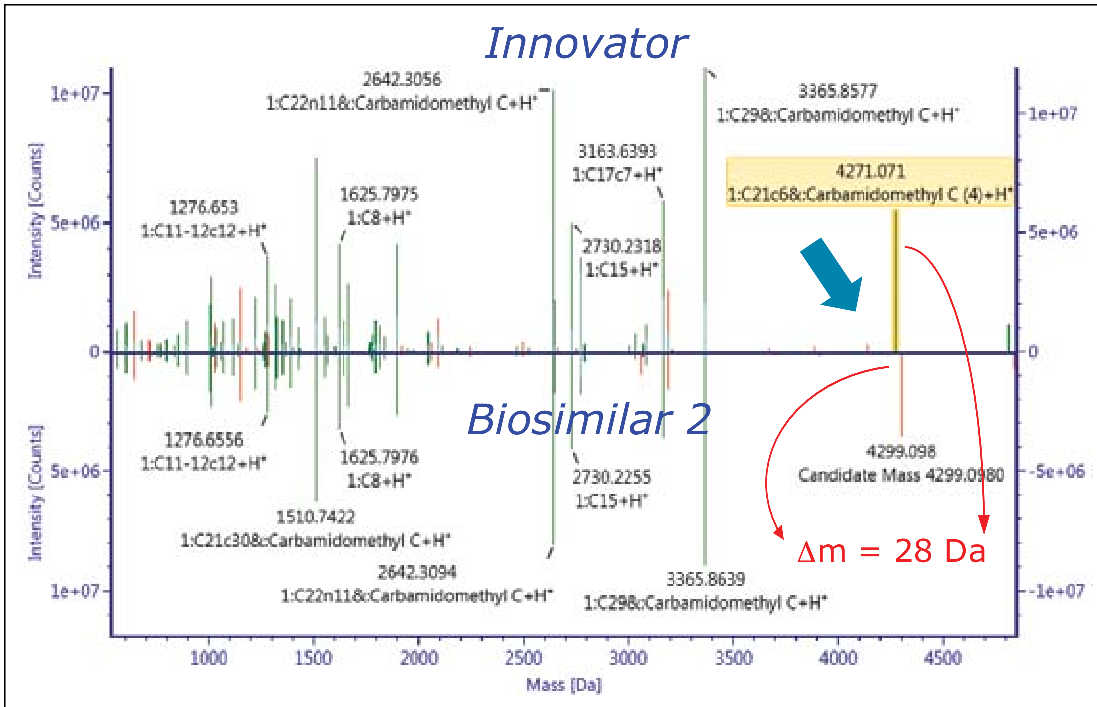

Figure 6. Chymotryptic peptide map analysis where components plot in compare mode, revealing 28 Da mass shift of the chymotryptic fragment in Biosimilar 2.

The reason that the tryptic map failed to pinpoint the amino acid substitution is that proteolytic cleavage occurs at Lys_{217}, Lys_{218} or Arg_{218}. So, the very amino acid of question gets cleaved as a single amino acid entity. Chymotryptic digest, on the other hand, captures the mutation within a single peptide. Finally, $Lys_{218} \longrightarrow Arg_{218}$ substitution was confirmed with MS^E data, as seen in Figure 7, which displayed a_{16}-ion fragment characteristic of Arg. UNIFI peptide map workflow proved the capability to confirm sequence mutation or other suspected PTMs.

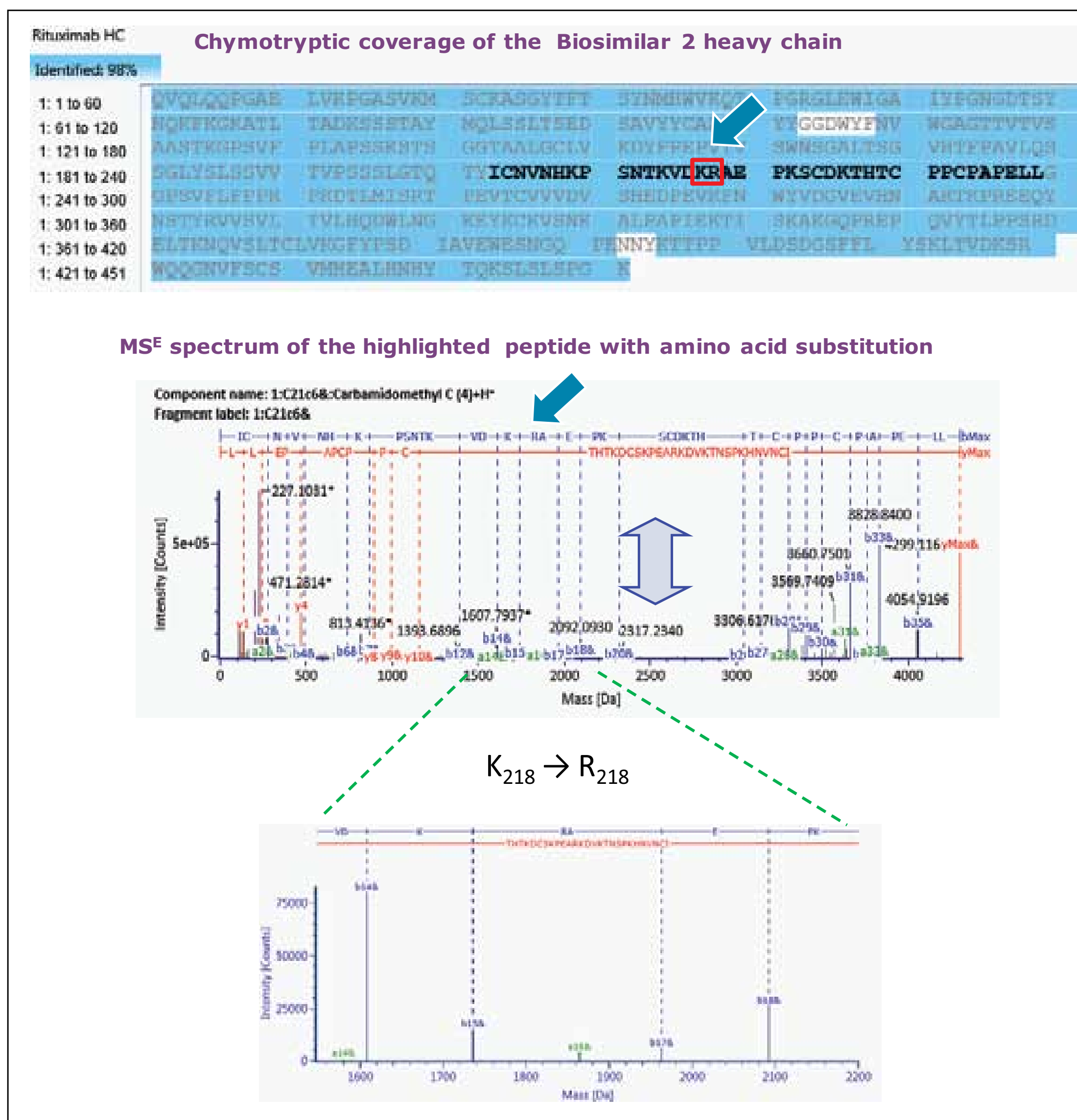

Figure 7. Chymotryptic digest analysis shows MS^E data confirm a single amino acid substitution (K ⟶ R) in Biosimilar 2.

CONCLUSIONS

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

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

References

1. Schiestl M, Stangler T, Torella C, Cepeljnik T, Toll H, and Grau R. Acceptable changes in quality attributes of glycosylated biopharmaceuticals. *Nature Biotechnology*. 2011; 29 (4): 310-312.
2. Jefferis R, Lefranc MP. Human immunoglobulin allotypes. *mAbs*, 2009; 1 (4): 1-7.

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

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BiopharmaLynx: A Bioinformatics Tool for Automated LC/MS Peptide Mapping Assignment

Joomi Ahn, Beth Gillece-Castro, and Scott Berger
Waters Corporation, Milford, MA, U.S.

OVERVIEW

The productivity of many biopharmaceutical characterization groups is limited by the manual and repetitive process of data analysis. LC/MS peptide mapping analysis is an archetype of this problem, as a LC/MS peptide map can take days to weeks to fully characterize for the first time. As peptide mapping remains a common technique to confirm the primary structure and modified forms of a recombinant protein, a solution for data analysis, which is often the bottleneck, is needed. This work features a software tool capable of handling the combinatorial complexity of peptides and their potential modifications.

BiopharmaLynx™ Application Manager, an informatics package for MassLynx™ Software, significantly reduces data analysis times for LC/TOF-MS peptide mapping studies. The automatically annotates peptides detected from LC/TOF-MS data. This software package also automates batch data processing of multiple peptide maps. Faster data processing and a user-friendly interface are some of the attractive features designed to reduce the burden of data analysis.

In this application note, the practical application of this innovative software is described for automated processing and map interpretation of a bovine hemoglobin digest analyzed by LC/MS.

INTRODUCTION

Several key features of the BiopharmaLynx Application Manager for qualitative peptide mapping are:

- Automated data processing enables annotation of peptide sequences to LC/MS peaks using accurate mass assignments.
- These peak assignments are displayed in the form of chromatograms, spectra, and coverage maps.
- Automated peak annotations include recognition of modified peptides present within the sample.
- Graphical tools for easy visualization of raw and processed chromatographic and mass spectral features.
- Tabulated results permit interactive data sorting and editing.

Figure 1 illustrates the overall workflow of BiopharmaLynx Application Manager and the effectiveness of key features proceeding automated peptide map data analysis. The software is also capable of processing and comparing a batch of LC/MS data to identify the differences among peptide samples.

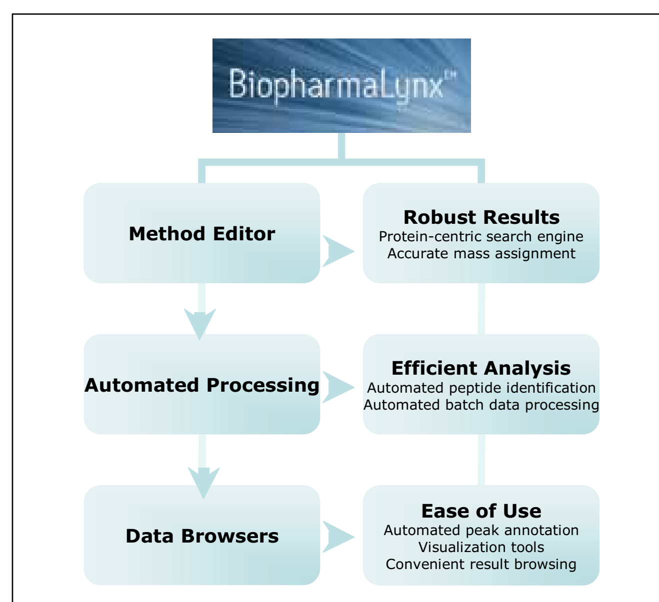

Figure 1. Key capabilities of BiopharmaLynx Application Manager for peptide mapping analysis.

Method editor

A streamlined wizard interface creates methods that can be used to batch process LC/TOF-MS peptide map data. The method editor includes the ability to:

- Define a list of protein sequences to be searched.
- Set search criteria for producing peptide map assignments (mass tolerance, proteolytic enzymes).
- Select fixed and variable modifications from a comprehensive list, with support for customized modifications.
- Specify lockmass parameters for increased mass accuracy.

An example hemoglobin map processing method (Figure 2A) defines a targeted search against the individual alpha and beta chain sequences. The products of cysteine alkylation by iodoacetamide, asparagine deamidation, and methionine oxidation were searched as variable modifications against these sequences.

Automated processing

Raw data processing is facilitated by linking a set of MassLynx data files with a processing method created in the editor. Data files from multiple MassLynx projects (on local and networked drives) can be processed by batch analysis (Figure 2B). LC/MS maps process in minutes on typical desktop workstations.

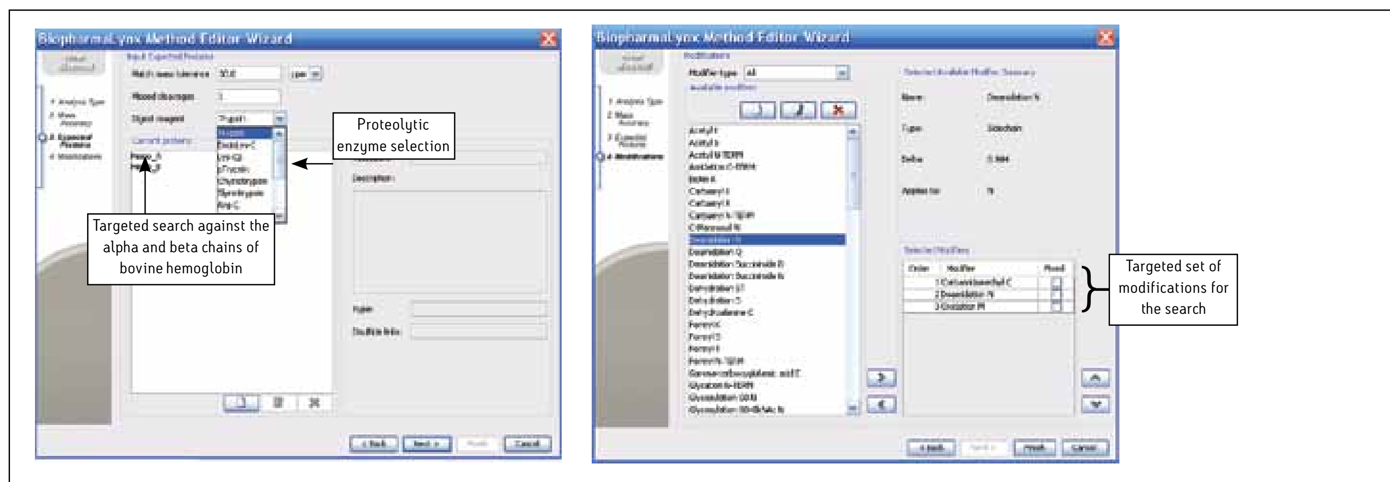

Figure 2A. The method editor created for a bovine hemoglobin LC/MS peptide map.

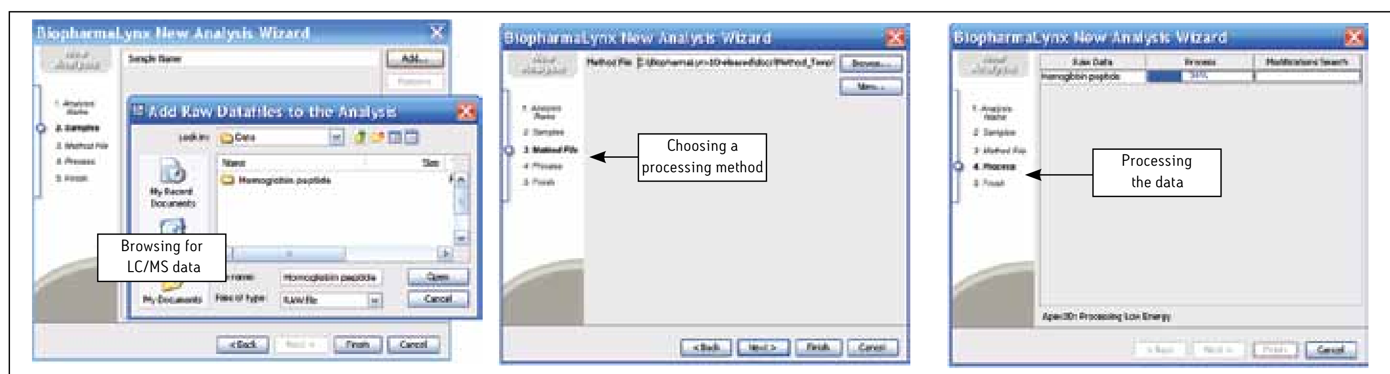

Figure 2B. Automated data processing.

Data browsers

Peptide mapping results can be browsed in several graphical and tabular presentation formats:

- Annotated displays of raw and processed chromatograms.
- Annotated raw and processed mass spectral views.
- Tabular result views that can be customized.
- Protein-centric views of the processed data can be superimposed on coverage maps or a list of digested peptides.

The sequence coverage shown in Figure 3 for the hemoglobin peptide mapping indicates high protein coverage for both alpha and beta chains (95.0% and 94.4%, respectively). The color-coded coverage view provides additional information, such as peptides that have been matched, modified, and not detected. The unmatched peptides highlighted in gray were usually in low intensity signals or short peptides, which were not well retained on reversed phase columns.

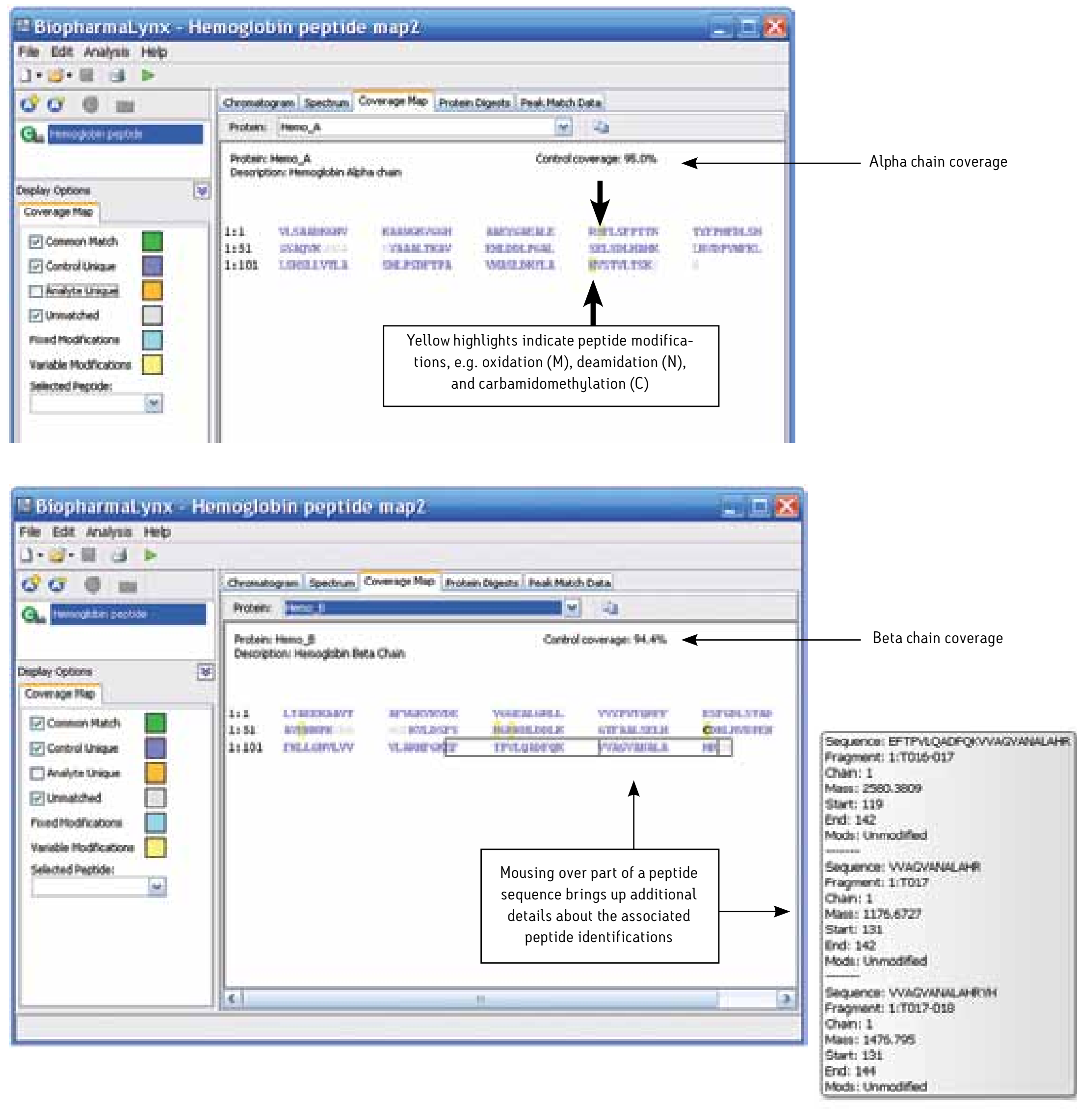

Figure 3. The coverage map overlays LC/MS mapping results on the searched protein sequences.

Displays of the raw and processed chromatogram views in Figure 4 depict annotation of the peptide identifications on the LC/MS data. Tryptic peptides in both chromatograms are labeled as protein digest fragment numbers (T_n) along with their retention times. Additional peptide information is accessible by mousing over any given peak.

In the processed chromatogram in Figure 4B, the peptides are shown as centroided "sticks" at the peak apex retention time. Each centroid represents the summed intensity (ion counts) of all isotopic peaks for all detected charge states of that peptide over the full chromatographic elution profile.

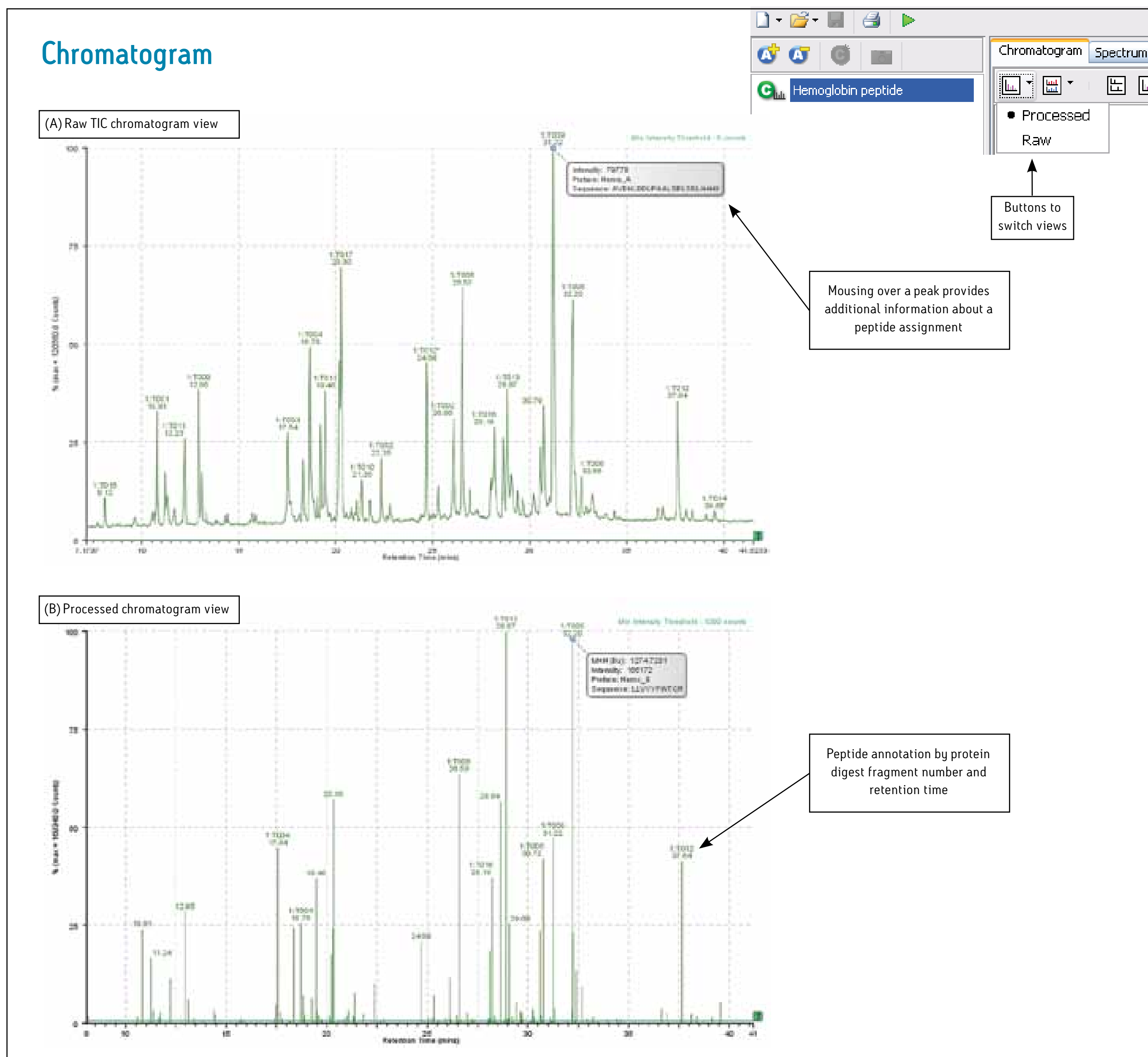

Figure 4. The raw (A) and processed (B) chromatogram views of the bovine hemoglobin peptide map.

The peak match data table display (Figure 5) contains processed results for all detected LC/MS components, including protein and peptide annotations produced by the targeted search. This tabular display of peptides can be customized, sorted by any category, and thresholded to suppress false identifications from low intensity background ions. The results in this table are exportable to Microsoft Excel or other spreadsheet applications. Scientists can also use this view to select alternative assignments for peptides in question or manually annotate an entry.

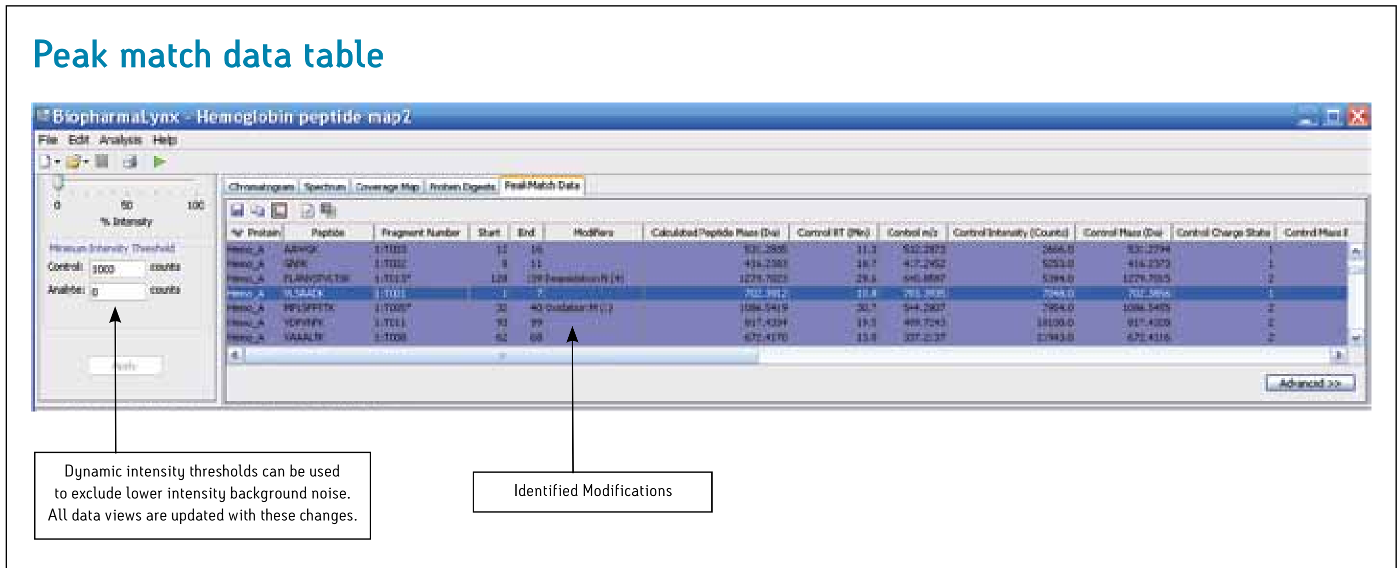

Figure 5. The peak match data table.

EXPERIMENTAL

MassPREP™ Hemoglobin (alpha and beta chains) Digestion Standard (P/N 186002327) was prepared (2 pmol/µL in 0.1 % formic acid). This standard is a purified tryptic digest of bovine hemoglobin (Swiss Protein database files HBA P01966 and HBB P02070) that was reduced with DTT and alkylated by iodoacetamide. 20 pmol of the standard digest was injected for LC/MS peptide mapping analysis.

LC conditions

LC system:	Waters® ACQUITY UPLC®
Column:	ACQUITY UPLC BEH 300 C_{18} 2.1 x 150 mm, 1.7 µm, 300 Å
Flow:	200 µL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	0 to 50% B over 60 min.
Column temp.:	40 °C
Weak wash:	95% buffer A/5% buffer B
Strong wash:	20% buffer A/80% buffer B

MS conditions

MS system:	Waters SYNAPT™ MS
Ionization mode:	ESI Positive
Capillary voltage:	3.0 kV
Cone voltage:	35 V
Desolvation temp.:	250 °C
Desolvation gas:	350 L/Hr
Source temp.:	120 °C
Acquisition:	50 to 1700 m/z
Lockmass:	100 fmol/µL Glu-Fibrinopeptide B $(M+2H)^{2+}$ =785.8426

CONCLUSION

The BiopharmaLynx Application Manager is a powerful bioinformatics tool that automates data processing and peptide annotation for LC/TOF-MS peptide mapping data sets. Data browsing with graphical and tabular tools quickly provides the information needed to make decisions about protein quality or effectiveness of an analytical method or process. Obtaining better information faster can decrease development costs and streamline development timelines.

Waters provides a total system solution for peptide map analysis that includes ACQUITY UPLC-based separations to enable faster more resolving peptide maps, multiple UPLC® chemistries to exploit map selectivity differences, and sensitive TOF mass spectrometers for accurate mass and modified peptide identifications. BiopharmaLynx complements Waters peptide mapping solutions, allowing for integrated peptide mapping data analysis with greater speed and confidence. Automated processing and annotation of peptide maps can be achieved in minutes, removing the most tedious and productivity-limiting element of a peptide mapping analysis.

In conclusion, BiopharmaLynx decreases the expense and time-to-market of protein therapeutics by reducing data analysis time for peptide mapping studies. This software offers fast data processing and an easy-to-use interface that saves time and allows scientists to engage in other high-value tasks.

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

[APPLICATION NOTE]

Efficiently Comparing Batches of an Intact Monoclonal Antibody using BiopharmaLynx

Scott J. Berger and Weibin Chen
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Intact protein LC/MS analysis of a biotherapeutic provides a holistic view with a much simpler set of data than "divide and conquer" methods such as peptide mapping. The major tradeoffs for intact protein MS analysis are that the types of detectable modifications become more limited as protein mass increases and that modification sites cannot be deduced from the intact mass alone.

Assessing clone-to-clone or batch-to-batch variation for glycosylated therapeutic proteins is a common and routine task that often requires significant resources for manual data processing and results integration. When high throughput or rapid sample turnaround is desired, analytical approaches that provide rapid global information about a molecule are preferred over slower approaches that generate more detailed information.

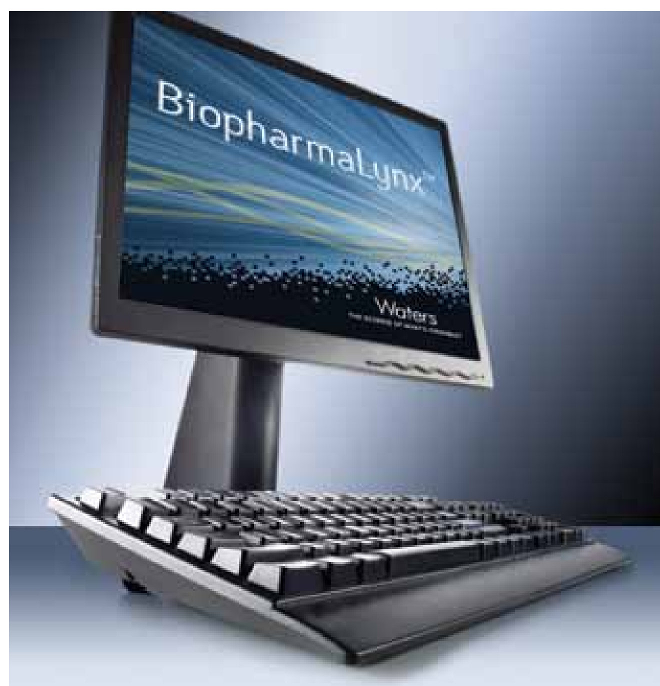
For modifications such as oxidized methionines, there is roughly a 16 Da increase over the unmodified protein mass. These modifications can be readily detected at lower levels in the TOF mass spectra of smaller proteins (30 kD and below, e.g., antibody light chains), but require significant stoichiometry to be detected on a 50 kD protein (e.g., antibody heavy chains), and are not readily detectable on large proteins such as a 150 kD intact antibody. For this reason, intact antibody analysis is almost always used in combination with reduced antibody (LC/HC) analysis and peptide mapping studies for fully characterizing batches of a therapeutic antibody.

In general, there are two classes of protein variants that are typically scrutinized using mass analysis of an intact antibody: glycan structure heterogeneity where variation of core glycans on each heavy chain is extended by a series of 146 to 291 Da carbohydrate units and, the potentially inefficient proteolytic processing of heavy chain carboxy-terminal lysine residues (+128 Da).

Assessing this heterogeneity using intact antibody mass profiles can be useful for selecting a clonal expression cell line with desirable product attributes and monitoring the effects of process changes on a biotherapeutic. It is also a useful approach for demonstrating the consistency and comparability of individual batches of drug product.

Comparative LC/MS analysis of intact antibodies can be accomplished using rapid LC/MS methods.[1-3] Using proper methodology, this type of analysis is robust, and pharmaceutical companies have acquired intact mass data on literally thousands of proteins using OpenLynx™ enabled open access LC/MS analysis stations.[4-6] The resulting deconvoluted intact mass information found in an OpenLynx report is usually sufficient for routine mass confirmation of a recombinant protein, but often proves limiting for in-depth characterization or comparative profiling studies of biotherapeutics.

BiopharmaLynx™ intact protein analysis workflows are designed to expand functionality for automated batch processing of data by providing additional processing capabilities and related deconvoluted masses to targeted proteins and their variants.



In addition, the graphical graphical and tabular tools necessary for comparisons of experimental samples against a "gold" reference standard are present in the application.

This application note discusses key elements of the intact mass workflow, and applies them in the comparative analysis of two batches of a therapeutic monoclonal antibody. Overall, the use of such automated data processing and annotation workflows should allow biopharmaceutical organizations to screen more samples in less time, and make better decisions faster for the development of their biotherapeutic molecules.

EXPERIMENTAL

LC conditions

LC system:	Waters nanoACQUITY UPLC® System
Column:	MassPREP™ Micro desalting 2.1 x 5 mm
Column temp.:	65 °C
Flow rate:	20 µL/min
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	ACN with 0.1% formic acid
Gradient:	10% B for 5 min, 10% B to 90% B over 5 min

MS conditions

MS system:	SYNAPT™ High Definition Mass Spectrometry™ (HDMS™) System
Ionization mode:	ESI+
Capillary voltage:	2.0 kV
Cone voltage:	65 V
Desolvation temp.:	250 °C
Desolvation gas:	100 L/hr
Source temp.:	105 °C
Acquisition range:	1000-4600 m/z
Calibration range:	1000-4600, CsI, external
Sample:	0.5 mg/mL of a monoclonal IgG1 in 25 mM ammonium bicarbonate
Sample volume:	3 µL

The LC/MS configuration included an additional post-column desalting valve (system controlled) as detailed in the Care and Use document for the MassPrep Intact Protein Desalting Kit.

Acquisition and processing methods

The data were acquired using BiopharmaLynx v.1.1, a MassLynx™ Software application manager that allows laboratories to streamline and automate the processing, bioannotation, and comparison of peptide map and intact protein LC/MS data sets.

RESULTS AND DISCUSSION

Screening of intact antibody variants was accomplished using a simple desalting LC/MS approach. We have previously described[1-3] rapid, reversed phase desalting methods that use short four-minute cycle times for an intact antibody to facilitate post-column salt diversion during sample loading. Such desalting strategies are advantageous when fast sample turnaround or higher sample throughput is desired.

Residual salts on the bound protein were washed away at low organic modifier; rapid gradients were generated to elute proteins as concentrated peaks for mass analysis; and additional sawtooth gradients were applied to ensure that the column was in pristine condition for the subsequent injection. In this work, a variant of this methodology was optimized for the nanoACQUITY UPLC System, and applied for the analysis of two commercial monoclonal antibody preparations.

Automating the data processing and interpretation of two intact antibody desalting runs in BiopharmaLynx was accomplished using spectral summation over the chromatographic elution profile of the antibody, subjecting the summed spectrum to MaxEnt1 spectral deconvolution, and searching the resulting deconvoluted masses against the antibody sequence and a series of common biantennary glycan modifications. The following sections will describe this processing workflow in greater detail.

Our standard approach for processing very large proteins, such as intact antibodies, is to deconvolute a selected high signal-to-noise m/z range within the summed mass spectrum. This "heartcut" m/z region (typically comprising at least six charge states) contains the best population of peaks with good signal-to-noise that are not artificially broadened by the presence of adducts, (which is an issue at higher m/z). These higher m/z peaks, (fewer charges) represent more folded structures that can preferentially maintain neutral and salt adduct associations during the electrospray process.

Our method (Figure 1) specifies the spectral deconvolution of peaks between 2500 and 3000 m/z in the raw data summed between 10 and 12 minutes to produce a MaxEnt1 zero-charge-state deconvolution spectrum in the range of 147,000 to 150,000 Da. This selected retention window and mass range are highlighted by shaded regions on the mirrored differential plots of the total ion chromatogram (Figure 2) and summed raw mass spectra (Figure 3A). Enlarging several charge-states within this region (Figure 3B) shows the complex nature of each charge-state arising from the various glycovariants of the antibody, while the differential plot shows consistent relative intensity differences of individual glycovariants across charge-states. From this repeating pattern of differential intensity, it would be expected that the final deconvoluted result would reflect a similar pattern of relative glycovariant intensity differences between the two samples.

Additional settings for MaxEnt1 deconvolution of the antibody data are accessible through the advanced deconvolution settings button (Figure 1, red box highlight). These settings (Figure 4) show that the summed spectrum was background subtracted prior to applying 15 cycles of MaxEnt1 spectral deconvolution, and that the MaxEnt1 deconvoluted spectrum was background subtracted prior to measuring the height centroid of each peak. It is these centroid mass-intensity values that populate the results table and that are used for annotating protein variant structures.

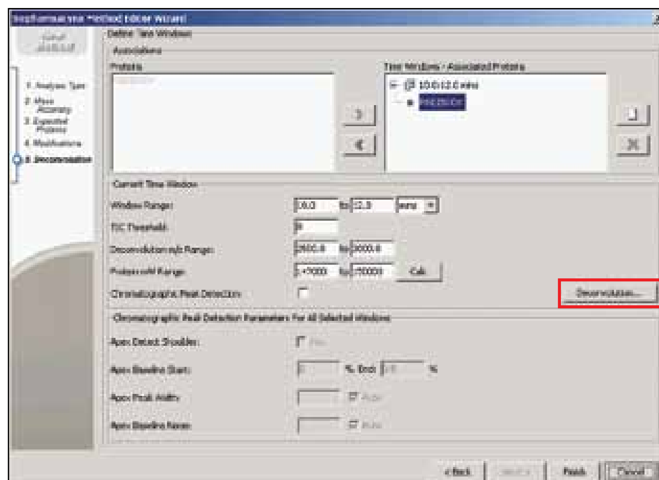


Figure 1. BiopharmaLynx Method Editor screen detailing protein search, spectral summation, and spectral deconvolution parameters. BiopharmaLynx allows users to custom define time regions within an LC/MS run, associate each of those regions with optimal spectral deconvolution conditions, and target proteins for annotation against the observed masses.

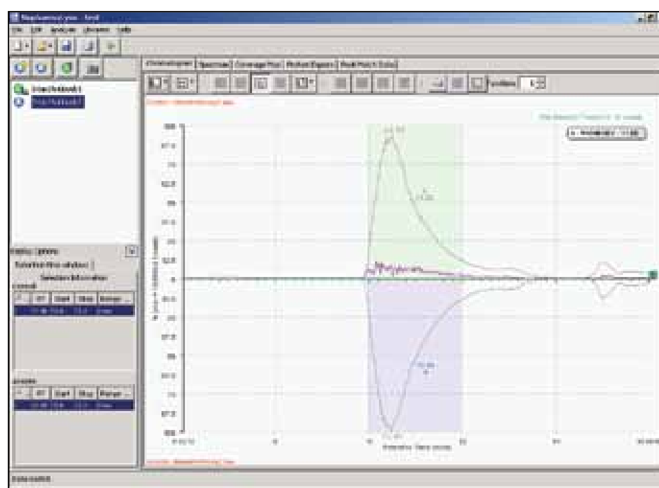


Figure 2. Results Browser differential Total Ion Chromatogram mirror plot for two intact antibody LC/MS analysis runs.

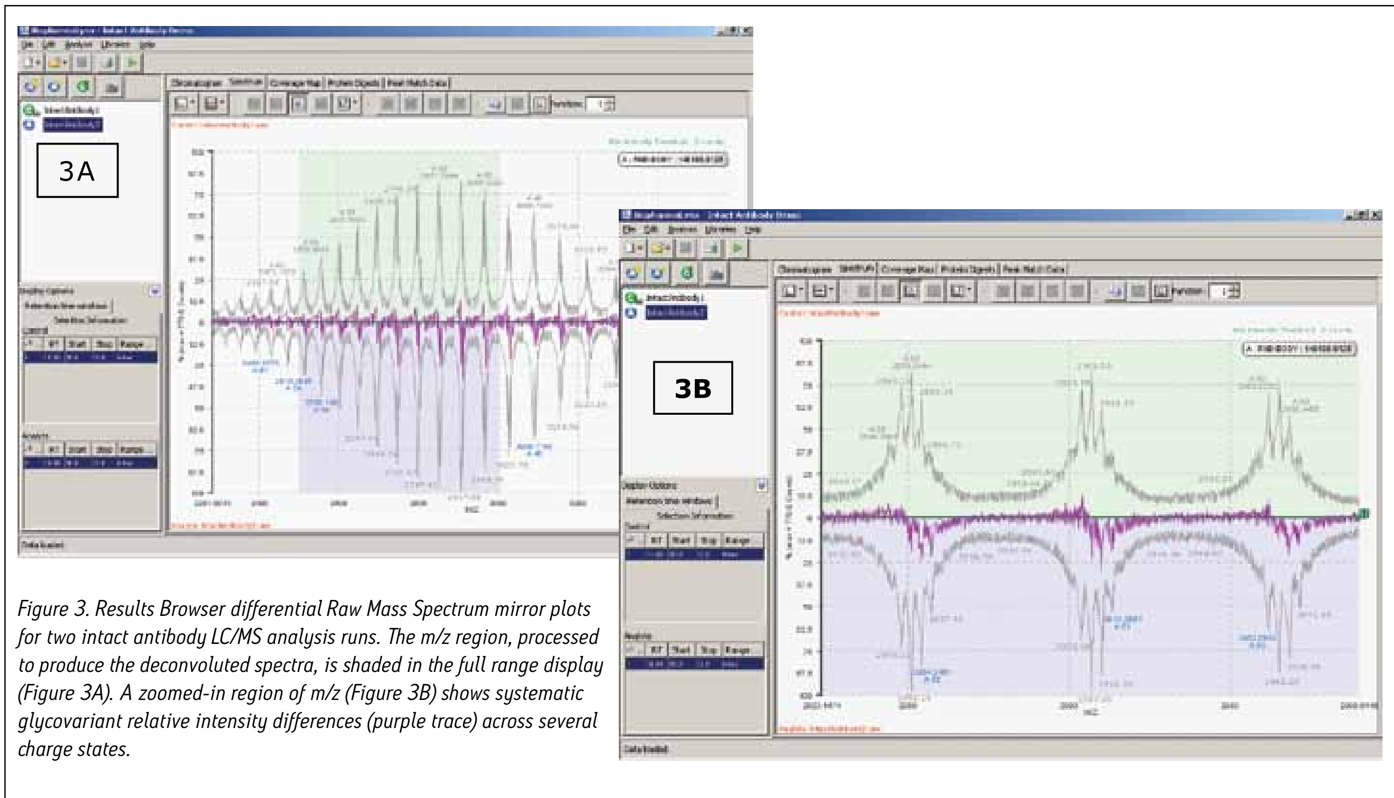


Figure 3. Results Browser differential Raw Mass Spectrum mirror plots for two intact antibody LC/MS analysis runs. The m/z region, processed to produce the deconvoluted spectra, is shaded in the full range display (Figure 3A). A zoomed-in region of m/z (Figure 3B) shows systematic glycovariant relative intensity differences (purple trace) across several charge states.

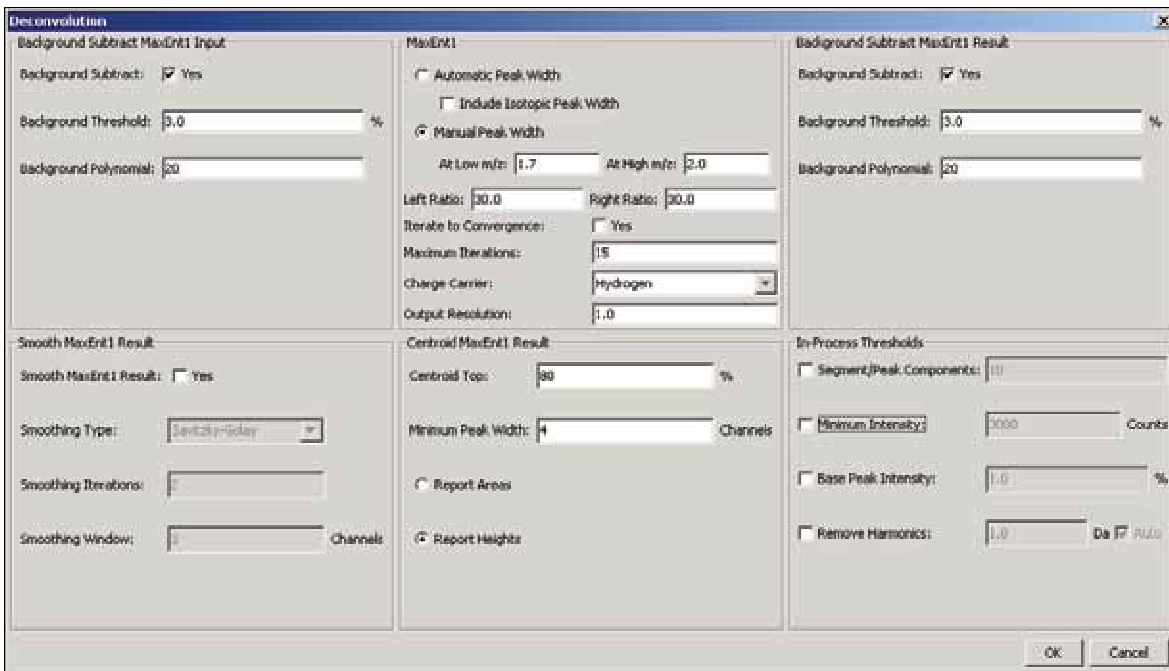


Figure 4. BiopharmaLynx Method screen detailing advanced deconvolution parameters.

BiopharmaLynx contains the latest generation MaxEnt1 spectral deconvolution algorithm. While a full discussion of this improved algorithm will appear in a separate technical note, it is important to focus on several settings (Figure 4) selected for processing of the antibody LC/MS data. First, the "automated peak width" function is not selected. This new capability automatically populates the MaxEnt1 spectral peak width setting based on the entered instrument resolution and average output mass range. This width value can be modeled effectively for smaller proteins (under 40 kD) that do not have significant charge-state peak broadening due to adducts or micro-heterogeneity. However, the width value cannot be modeled effectively for larger proteins due to the greater occurrence and magnitude of these effects. In both the automated and manual peak width modes, the improved MaxEnt1 algorithm now better models TOF instrument data by recognizing that peak width on TOF instruments systematically varies with m/z.

Thus, two spectral peak width settings (typically measured for the best resolved or most intense variant at half peak height) that correspond to the highest (low m/z) and lowest (high m/z) charge-states used for deconvolution can be entered in the manual mode. For the two antibody data sets, these values were determined as 1.7 and 2.0, respectively. Selecting a common value for both entries would mirror what is implemented in the current MassLynx Software v.4.1 implementation of MaxEnt1, when the "Uniform Gaussian" peak width model is used.

This method applies 15 iterations of MaxEnt1 processing to the data rather than taking the deconvolution process to full convergence of the mass spectral and model ("mock")-spectral data sets. This choice is made in recognition that the peak models used by MaxEnt, even with variable spectral peak width, cannot perfectly match data for large proteins (>100 kD). There is unresolved micro-heterogeneity within each charge-state, and adducts preferentially interact with lower charge-state/structures of a protein. Both effects produce additional variance in peak width and peak asymmetry that are not directly predictable. This unaccounted-for spectral signal can contribute to additional peak structure in the deconvoluted spectrum, which is not evidenced within the raw spectral data, and which typically begins to appear in later MaxEnt1 iterations. The improved TOF model has reduced the occurrence and magnitude of such peaks, but they may still be generated during large protein deconvolution studies.

Selecting the number of iterations provides proper resolution and relative quantitation of protein variants, while avoiding these potential artifacts, is an important part of developing a robust data processing workflow for large proteins. The proper balance can be achieved for biotherapeutic antibody analysis, as indicated by the recent published work of Gadgil and colleagues.[8]

The resulting deconvoluted spectrum produced by MaxEnt1 processing of the two data sets (Figure 5) displays systematic differences between antibody samples. Consistent with the raw spectra differential display (Figure 3B), the lower sample is enriched with higher mass glycovariants and deficient in some lower mass glycovariants. As a simple validation exercise, true differences between deconvoluted protein spectra should be observable in the underlying raw charge-state data, although these differences can be subtle. Additional information about a selected peak in the deconvoluted data (corresponding to the G0F/G1F glycoform) was obtained by mouse-over at the peak apex. The centroided mass-intensity information derived from this deconvoluted spectrum was used to produce tabular results and processed spectra/chromatogram views (not shown) of the data.

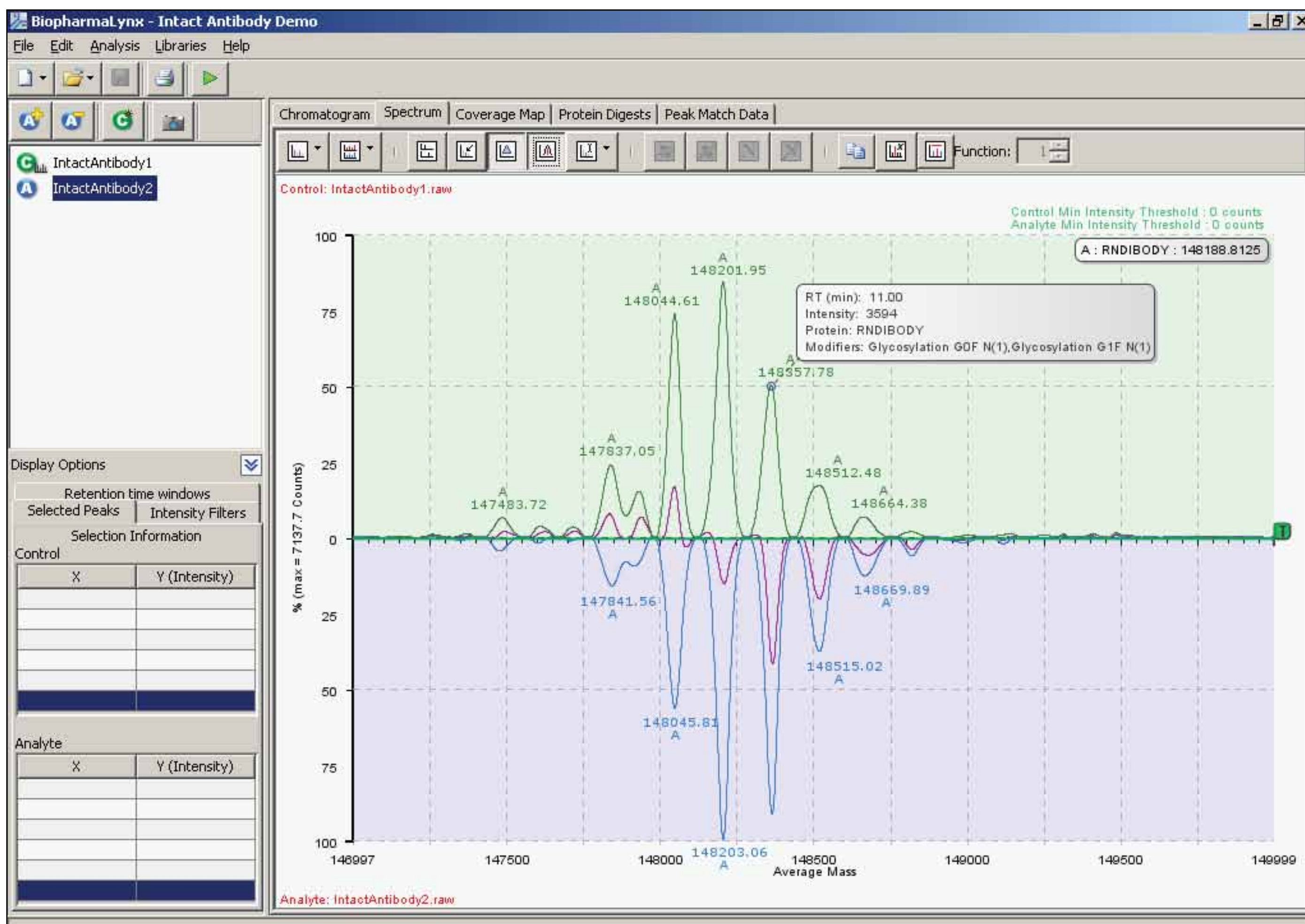


Figure 5. Results Browser differential MaxEnt1 deconvoluted mass spectrum mirror plots for two intact antibody LC/MS analysis runs. The glycovariant intensity differences apparent in the raw data are also evident in the differential deconvoluted spectrum (purple line).

The bioinformatic assignment of antibody masses to protein structures was also accomplished as part of the processing method. The recombinant IgG1 antibody sequence was defined in the method (Figure 6) as a single protein with four protein chains (two heavy, two light) containing 16 intermolecular and intramolecular disulfide bonds. The sequence has been randomized to obscure the identity of the biotherapeutic molecule used in this comparative analysis. Defining the correct number of disulfide linkages is important, as each disulfide will increment the predicted protein mass downwards by 2 Da, or roughly a -32 Da mass difference for the intact antibody.

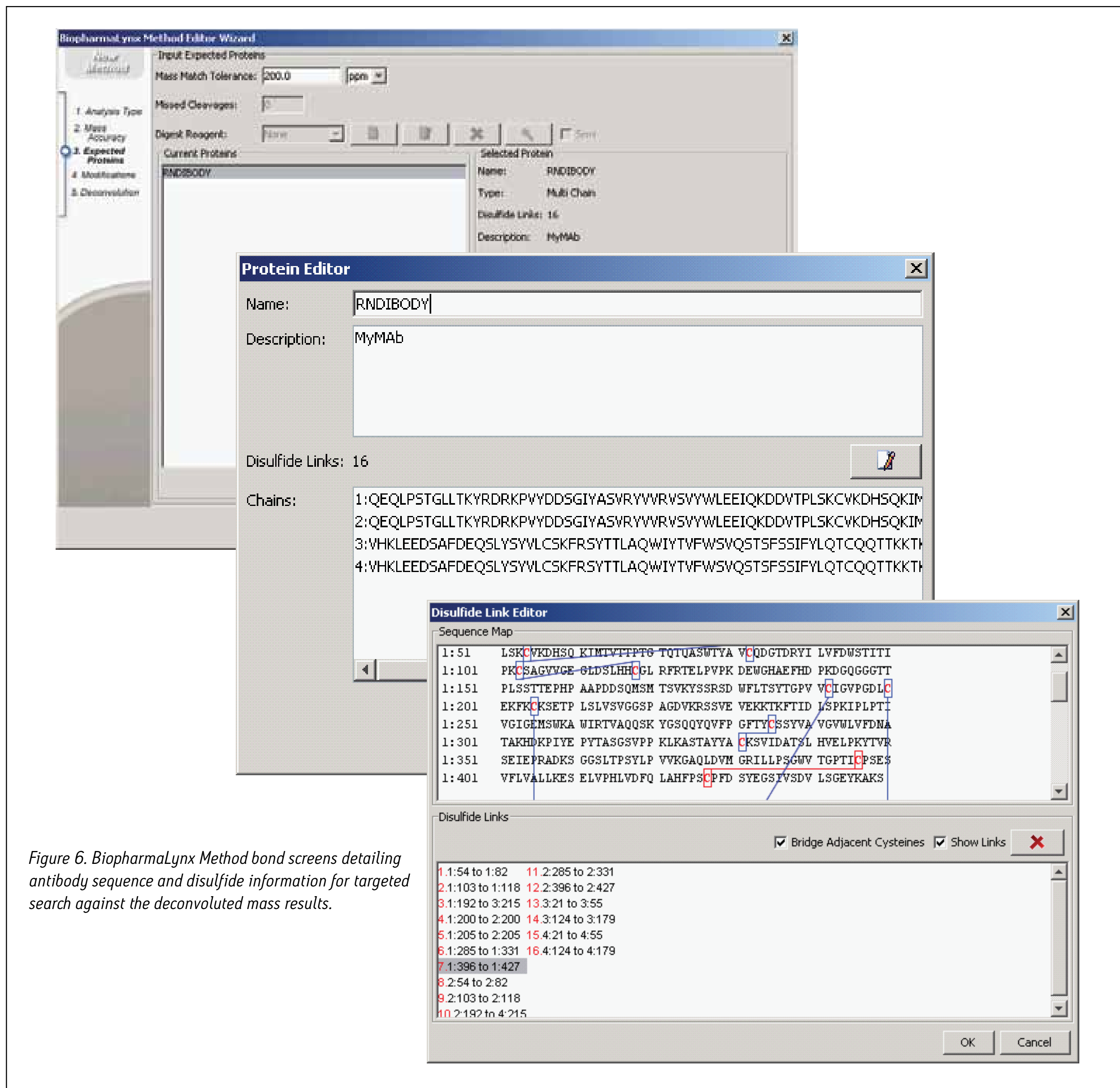


Figure 6. BiopharmaLynx Method bond screens detailing antibody sequence and disulfide information for targeted search against the deconvoluted mass results.

Modifications searched in the method (Figure 7) included a set of seven common biantennary glycan structures targeted to canonical NX(S/T) N-glycosylation sites (e.g. G0F in Figure 7, inset).

BiopharmaLynx Method Editor Wizard

New Method

1. Analysis Type
2. Mass Accuracy
3. Expected Proteins
4. Modifications
5. Deconvolution

Modifications

Modifier Type: All

Dehydroalanine C
Formyl K
Formyl S
Formyl T
Formyl N-TERM
Gamma-carboxyglutamic acid E
Glycation N-TERM
Glycosylation G1 N
Glycosylation G2 N
Glycosylation G1F+SA N
Glycosylation G2F+SA N
Glycosylation O-GlcNAc ST
Homoserine M
Homoserine-H20 M
Hydroxyl DKNP
Hydroxyl K
Hydroxyl W
Hydroxyl P
Hydroxyl D
Lipoyl K
Methyl CDEHKNRQ
Methyl K
Methyl N
Methyl S
Methyl T
Methyl x2 R
Methyl x2 K
Methyl x3 K
Methyl N-TERM

Order	Modifier	Fixed	Max Mods
1	Glycosylation G0 N		No Limit
2	Glycosylation G0-GlcNAc N		No Limit
3	Glycosylation G0F N		No Limit
4	Glycosylation G1F N		No Limit
5	Glycosylation G2F N		No Limit
6	Glycosylation Man5 N		No Limit
7	Glycosylation G0F-GlcNAc N		No Limit

Modifier Reagent Viewer

Name: Glycosylation G0F N
Type: Sidechain
Delta Mass (Da): 1444.5339
Applies To: N
Look Behind:
Look Ahead: (?=.[ST])
OK

< Back   Next >   Finish   Cancel

Figure 7. BiopharmaLynx Method screen detailing modifications considered during the protein search. Note that Glycosylation modifications are searched with NX(S/T) sequence specificity, and users can limit the maximum number of a modification that can be present on a protein.

The peak match data table (Figure 8) links the processed data to the proteins and modifications assigned through a targeted bioinformatic search. Shown are the top 10 most intense components identified in the control sample, sorted from the most to least intense.

Figure 8. Results Browser Tabular Results View (Focus on control sample results) shows that the Top 10 most intense peaks correspond to glycovariants of the searched antibody. The green color of each row indicates that the variant was observed in both the control and experimental analyte samples. Relative levels of a variant within a sample are seen in the (%Total) column, while differences between the samples are displayed in additional columns in the table (not shown).

The table also contains information about the detection of these components in the analyte, and mass/intensity differences between the two samples (not shown for figure legibility). All 10 components were assigned to antibody glycovariants structures, and the green shaded row indicates that the components were all detected within the deconvolution results generated for both samples.

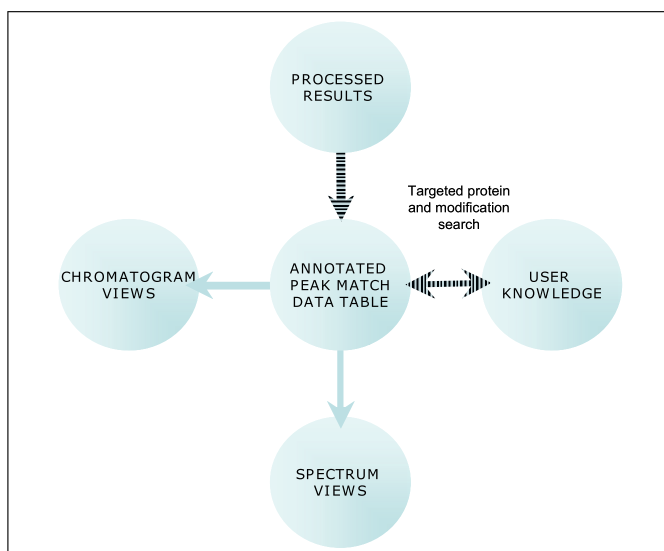


Figure 9. The overall BiopharmaLynx results workflow is component- and protein-centric. The peak match data table links the processed data (detected components with an RT, mass, intensity) to proteins and associated modifications found through the targeted bioinformatic search. These bioinformatics-assigned annotations can be revised by the user, and such changes will be propagated throughout the chromatogram/spectral viewing and reporting tools.

- BiopharmaLynx results are protein-centric. The peak-match data table links the processed data to proteins and modifications found in a targeted bioinformatic search.
- These annotated data interactively supply information for the various viewing tools that enable effective data analysis.
- A software user can select between alternative annotations produced by the search, or use their knowledge to manually annotate data table results.
- All data views are sensitive to changes in the data table and are updated with these changes.

The control intensity (% Total) value is linked to the selection column to its right, and generates on-the-fly calculations of relative composition of the selected components within an individual sample.

This peak-match data table represents a central location for processed data that supplies annotation information to the various tools that facilitate graphical result viewing and sample-sample comparisons. As depicted in Figure 9, a central BiopharmaLynx paradigm is that scientists are the ultimate arbiters for interpreting results generated by the program. This may include aligning and normalizing data, altering minimum intensity thresholds for peak detection, adjusting incorrect assignments produced by a search, choosing between alternative assignments fitting the protein search criteria, or using their knowledge to manually annotate components within a sample. Chromatographic and spectral displays are immediately updated with any changes to annotations, and user-comments associated with individual components can be included when constructing reporting templates.

CONCLUSIONS

This application note has detailed how the BiopharmaLynx Application Manager can demonstrate batch-to-batch differences in the glycovariant profile of a recombinant therapeutic monoclonal antibody. Important points in this note cover many of the best practices we employ to build processing methods capable of recognizing and comparing LC/TOF-MS profiles of therapeutic antibody variants, and a rational framework for assessing that deconvolution conditions have been properly selected. Today, LC/MS data on intact antibodies can be routinely collected using fast and robust analytical methodologies. The productivity-limiting step for many analytical groups has become the processing and synthesis of useful information from the abundance of raw data generated by these studies. The BiopharmaLynx Application Manager has been designed to address this productivity limitation by automating processing and bioannotation of such data, while providing tools for sample comparison, data export, and report generation. Overall, these improved workflow efficiencies facilitate the rapid communication of results to other scientists and organizations, and help these groups make better decisions faster about the development of their biotherapeutics.

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

[APPLICATION NOTE]

An Automated Data Analysis of Therapeutic Interferon Protein using BiopharmaLynx

Joomi Ahn, Asish B. Chakraborty, Ying Qing Yu, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The combination of reversed phase liquid chromatography (RPLC) and electrospray ionization mass spectrometry (ESI-MS) has emerged as one of the most informative analytical platforms for biopharmaceutical characterization. Two complementary workflows have developed based on this technology platform.

- The so called “top-down” approach applies LC/MS for profiling a biopharmaceutical protein at the intact protein level, obtaining information that confirms molecular weight (MW) and reveals evidence for product heterogeneity or the presence of non-product impurities.
- A second workflow, based on peptide map analysis of an enzymatic digested protein, typifies a “bottom-up” analysis workflow. The analysis complexity introduced by the enzymatic digestion is offset by the large increase in information about the biopharmaceutical sample. This workflow reveals and localizes sites of heterogeneity and rapidly quantifies structural differences between samples.

As HPLC has evolved with UPLC® Technology, and time-of-flight (TOF) mass spectrometry has become more powerful and easier to use, both data quality and quantity have dramatically increased. However, this continued evolution in data acquisition technology results in many laboratories becoming productivity-limited not by LC/MS data acquisition delays, but by the processing of this data.

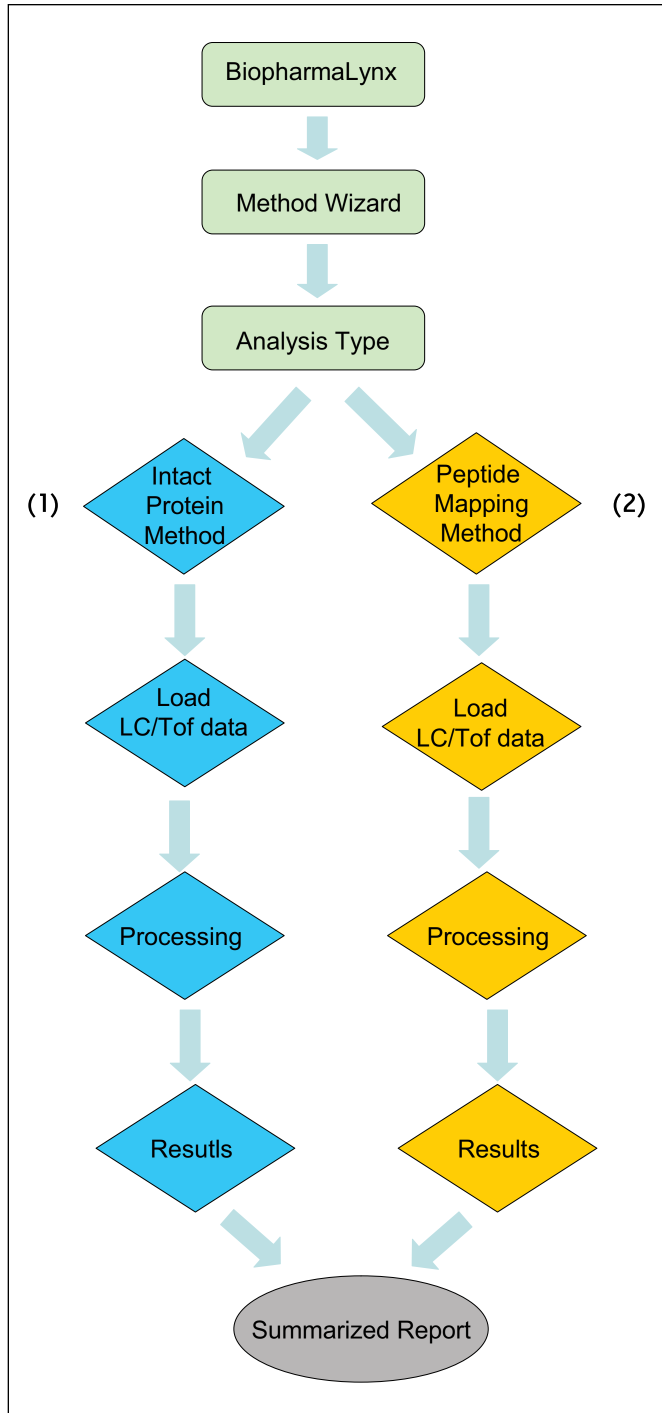
Intact protein LC/MS analysis has traditionally been a repetitive task of spectral summation, spectral deconvolution, and manual assignments of deconvoluted masses to product variants. As the number of routine intact mass analyses grows, so does the desire for batch processing of intact protein LC/MS data and automated annotation of protein structures to the data.

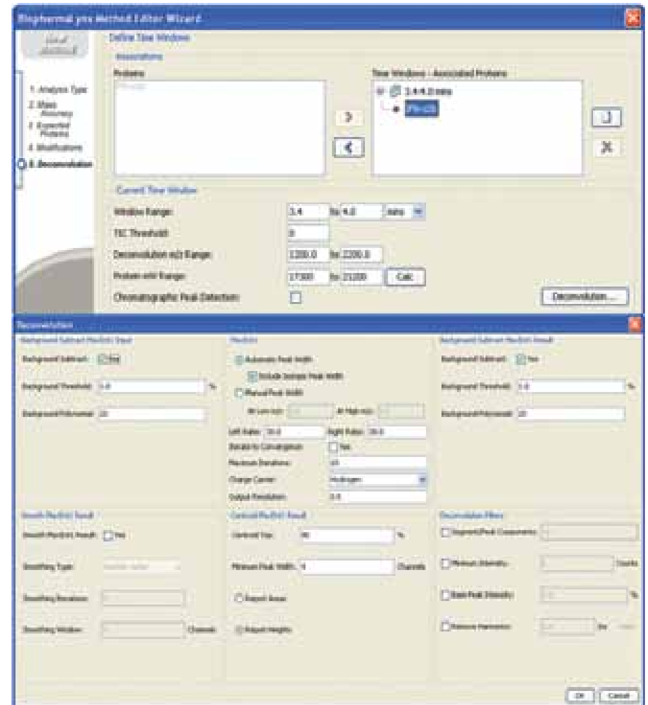
LC/MS peptide mapping analysis involves much greater levels of repetitive data processing for each run of acquired data, and presents significant challenges when inter-run comparisons are required. Many labs have turned to proteomic software tools to automate data processing, only to find that these tools are rather blunt for biopharmaceutical analysis. The proteomic focus on protein-level identification rather than peptide-level characterization results in overall poor characterization of a protein, and a significant lack of information about the sample. Proteomic software also lacks appropriate functionality for structural investigations, such as disulfide mapping, glycopeptide characterization, and quantitative investigations into sample-sample differences.

In this application note, we focus on the application of BiopharmaLynx™ Software, an informatics tool that automates processing and analysis of both intact mass and peptide mapping biopharmaceutical workflows. A case example is presented for recombinant Interferon produced by two cell culture conditions, and the ability of the software to process, annotate, and compare the results from the LC/TOF-MS analysis of these two samples. The benefits of this combined intact mass-peptide mapping approach for readily detecting and highlighting the structural differences between these samples is illustrated.

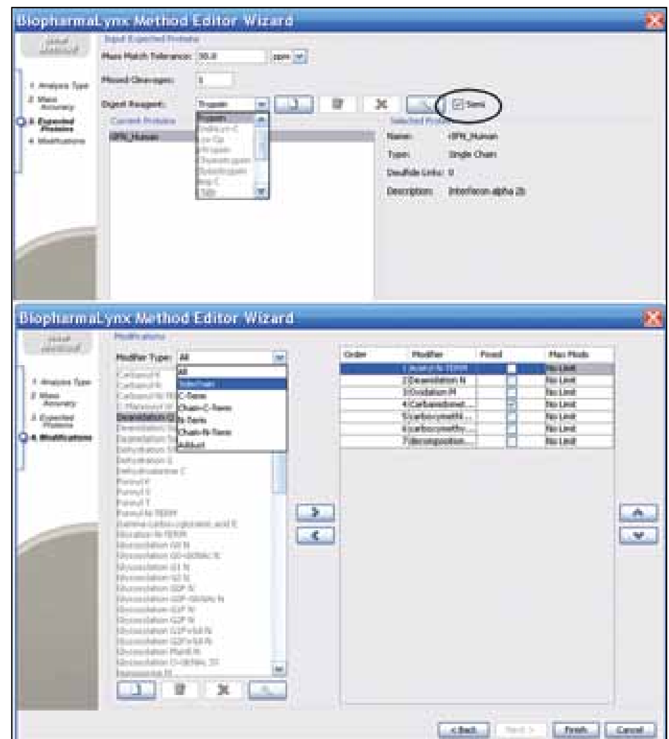
METHODOLOGY

The biopharmaceutical workflow using BiopharmaLynx 1.1





(1) Method wizard: Intact protein.



(2) Method wizard: Peptide mapping.

RESULTS

Intact Interferon protein analysis

Our experimental goals are to obtain intact protein MW, compare the MW of Interferon expressed from two cell culture conditions (control vs. analyte), provide purity assessment, and search for post-translational modifications (PTMs). Details of the LC/MS experiments are listed in the Experimental Details section. An example of the BiopharmaLynx Intact Protein Analysis Method Editor is shown in the BiopharmaLynx 1.1 workflow.

There are multiple ways to display results in the BiopharmaLynx browser. Each display reveals a different aspect of the data. For example, the display of Total Ion Chromatogram (TIC) gives the chromatographic retention time for each component, the Raw Spectrum View displays the charge envelope of the intact protein, the Processed Spectra View shows the deconvoluted mass spectrum and relative intensities of components, and the peak match data table summarizes all the above information in a spreadsheet format (Figures 1 and 2).

Figure 1. Results display for control and analyte Interferon protein.

A) Total Ion chromatogram (TIC) where the main Interferon peak is highlighted. The later eluting peaks represent product-related impurities..

B) Raw Spectrum view: m/z of multiply charged ions of Interferon (+9 to +15 ion series were selected and summed for mass deconvolution).

C) Deconvoluted intact Interferon mass spectra in mirror view. The Δmass of + 42 Da observed between control and analyte results from mono-acetylation of the analyte sample. Also, minor components (~6%) representing oxidation (+16 Da) of the major form
were observed for both samples.

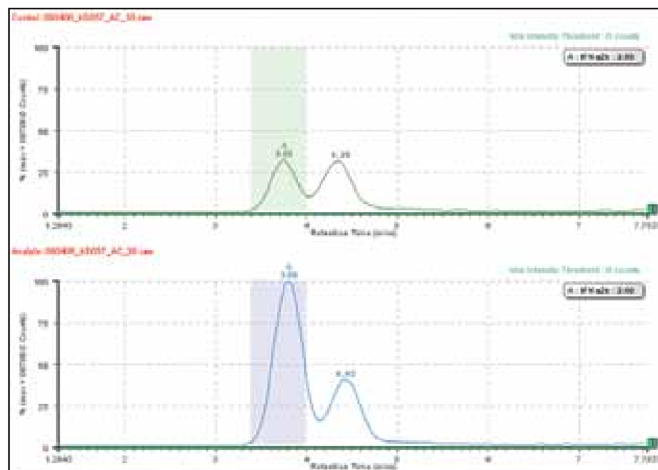


Figure 1A. Total Ion Chromatogram (TIC) View.

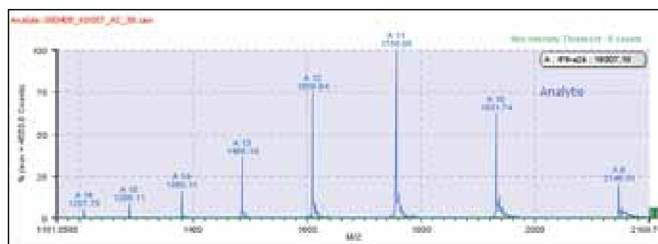


Figure 1B. Raw Spectrum View.

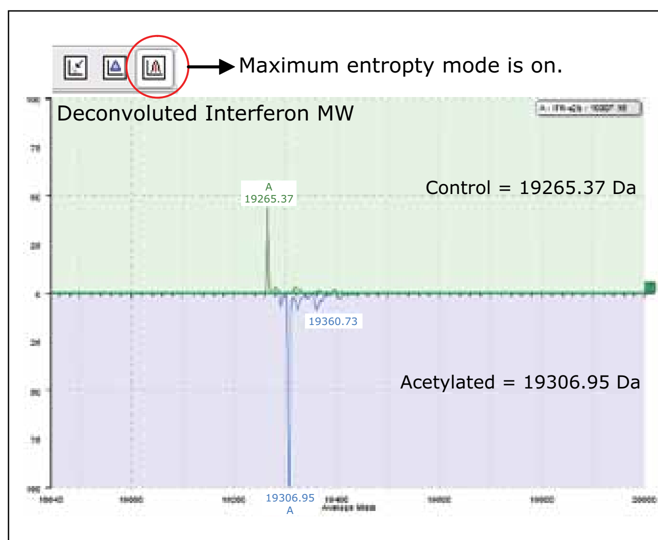


Figure 1C. Processed Spectra View.

Chromatogram | Spectrum | Coverage Map | Protein Digests | Peak Match Data

Protein	Modifiers	Calculated Protein Mass (Da)	Control Intensity (Counts)	Control Mass Error (ppm)	Analyte Intensity (Counts)
IFN-α2b		19265.1543	24497.1	11.3	
IFN-α2b	Oxidation M(1)	19281.1484	1409.8	3.9	
IFN-α2b	Acetyl N-TERM(1)	19307.1641			55893.1
IFN-α2b	Acetyl N-TERM(1),Oxidation M(1)	19323.1602			5362.1
IFN-α2b	Acetyl N-TERM(1),Oxidation M(4)	19371.1445			2841.1

Figure 2. Peak match data table displays the mass spectral results annotated with the targeted protein search results. Information such as modifications, intact protein mass, and component are listed. These search results can be customized, filtered, and sorted by any category. Intensity thresholds can be adjusted to prevent false assignment to lower level spectral noise peaks.

Peptide mapping analysis

With the knowledge gained from the intact protein analysis, peptide mapping analysis in BiopharmaLynx can focus on more specific questions. A common goal for peptide mapping is to confirm protein identity and characterize the primary protein structure with a high coverage LC/MS peptide map. Secondary goals usually involve annotating modifications (e.g., finding the acetylation site suggested by analyte intact protein data), and producing comprehensive sample-to-sample comparisons. Again, these results can be viewed and compared in different formats (sequence coverage map, TIC, spectrum or a data table).

Figure 3 shows results in a protein sequence coverage map display. High protein coverage (97.0% and 96.4% in control and analyte respectively) was achieved. BiopharmaLynx also identified the acetylation site as the N-terminus cysteine in the analyte. This finding correlates the intact protein results (Figure 1C). The color coding scheme depicted in the left panel of Figure 3 allows users to readily visualize control vs. analyte sample differences. As an example, the acetylation unique to the analyte sample is highlighted with an orange hue.

The high mass accuracy from TOF MS data (< 10 ppm routinely with lock mass correction) enables confidence in producing assignments of ions to peptides generated by proteolytic digests. To more comprehensively assign all the ions detected during the LC/MS analysis, the analysis of semi-digested can be enabled. Even under optimal conditions, the electrospray process can generate in-source fragments of labile peptides, and other ions representing neutral loss of water and ammonia. BiopharmaLynx 1.1 now recognizes and annotates these ions, and allows users to expand the range of modifications and MS adducts beyond those found in the default library.

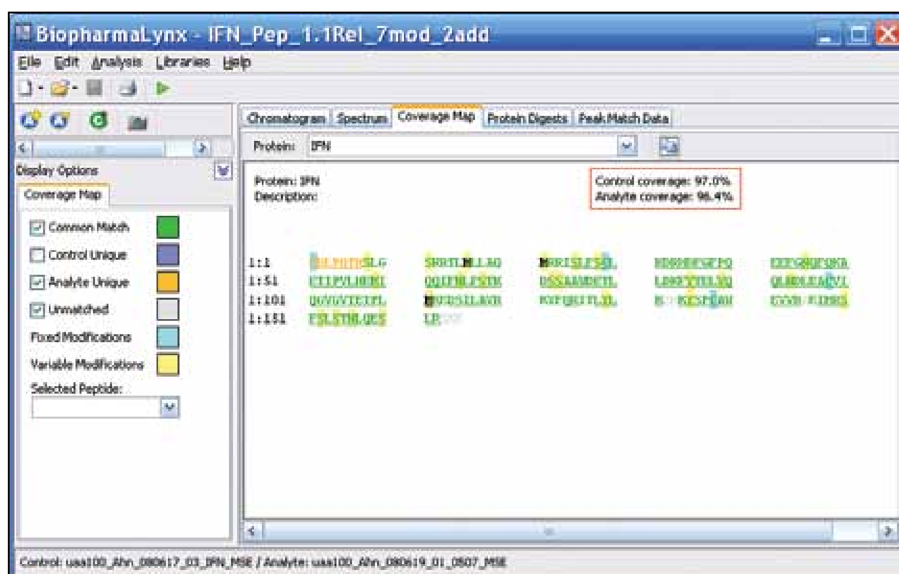
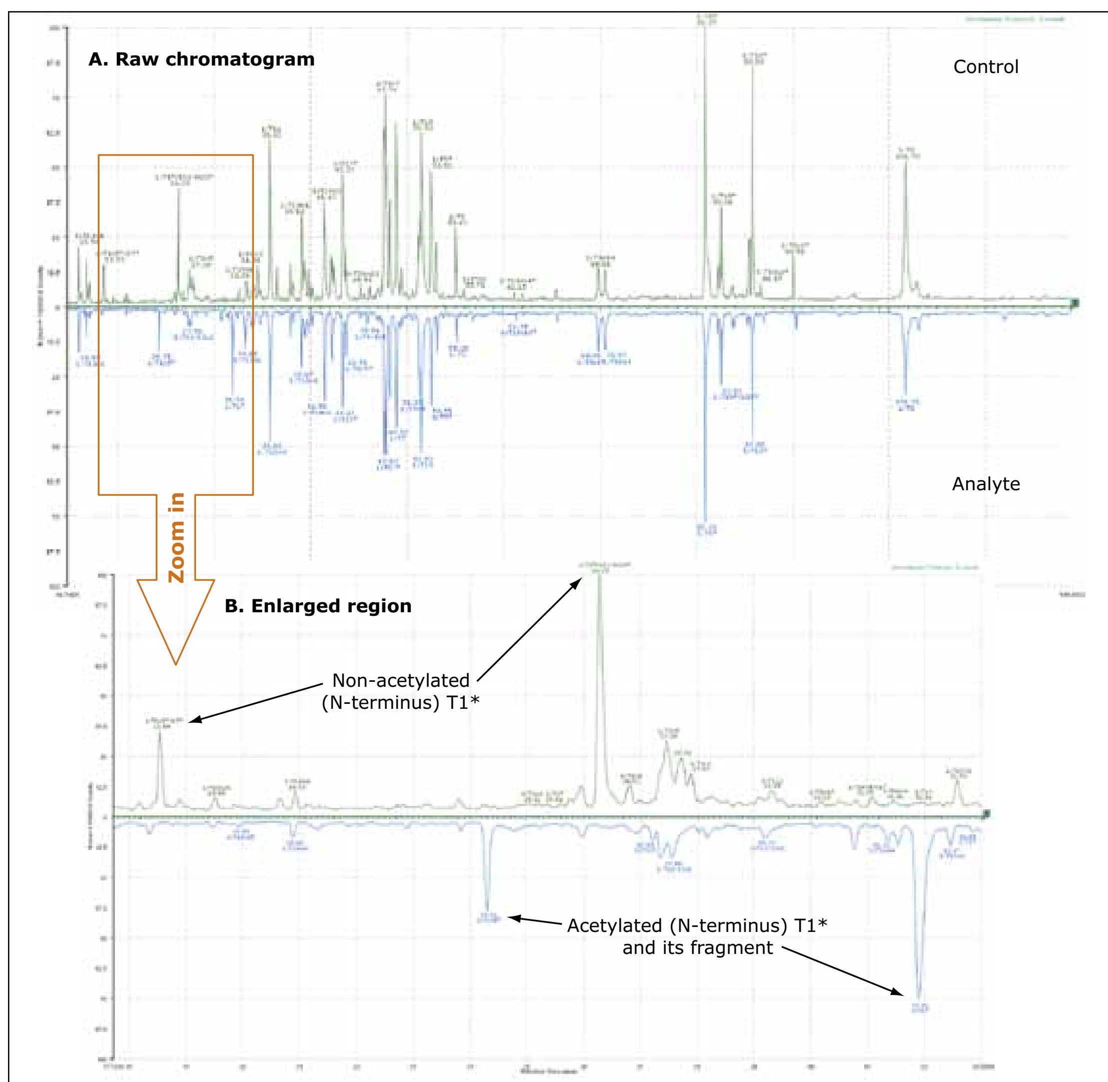


Figure 3. Protein sequence coverage for control and analyte Interferon samples.

The raw TIC display depicts the overall elution pattern for all peptides detected during the LC/MS map analysis. Visualizing significant differences between samples can often be accomplished using the mirror plot view of the data (Figure 4A). Tryptic peptide peaks are automatically annotated using Tryptic peptide fragment numbers (T_n) and retention time in chromatograms.

A segment of the chromatogram in Figure 4A was enlarged to show peaks that do not align between the control and the analyte samples (Figure 4B). These peaks reflect T1 (CDLPQTHSLGSR) modification differences: the control T1 peptide was carbamidomethylated (during protein reduction/alkylation step) while the analyte T1 peptide was carbamidomethylated and acetylated.



(e.g., T --- Tryptic peptides (T1, first peptide from N-terminus)
** --- post translational modifications*
n/c --- partial enzymatic cleavage from C- terminus (or N-terminus), e.g., T1/c5 or T1/n2
y/b --- y (or b) series of in-source fragment ions of T1, eg. T1/y11 or T1/b3

Figure 4. (A) TIC (mirror view) of the two Interferon peptide maps. A region of the chromatogram that shows unique peptides for each sample is highlighted. (B) The enlarged chromatogram region indicates that these unique peptides were all generated from Interferon T1 tryptic peptide and a semi-tryptic fragment of this peptide that differ between samples by acetylation of the N-terminus.

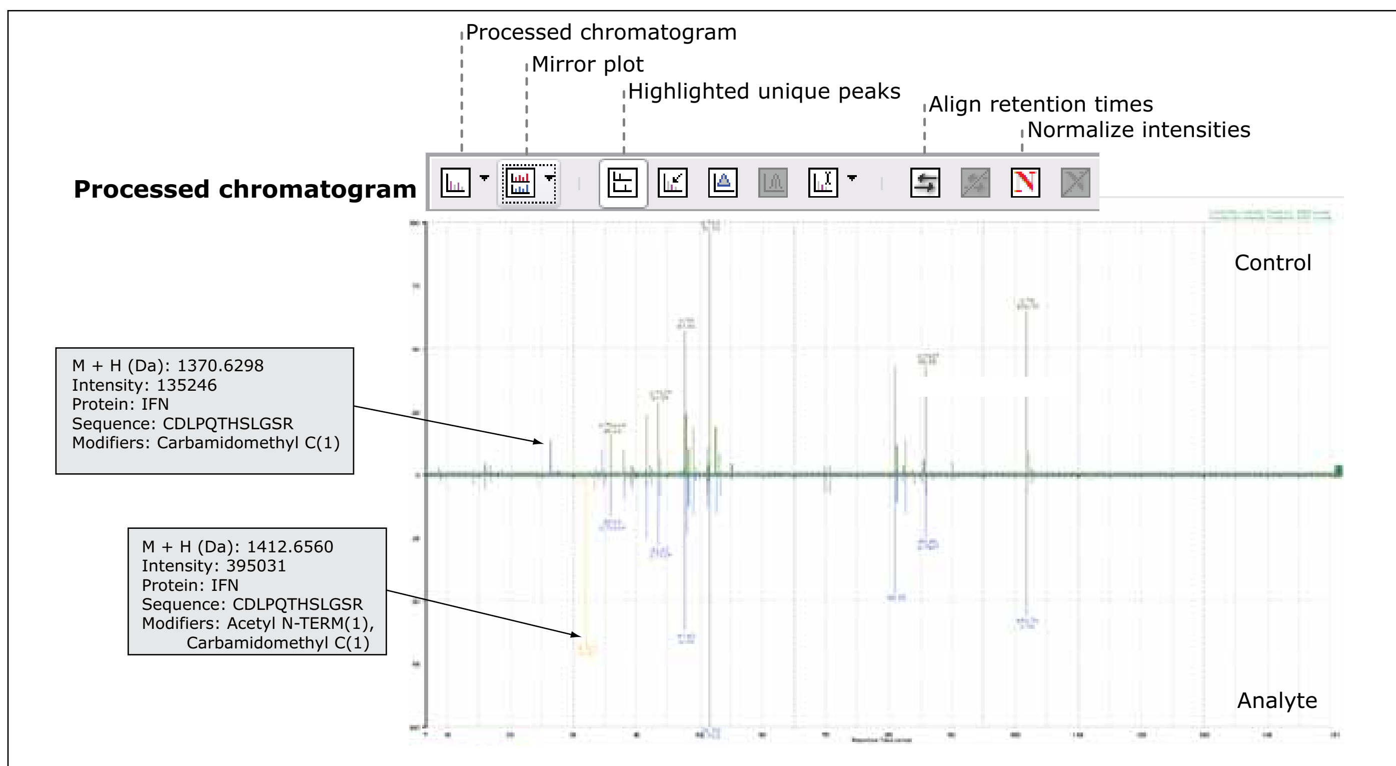


Figure 5. The processed chromatograms displayed in mirror plot with highlighted unique peaks in purple and orange colors for control and analyte interferon peptides.

More sample information is highlighted in Figure 5, which displays the mapping results as a component-centric processed chromatogram view. Each centroided "stick" in the processed chromatogram represents the summed intensity (ion counts) of all isotopes from all detected charge states of a peptide over its full chromatographic elution profile. This simplified view enables several useful tools potentially needed for efficient sample-sample comparisons, including alignment of LC retention times and normalization of ion intensities for better relative quantitation results. Additional information about peptide assignment is accessible by moving the mouse over any given peak.

CONCLUSION

In this study, we have demonstrated how automated BiopharmaLynx data processing promotes rapid comparisons of two batches of Interferon produced by different expression systems. Both the top-down intact protein analysis and the bottom-up peptide mapping analysis supported the finding that the two batches differed primarily by the presence of N-terminal acetylation in one sample.

BiopharmaLynx is a key addition to the Waters UPLC/TOF-MS system solution for biotherapeutic protein characterization.

BiopharmaLynx automates most aspects of data processing, protein/peptide annotation, sample comparison, and report generation, which are currently viewed as the major productivity bottlenecks for most biopharmaceutical characterization laboratories. Routine characterization studies that take well-trained scientists several days to weeks to manually process can be batch processed by BiopharmaLynx in minutes. The laboratory productivity gained by allowing scientists to focus on higher-value activities rather than routine data analysis should permit development organizations to expand the breadth of their activities and streamline timelines existing investigations.

EXPERIMENTAL DETAILS

Intact Protein LC/MS

Sample preparation

Two recombinant Interferon expressed under different cell conditions were used (control and analyte). Interferon samples were reconstituted in 50 mM ammonium bicarbonate buffer, micro-centrifuged, and injected onto the column for LC/MS analysis. Additional details on the method can be found in Waters Application Note 720002107en.

LC conditions

LC system: Waters® ACQUITY UPLC® System
Column: MassPREP™ Micro Desalting Column (2.1 x 5 mm)
Mobile phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% Formic acid in acetonitrile
Gradient: Total run time: 10 min
5-10 %B in 0.5 min, 0.2 mL/min (waste)
Then 10-20 %B in 0.01 min, 0.2 mL/min (MS)
Then 20-45 %B in 7.1 min (MS), 0.2 mL/min
Then 3-sawtooth 90-5 %B in 0.5 min, 0.5 mL/min
Column temp.: 80 °C

MS conditions

MS system: Waters LCT Premier™ ESI-TOF MS
Ionization mode: ESI Positive, V mode
Capillary voltage: 3200 V
Cone voltage: 40 V
Desolvation temp.: 350 °C
Source temp.: 150 °C
Desolvation gas: 800 L/Hr
Ion guide 1: 5 V
Acquisition range: 600 to 3000 m/z

Peptide mapping LC/MS

Sample preparation

The control and analyte were dissolved in 50 mM ammonium bicarbonate and denatured with 0.025% RapiGest™ SF (Waters), then reduced and alkylated with DTT and iodoacetamide (Sigma) respectively. Finally the protein was digested with porcine trypsin (Promega).

LC conditions

LC system: Waters ACQUITY UPLC System
Column: ACQUITY UPLC BEH 300 C_{18}, 2.1 x 150 mm, 1.7 µm 300Å
Flow: 200 µL/min
Mobile phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% Formic acid in acetonitrile
Gradient: 0-50 %B over 120 min
Column temp.: 60 °C
Weak wash: 95% buffer A / 5% buffer B
Strong wash: 20% buffer A / 80% buffer B

MS conditions

MS system: Waters SYNAPT™ MS
Ionization mode: ESI Positive
Capillary voltage: 3.0 kV
Cone voltage: 35 V
Desolvation temp.: 250 °C
Desolvation gas: 350 L/Hr
Source temp.: 120 °C
Acquisition: 50 to 1700 m/z
Lockmass: 100 fmol/µL Glu-Fibrinopeptide B $(M+2H)^{2+}$ =785.8426

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34 Maple Street
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Improving Peptide Mapping Productivity with UPLC/MS^E and BiopharmaLynx: Comprehensive Protein Characterization without Time-Consuming Chromatographic Method Optimization

Hongwei Xie, St John Skilton, Scott J. Berger, and Martin Gilar
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The fundamental ethic of the well-characterized biopharmaceutical is that drug developers can define and monitor the critical attributes of a biotherapeutic, and that they can produce a process that maintains control over these characteristics. The faster you can define the underlying structural variation in a product, the faster you can proceed through product commercialization and implement process improvements.

Peptide mapping by LC/MS is an essential technique used by the biopharmaceutical industry to examine the primary structure of a protein. Mass spectrometry provides an extra dimension of separation over traditional UV detection methods. This removes the absolute reliance on effective chromatographic resolution to enable comprehensive qualitative and quantitative peptide map analysis. In an LC/MS peptide map, peptides can partially or fully coelute without impairing the ability to detect, assign, and quantify them. Thus, an MS detection workflow enables product characterization to proceed independently of the time-consuming efforts to develop the more resolving LC/UV peptide maps that are required for later development and quality control activities. The challenge with implementing LC/MS peptide mapping has been that data processing and interpretation were productivity-limiting.

The introduction of data-independent acquisition approaches such as UPLC®/MS^E has enabled shorter peptide mapping runs, and simultaneously increased the amount and quality of information available for biotherapeutic characterization. MS^E is a technique that acquires accurate peptide and fragment mass data for all peptides within a sample within a single run. The MS^E fragmentation data can be used to validate the accurate mass assignment of a peptide and localize assigned modifications within the peptide sequence. Fragmentation information is collected in parallel for all peptide ions, avoiding the bias and analytical irreproducibility that occurs with data-dependent approaches that require serial pre-selection of peptide precursor ions. LC/MS^E datasets are acquired with two alternating MS functions: one for MS of peptide precursors acquired at low collision cell energy, and one (MS^E) for collecting peptide fragmentation data at elevated collision cell energies.

Several publications[1-5] have detailed the power of processing such datasets using the chromatographic profiles of detected ions to determine isotopic cluster, charge state, and precursor ion/fragment ion relationships in MS^E datasets. Using this methodology, the resulting peptide information included retention time, accurate mass, intensity, and fragmentation profiles for all detected peptides within a peptide map.

In this application note, we show how BiopharmaLynx™ software quickly automates assignment of peptide sequence information from MS^E fragmentation data, even for cases of coeluting peptides. The ability of the LC/MS^E approach to reduce mAb peptide mapping run times from 180 min (300-mm column) to 60 minutes (100-mm column) without affecting peptide sequence coverage and fragment pattern interpretation is demonstrated.

Automating data analysis enables analysts to realize the increased productivity gained from using shorter LC/MS methods for product characterization studies. Furthermore, the ability of the underlying MS^E methodology to deal with high-complexity data enables the production of generic LC/MS screening methods for a wide array of biotherapeutic products. Productivity gains on the order of weeks to months can be expected compared to time spent optimizing and analyzing LC/UV peptide maps.

EXPERIMENTAL

Antibody digest

A commercially available monoclonal antibody (mAb) of approximately 148 kDa was digested with trypsin using a protocol described elsewhere.[6]

Chromatographic system

The Waters ACQUITY UPLC® System was configured with a standard peptide mapping mixer (425 µL) and a BEH300 C_{18} 1.7-µm Peptide Separation Technology (PST) Column (2.1 x 100 mm, 2.1 x 150 mm, or two coupled 2.1 x 150 mm columns).

Mass spectrometer

The Waters SYNAPT™ MS System was operated in the ESI+ mode with V ion geometry. Conditions: Source temp.: 100 °C, cone voltage: 37 V. MS^E acquisition: two alternating MS data functions were collected (1 sec total cycle time) with the collision cell at low energy (4 V) for acquisition of peptide mass spectra, and at elevated energy (linear ramp 20 to 40 V) for the collection of peptide fragmentation spectra. LockSpray™: 100 fmol/µL GFP in 50:50 acetonitrile/water containing 0.1% formic acid, sampled once every min.

Informatics

BiopharmaLynx 1.2 Application Manager for MassLynx™ Software.

Methodology

Triplicate analysis of an mAb tryptic digest was conducted using UPLC configurations with column lengths of 100, 150, and 300 mm. A linear gradient (1 to 40% acetonitrile in 0.1% TFA) was scaled with column length for run times of 60, 90, and 180 min, respectively.

RESULTS AND DISCUSSION

Peptide maps are inherently complex. An mAb tryptic digest contains fully-digested peptides, the products of over- and under-digestion, modified peptides, and non-product impurities. The combinations of these factors yield hundreds of unique peptide species for analysis. Coupling high-quality UPLC separations with mass spectrometry offers the ability to detect and monitor these species, even if they possess overlapping chromatographic profiles. Figure 1 shows that peptide coverage of 97%+ of the mAb sequence can be maintained, even when peptide map gradient length is reduced by two-thirds from 180 to 60 min.

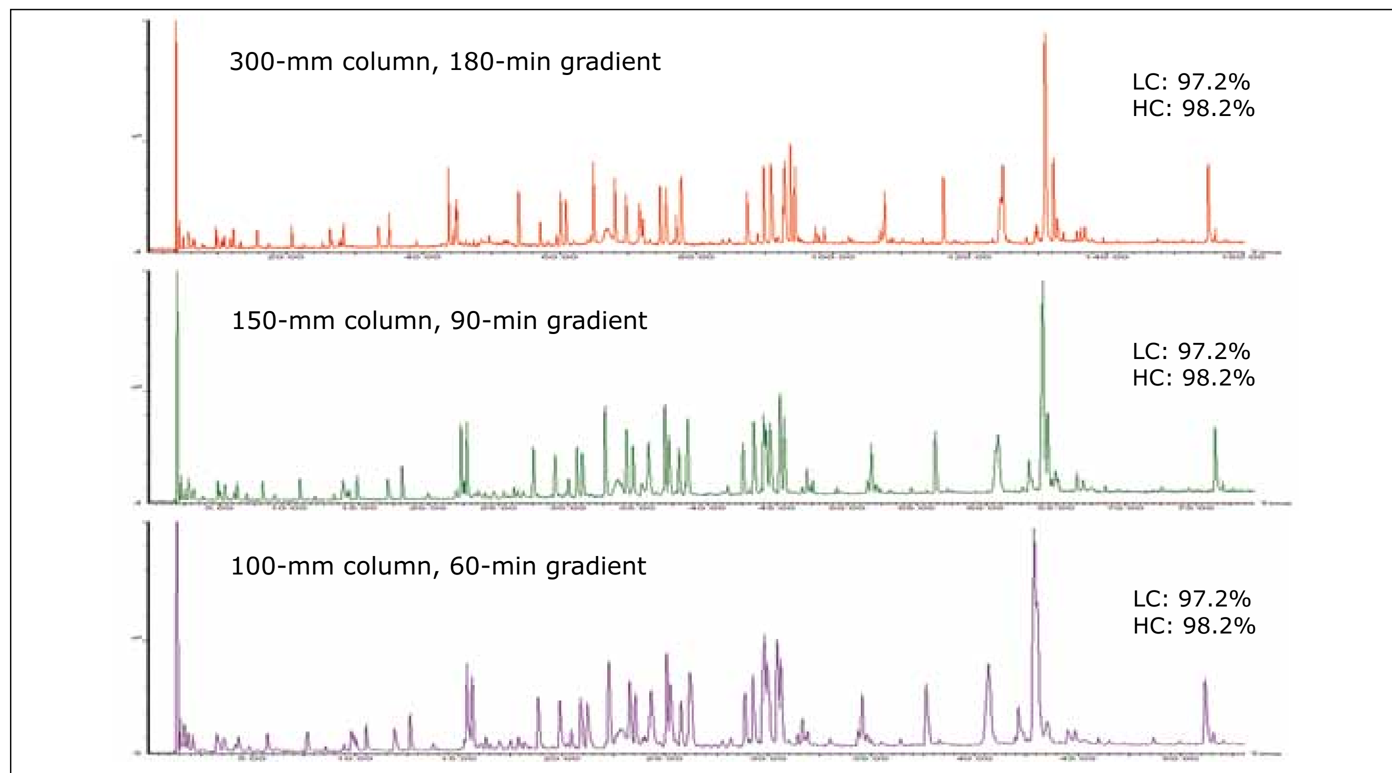


Figure 1. Chromatograms (TIC) for the tryptic digest of an mAb performed on three different column lengths at constant gradient slope. The overall protein sequence coverage of the Light Chain and the Heavy Chain are not affected.

In this example, column length was scaled in concert with gradient length, maintaining a consistent gradient slope over all three conditions, which preserves the overall peptide separation selectivity across the three methods. Even though separation capacity is reduced using the shorter column methods, and many peaks now partially or fully coelute, protein sequence coverage was not sacrificed.

Example peptides from this antibody peptide map are the Heavy Chain tryptic peptides HT11* (modified with an alkylated Cys) and HT6, which are partially resolved chromatographically (10 sec difference at peak apex) using the most resolving separation configuration (300 mm column length and 180-minute run time). The partially coeluting peptides (Figure 2a) were observed to fully coelute using the 90 min gradient (Figure 2b), and slightly resolve using the 60 min gradient (Figure 2c). Interestingly, the selectivity of the separation was sufficiently altered by the changes in column volume and gradient length to reverse the elution order of the two peptides.

BiopharmaLynx 1.2 was able to correctly assign both peptides and apply MS^E fragmentation data for sequence validation when partial and complete peptide coelution were observed. During processing of the MS and MS^E data (see reference 5 for greater detail), the chromatographic profiles of all detected ions were used to establish relationships within isotopic clusters, between charge states, and ascertain the correspondence of precursor ions in the MS scan with their cognate fragments in the MS^E scan.

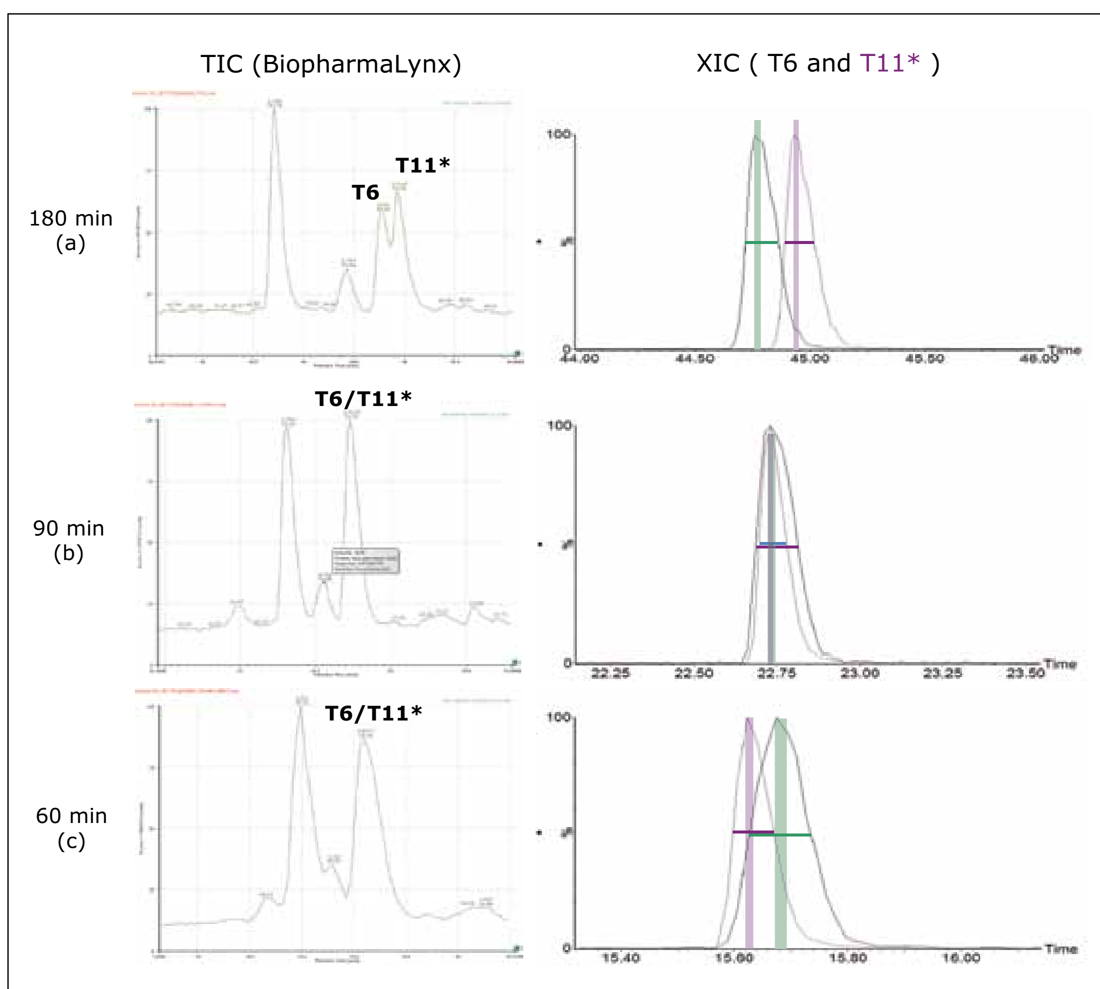


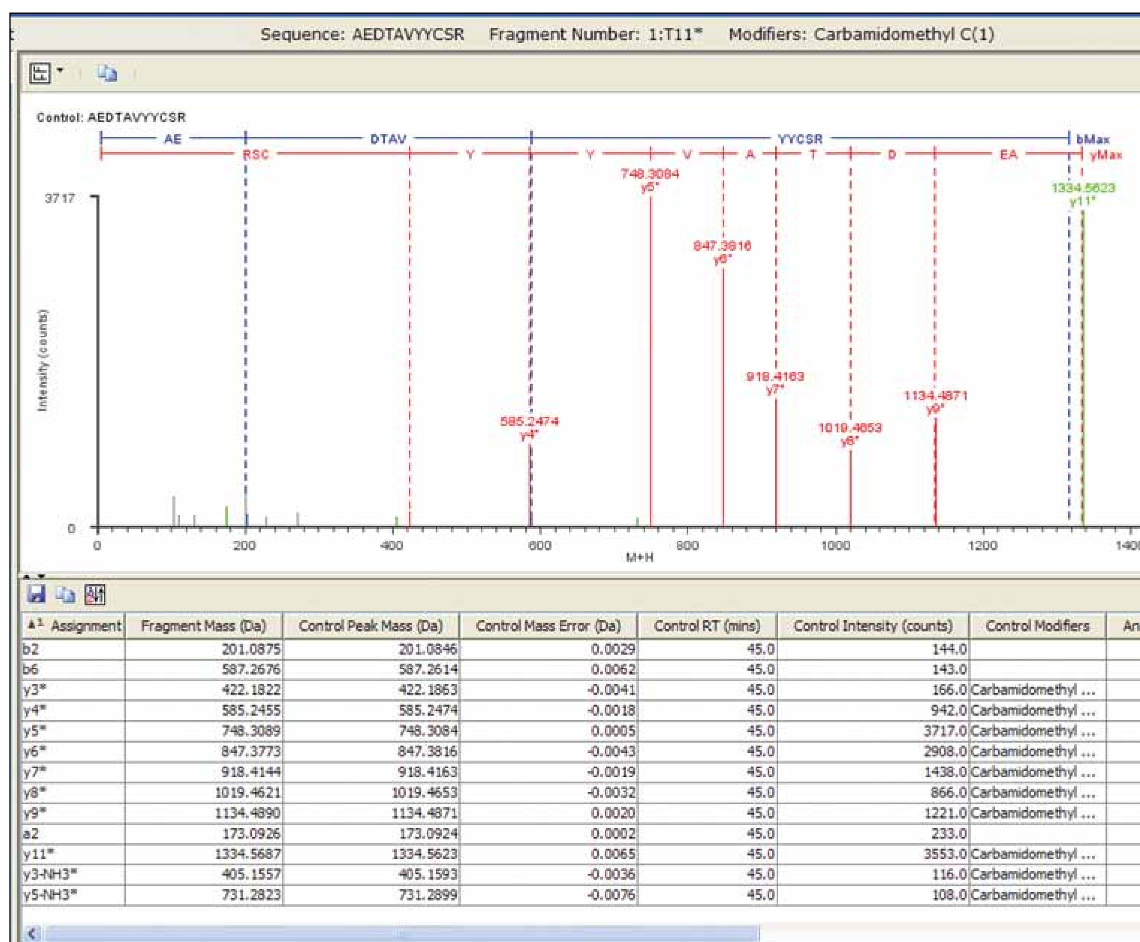
Figure 2. BiopharmaLynx Total Ion Chromatograms (left) and MassLynx eXtracted Ion Chromatograms. Panel (a) 180-min map showing partial resolution of HT11 (44.96 min) and HT6 (44.80 min) peptides. Panel (b) 90-minute map showing the co-elution of HT11* and HT6 (22.75 and 22.76 min) peptides. Panel (c) 60-minute map showing the partial coelution of HT11* and HT6 (15.64 and 15.69 min). Peak width at half height is represented as a horizontal line, while the time region for detecting associated MS^E fragments is represented as a vertical bar. Green=T6, Purple=T11*.*

The most basic of these relationships is that MS^E fragments will exhibit an apex retention time within one-tenth of a chromatographic peak width of the MS precursor ion from which they were generated. Thus, fragments from partially coeluting peptides with peak apex retention times outside this limited time window have no contribution to the fragment list used to validate a peptide assignment. This is visually displayed (Figure 2) as green (T6) and purple (T11*) vertical bars representing the RT range where the chromatographic apex of associated MS^E fragments would be detected. For the 180 and 60 min maps, these time regions are clearly distinguished, and no overlapping fragmentation data should be observed. The 90-min map represents a case of near absolute coelution, and it would be expected that these peptides would share a subset of MS^E fragment ions.

The MS^E data for the MS and MS^E traces is automatically processed in BiopharmaLynx 1.2 so that the user is presented with an appropriate time-aligned fragmentation spectra for each peptide (Figure 3). Using this methodology, the validation of peptide sequence in peptide maps becomes automated, simpler, and faster. It is also important to note that the experiment to acquire this confirmatory fragmentation data is no more complex than acquiring the MS data alone. All that was required was the addition of the second MS^E acquisition data channel within the MS acquisition method.

The fragmentation spectrum of HT11* from the 180-min separation depicts a series of y-ions and b-ions that sequence through the site of Cys modification, confirming both the peptide and modification site. As expected, no fragment overlap is observed with the partially coeluting T6 peptide, and all major ions assign to the T11* sequence.

In the 90-min mapping run, the T6 peptide was observed to fully coelute with the HT11* peptide. The use of accurate mass



Assignment	Fragment Mass (Da)	Control Peak Mass (Da)	Control Mass Error (Da)	Control RT (mins)	Control Intensity (counts)	Control Modifiers	Anal
b2	201.0875	201.0846	0.0029	45.0	144.0		
b6	587.2676	587.2614	0.0062	45.0	143.0		
y3*	422.1822	422.1863	-0.0041	45.0	166.0	Carbamidomethyl ...	
y4*	585.2455	585.2474	-0.0018	45.0	942.0	Carbamidomethyl ...	
y5*	748.3089	748.3084	0.0005	45.0	3717.0	Carbamidomethyl ...	
y6*	847.3773	847.3816	-0.0043	45.0	2908.0	Carbamidomethyl ...	
y7*	918.4144	918.4163	-0.0019	45.0	1438.0	Carbamidomethyl ...	
y8*	1019.4621	1019.4653	-0.0032	45.0	866.0	Carbamidomethyl ...	
y9*	1134.4890	1134.4871	0.0020	45.0	1221.0	Carbamidomethyl ...	
a2	173.0926	173.0924	0.0002	45.0	233.0		
y11*	1334.5687	1334.5623	0.0065	45.0	3553.0	Carbamidomethyl ...	
y3-NH3*	405.1557	405.1593	-0.0036	45.0	116.0	Carbamidomethyl ...	
y5-NH3*	731.2823	731.2899	-0.0076	45.0	108.0	Carbamidomethyl ...	

Figure 3. BiopharmaLynx 1.2 fragment ion display for the T11 peptide (T11 peptide with a modification) of the Heavy Chain from the 180-min map.*

fragmentation data permits BiopharmaLynx 1.2 to automatically and unambiguously assign the correct fragments to each coeluting peptide (Figure 4). The MS^E fragmentation data was clearly able to validate the map assignments for both peptides, and with the same level of sequence detail for HT11* as was seen from the longer run. Ions corresponding to the assignments for the indicated peptide are colored in red (y-ions), blue (b-ions), and green (neutral loss ion), while ions assigned to the coeluting peptide are clearly distinguished as pink colored peaks. In both instances, the user can use the pattern of major fragment ions to confirm that the accurate mass assignment of each peptide is correct.

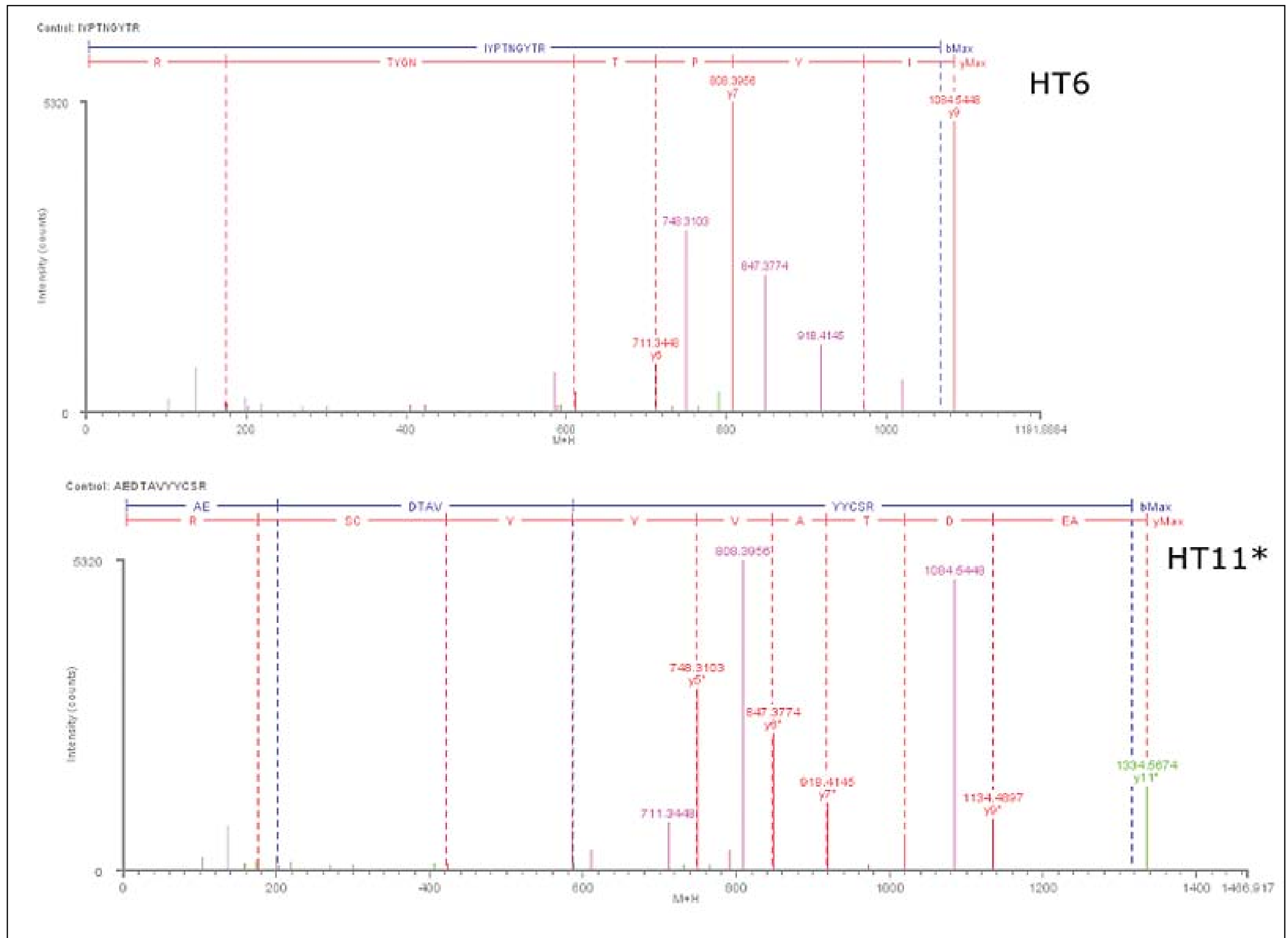


Figure 4. BiopharmaLynx 1.2 fragment ion display of peptide sequence of HT6 and HT11 from the 90-min map. The colors of the assigned peaks indicate that a peak is assigned to the indicated sequence (red y-ion, blue b-ion, green neutral loss ion), or assigned to a coeluting peptide (pink). Grey ions represent peaks that were not assigned.*

Even in shorter peptide maps, perfect coelution of peptides is rather rare. The data for the two peptides from a shorter 60-min map show that two peptide peaks are resolved by a ~10% valley (XIC plots in Figure 2c), and that no overlap of MS^E fragmentation data is observed (Figure 5.)

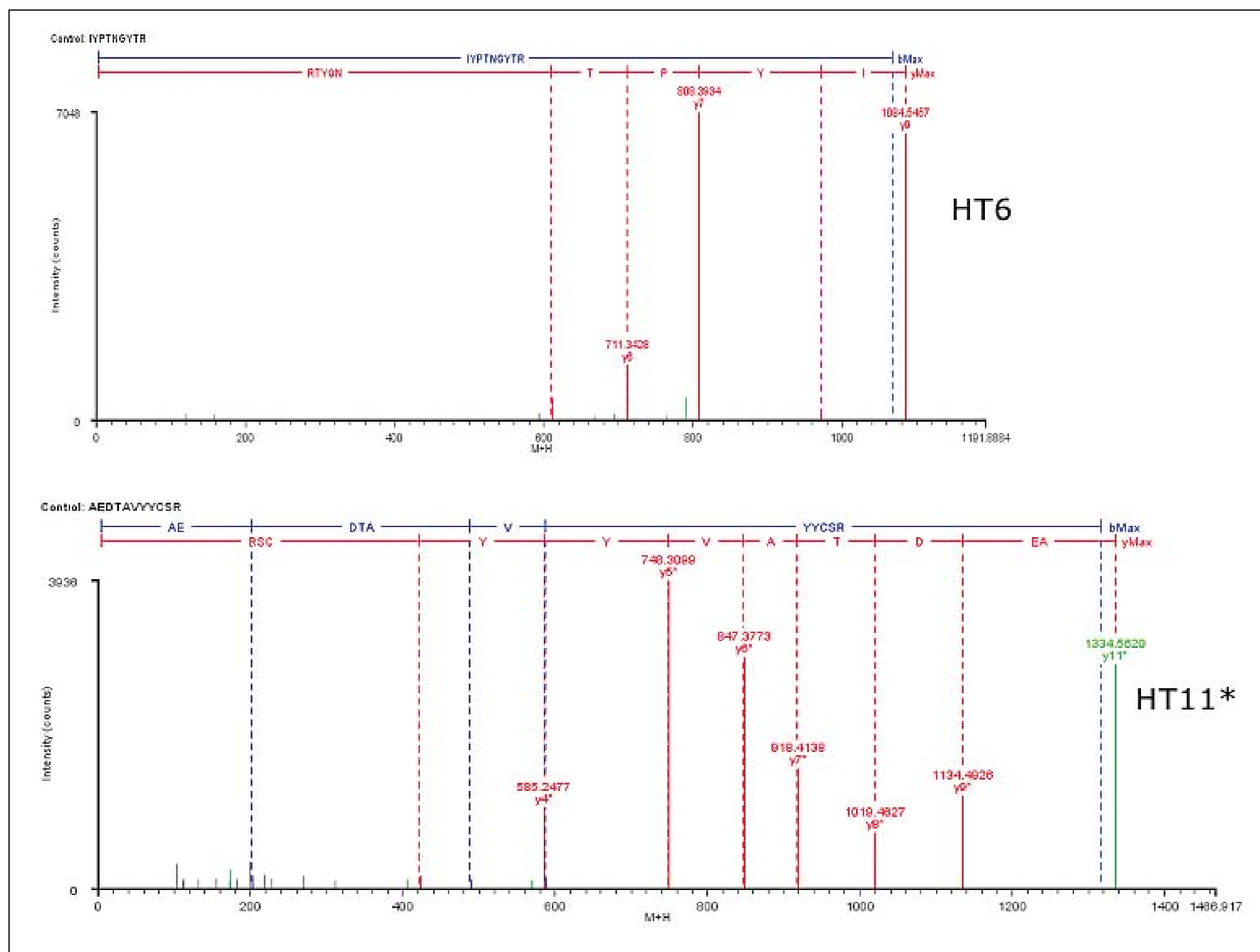


Figure 5. Panel (a) BiopharmaLynx 1.2 fragment ion display of peptide sequence of HT6 and HT11 from the 60-min map. The colors of the assigned peaks indicate that a peak is assigned to the indicated sequence (red y-ion, blue b-ion, green neutral loss ion), or assigned to a coeluting peptide (pink). Grey ions represent peaks that were not assigned.*

CONCLUSION

- The application of UPLC/MS^E methodology to peptide mapping enables universal and unbiased data acquisition that provides for comprehensive characterization of a therapeutic antibody.
- The tolerance of this approach for imperfect chromatographic resolution of components enables users to obtain qualitative and quantitative mapping results without significant investments in chromatographic method development.
- BiopharmaLynx 1.2 Software automates the processing of MS^E analyses, and presents the user with information that allows for better decisions to be made faster.
- The combination of these technologies permits scientists to acquire peptide mapping data using shorter analysis times without sacrificing protein coverage or peptide identification quality.

Acknowledgement

The authors wish to thank Aviva De Beer-Heidt for her insightful contributions to this application note.

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Monitoring Deamidation Progression in an Antibody Tryptic Digest using UPLC/MS^E with BiopharmaLynx 1.2 and Xevo QTof MS System. Waters. 2009; 720003168en.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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[APPLICATION NOTE]

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Monitoring Deamidation Progression in an Antibody Tryptic Digest using UPLC/MS^E with BiopharmaLynx and a Xevo QTof MS System

Hongwei Xie, Martin Gilar, Asish Chakraborty, Weibin Chen, and Scott Berger
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Monoclonal antibodies (mAb) represent roughly half of all therapeutic proteins in clinical and preclinical development. While overall antibody stability is an attractive feature of this class of biotherapeutics, they are subjected to a variety of chemical degradations that can occur during production and upon extended storage. Deamidation of asparagine (N), and to a lesser extent glutamine (Q), residues is a common pathway for mAb degradation. Efficient detection and sensitive monitoring of deamidation level and sites has become a routine challenge for bioanalytical scientists.

In previous studies,[1-2] we have demonstrated that peptide mapping with UPLC®/MS^E is capable of the separation and identification of peptides and their deamidated variants. Specifically, UPLC resolves the two common N-deamidated peptide products (where asparagine is converted to isoaspartic (isoD) or aspartic (D) acid) in addition to resolving a succinimide intermediate of the deamidation reaction. Using accurate mass UPLC/MS^E analysis, these modification sites can be unambiguously identified and quantified.

Furthermore, the processing and interpretation of this complex mapping data can be automated using BiopharmaLynx,™ an informatics tool developed for analysis of peptide mapping and intact protein data from therapeutic proteins.[3] This overall workflow allows annotation and quantitation of peptide maps with greater speed and confidence than manual data analysis or analysis with bioinformatics tools designed for proteomic investigations.

In this application note, we demonstrate the use of UPLC/MS^E and BiopharmaLynx for fast and effective monitoring of deamidation in an accelerated stability study of a monoclonal antibody tryptic digest. *In vitro* deamidations were introduced by exposing the peptide digest to elevated temperature (60 °C) and alkaline pH (~ 9). Deamidation changes over time were monitored by UPLC/MS^E analysis using a Xevo™ QTof MS System coupled with an on-line ACQUITY UPLC® peptide mapping system. The collected LC/MS^E data were analyzed using BiopharmaLynx 1.2 Software to identify and quantify the levels of various deamidated peptides.

EXPERIMENTAL

The detailed tryptic digestion procedure has been described previously.[2] Briefly: at presence of 0.05% RapiGest SF, the mAb was digested with trypsin for 4 hours after reduction with dithiothereitol and alkylation with iodoacetamide. The pH of the resulting digest was then adjusted to ~ 9 with 1 M NH_4OH. Aliquots of the alkalized digest were incubated at elevated temperature (60 °C) for 0, 5, 10, 30, and 60 min, respectively. Following incubation, each aliquot was diluted (to 1.5 pmol/µL) with 0.1% formic acid (FA) in 5% acetonitrile, and analyzed by LC/MS^E peptide mapping experiment.

LC/MS^E studies were performed using a Xevo QTof MS System coupled with an on-line ACQUITY UPLC® System. It was configured with a 1.7-µm Peptide Separation Technology (PST) BEH300 C18 Column, 2.1 x 150 mm. Peptides were eluted with a 90-min gradient (1 to 40% acetonitrile in 0.1% FA) at a flow rate of 0.2 mL/min and 60 °C column temperature.

MS^E data were acquired at 1 Hz in ESI positive ion mode, with collision cell energy alternating between low energy (4 V) to collect peptide precursor (MS) data and elevated energy (ramping from 15 to 45 V) to obtain peptide fragmentation (MS^E) data. A capillary voltage of 3.0 kV, source temperature of 105 °C, cone voltage of 25 V, and cone gas flow of 10 L/h were maintained during the analyses. Sampling of the LockSpray™ channel (100 fM GFP in 50:50 acetonitrile/water containing 0.1% FA) was performed every 1 min to ensure mass accuracy. The analyses were completed in triplicate.

Peptide mapping data were batch-processed by BiopharmaLynx 1.2, an application manager for MassLynx™ Software, using traditional tryptic cleavage rules and setting cysteine carbamidomethylation as a fixed modification and N-deamidation as a variable modification. Additional BiopharmaLynx method parameter settings were detailed in a previous publication.[3] Identifications of N-deamidated peptides were confirmed using the MS^E fragmentation data that also enabled the peptide deamidation

sites to be determined. The concentration of deamidations was calculated using reliably measured MS signal intensities.

RESULTS AND DISCUSSION

BiopharmaLynx processing of the UPLC/MS^E data acquired from the accelerated mAb degradation study identified 13 N-deamidation sites located within 10 tryptic peptides. Among them, three -NG- sequence motifs were observed to be hypersensitive to deamidation, with nearly 100% conversion following 10 min treatment. In contrast, two sites with a -NX- motif, where X is an aromatic amino acid, showed slow deamidation (< 10% even after 60 min treatment). Intermediate deamidation sensitivity was observed for the other detected sequence motifs (-NT- (2), -NN- (2), -NQ-, -NA-, -NH- and -NS-). These results are consistent with previous published studies.[4]

We choose a tryptic peptide NQVSLTCLVK (annotated as heavy chain tryptic peptide or HT36) with intermediate sensitivity to deamidation as an example to demonstrate the application of BiopharmaLynx 1.2 for monitoring peptide deamidation progression.

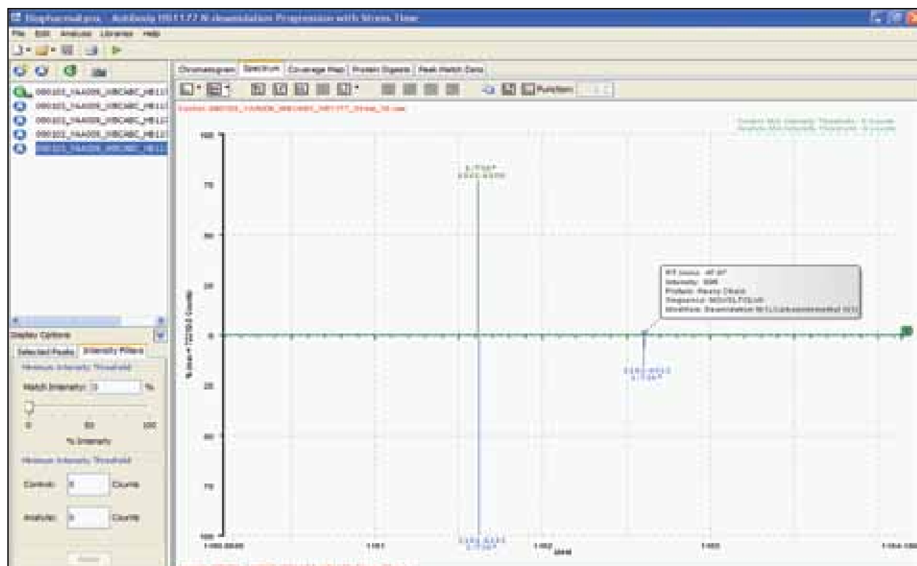

Figure 1. Comparative processed spectra display for HT36 and N364-deamidated HT36 from an antibody digest exposed to 0 (top) and 60 min (bottom) of incubation under stress (60 °C and pH ~ 9).

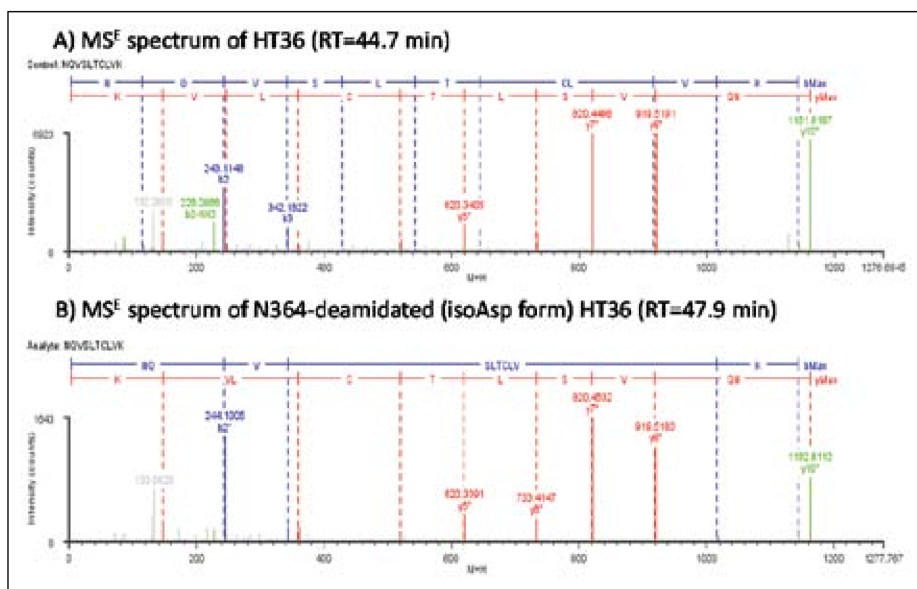

Figure 2. MS^E spectra confirmation for peptide HT36 and its N364-deamidated variant.

BiopharmaLynx - Antibody HB1177 N-deamidation Progression with Stress Time

	Protein	Peptide	Fragment...	Modifiers	Calculated Peptide...	Control RT...	Control Intensity...	Control % Modified	Analyte % Modified
1	Heavy Chain	NQVSLTCLVK	1:T36*	Carbamidomethyl C(1)	1160.6223	44.72	55503.0	97.2	81.7
2	Heavy Chain	NQVSLTCLVK	1:T36*	Deamidation N(1),Carbamidomethyl C(1)	1161.6063	46.66	627.0	1.1	5.6
3	Heavy Chain	NQVSLTCLVK	1:T36*	Deamidation N(1),Carbamidomethyl C(1)	1161.6063	47.87	996.0	1.7	12.7

Figure 3. Comparative tabular view of peptide HT36 and its deamidated products from the 0 min (control) and 60 min (analyte) stress samples.

Cross Analyte Comparison

	Analytes	Sequence	Modifiers	RT (Min)	m/z	Charge	Mass (Da)	Intensity (Counts)	Mass Error (Da)	Mass Error (PPM)	% Modified
1	090103_YAA009_WBCABC_HB1177...	NQVSLTCLVK	Deamidation N(1),Carbamidomethyl C(1)	46.7	581.8073	2	1161.5986	627	-0.0077	-6.6288	1.10
2	090103_YAA009_WBCABC_HB1177...	NQVSLTCLVK	Deamidation N(1),Carbamidomethyl C(1)	46.7	581.8073	2	1161.5988	626	-0.0075	-6.4566	1.43
3	090103_YAA009_WBCABC_HB1177...	NQVSLTCLVK	Deamidation N(1),Carbamidomethyl C(1)	46.8	581.8106	2	1161.6053	1442	-0.0010	-0.8609	2.01
4	090103_YAA009_WBCABC_HB1177...	NQVSLTCLVK	Deamidation N(1),Carbamidomethyl C(1)	46.8	581.8119	2	1161.6079	2268	0.0016	1.3774	3.52
5	090103_YAA009_WBCABC_HB1177...	NQVSLTCLVK	Deamidation N(1),Carbamidomethyl C(1)	46.9	581.8092	2	1161.6025	4997	-0.0038	-3.2713	6.63

Figure 4. Cross Analyte Comparison Table for N364-deamidated HT36 showing increasing deamidation in response to increasing exposure to the stress condition.

Figure 1 contains a comparison of HT36 between two digests subjected to 0 (top panel) or 60 (bottom panel) minutes of the stress condition. The major and minor peaks represent the processed (deisotoped, charge-reduced, and centroided) mass and intensities of tryptic peptide HT36 before (1161.62 Da) and after (1162.60 Da) deamidation. The MS intensity of deamidated HT36 peptide is about 500 times higher in the 60 min treatment condition than in the control digest. This graphical view readily provides information on:

- The identification of the peptide and its deamidated form
- The relative amount of deamidated peptide in each digest
- The different levels of deamidation in the two samples.

MS^E spectra of the unmodified and deamidated peptides (Figure 2) confirm the proper assignment of the ions by BiopharmaLynx. The 1 Da mass difference observed for the y10 and b-series ions in the MS^E spectra, before and after N-deamidation, allows unambiguous determination of the modified asparagine. By clicking on each sample listed in the left panel of BiopharmaLynx, comparable information can be generated for each sample compared to the control digest.

BiopharmaLynx can also present a tabular view to display the identified HT36-related peptides. Figure 3 tabulates the identification of alkylated HT36 peptide (RT 44.72 min) and two deamidated products (RT 46.66 and 47.87 min, representing two isoforms of N-deamidation – isoD and D, respectively). An additional peak at 47.0 min was also observed for the 60 min treated digest (data not shown) showing 1-Da mass increase compared to unmodified HT36, and was due to deamidation of the glutamine adjacent to the N-terminal asparagine. At shorter treatment time, the Q-deamidation species was below the limit of MS detection.

A Cross Analyte Comparison Table (available by clicking on each tabular identification in BiopharmaLynx) is available to track the absolute (in Counts) and relative (in percent of Total Counts) amounts across the samples collected from the whole stability study. For example, the Cross Analyte Comparison Table for the N-deamidated HT36 eluting at 46.66 min is shown in Figure 4. This identification and quantification was fully consistent with the results from manually obtained extracted ion chromatograms (Figure 5).

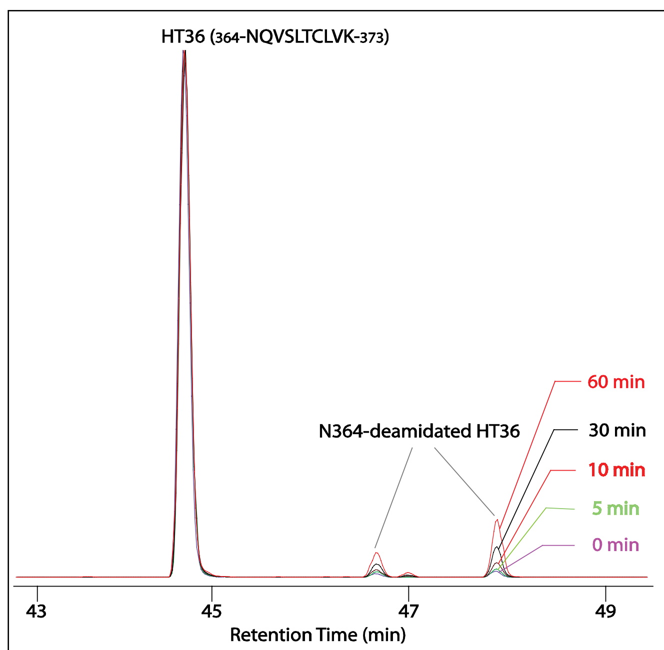

Figure 5. Extracted ion chromatograms (XIC) of peptide HT36 and deamidated products for samples from 0 to 60 min of stress.

Treatment Time	D364 + isoD364**	Unmodified	Standard Deviation
(min)	(%)	(%)	(%)
0	3.0	97.0	0.2
5	3.7	96.3	0.1
10	5.1	94.9	0.4
30	10.7	89.3	0.3
60	18.9	81.1	1.3

C* - Carbamidomethyl cysteine;
** - Averaged from triplicate analysis.

*Table 1. Summary of HT36 (364-NQVSLTC*LVK-373) N364-deamidation from increasing exposure to the stress condition.*

By combining BiopharmaLynx results for the unmodified HT36 peptide and both deamidated products from triplicate injections of each treatment condition, we determined the profile of N-deamination across the treatment time course (Table 1). Using BiopharmaLynx, these results were generated in minutes after the data were acquired.

CONCLUSION

UPLC/MS^E analysis has been shown as a sensitive and effective approach for detection and characterization of protein modifications on therapeutic proteins, including challenging modifications such as deamidation. The desire to detect low levels of multiple deamidated peptides differing by only one Dalton requires the combination of superior chromatography, sensitive and accurate mass spectrometry, and informatics capable of properly interpreting the resulting complex data. The workflow combination of UPLC, PST column chemistry, Xevo QTof, and BiopharmaLynx 1.2 has been shown to excel for this task.

Data analysis has typically been the limiting factor in these studies, and the addition of MS^E fragmentation processing in BiopharmaLynx 1.2 has enabled the interpretation of such data with greater speed and confidence. Automated processing and analysis of the large volume of LC/MS^E peptide mapping data using BiopharmaLynx enabled efficient identification of deamidated peptides, localization of individual deamidation sites, and monitoring of the extent of deamidation across this accelerated degradation study.

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Waters Corporation
34 Maple Street
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T: 1 508 478 2000
F: 1 508 872 1990
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[APPLICATION NOTE]

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Fast and Automatic Mapping of Disulfide Bonds in a Monoclonal Antibody using SYNAPT G2 HDMS and BiopharmaLynx 1.3

Hongwei Xie and Weibin Chen
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

In this study, an advanced LC/MS^E peptide mapping workflow is demonstrated for the detection and identification of disulfide linkages, including scrambled disulfide linkages, in a recombinant IgG1 mAb. We develop a viable, integrated approach for automatic mapping or monitoring of disulfide bonded peptides in a protein with multiple cysteines.

WATERS SOLUTIONS

ACQUITY UPLC

SYNAPT G2 HDMS

BiopharmaLynx Application Manager

MassLynx Software

KEY WORDS

Disulfide bonds, peptide map

INTRODUCTION

Disulfide bond formation is critical for establishing and maintaining proper three-dimensional folding and therefore functions of therapeutic proteins, e.g., monoclonal antibodies (mAbs). Localization and assignment of disulfide bonds are an important aspect of protein structural analysis.

However, the identification of disulfide pairing in a protein with multiple cysteine residues is generally more time-consuming and challenging than the determination of the protein sequence due to incomplete disulfide bond formation and disulfide bond scrambling.[1,2]

The combination of enzymatic digestion with liquid chromatography-mass spectrometry (LC/MS) is frequently applied as a routine method for the assignment of disulfide bonds.[3,4] However, peptide mapping via an LC/MS approach is traditionally labor-intensive and time-consuming in data processing and interpretation.

Furthermore, the assignment of disulfide linkages sometime is inconclusive when a protein contains multiple cysteines due to the significantly increased number of possible disulfide-bonded peptide isomers.[1] For example, there are totally 105 possible disulfide-bond pairing schemes for a protein containing eight cysteine residues (while an IgG1 mAb typically has 32 or more cysteines in its two light chains and two heavy chains).

This, plus the reality of potential disulfide bond scrambling, makes the disulfide bond assignment extremely difficult when the analysis is performed manually. An automated workflow is highly preferred for quick assessment of heterogeneities of therapeutic proteins caused by the disulfide linkages.

Recently, we have developed an integrated peptide mapping workflow for protein sequence confirmation and characterization using a UPLC®/MS^E method for data collection and a targeted bioinformatic tool, the BiopharmaLynx™ Application Manager for MassLynx™ Software, for automated data processing and annotation.[5]

The data independent MS^E approach alternatively collects mass spectrometric data of precursors and fragments of eluting peptides from protein enzymatic (e.g., tryptic) digests in an unbiased manner.[6] The integrated workflow overcomes most shortcomings of traditional peptide mapping methods[7] and has been demonstrated to be fast, robust and suitable for routine analysis of recombinant

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 x 150 mm (p/n 186004487) or ACQUITY UPLC BEH300 C_{18}, 1.7 µm, 2.1 x 150 mm (p/n 186003687)
Column temp.:	65 °C
Flow rate:	200 µL/min
Injection vol.:	5 µL (partial loop injection mode)
Mobile phase A:	0.1% FA in water
Mobile phase B:	0.1% FA in ACN
Gradient:	1–40% in 90 min

MS conditions

MS system:	SYNAPT HDMS G2
Ionization mode:	ESI+
Acquisition mode:	MS^E
Low collision energy:	4 eV
Elevated collision energy:	Ramping from 22 to 48 eV
Capillary voltage:	3.0 kV
Cone voltage:	30 V
Scan mode:	Resolution mode resolution ≥ 20000)
Scan time:	0.5 second
Source temp.:	100 °C
Desolvation temp.:	350 °C
Mass range:	100 to 2500 Da
Mass accuracy:	Lockmassed by GFP sprayed from lockmass channel every 30 seconds

Data management

BiopharmaLynx 1.3 Application Manager for MassLynx Software

Materials and reagents

Endoproteinase Lys-C (Wako) and iodoacetamide (Sigma) were used along with RapiGest (Waters, p/n 186001861). The IgG1 mAb Trastuzumab digests were prepared by incubating the sample (protein/Lys-C of 50:1) in 50 mM tris buffer (pH ~7.5) at 37 °C and the presence of 0.1% RapiGest SF for 18 hours. Prior to digestion, the protein was denatured at 80 °C for 10 min. For testing artificial scrambling potentially introduced during digestion, a digest prepared without alkylation was directly compared with a digest prepared in parallel with IAM alkylation before the digestion.

proteins such as therapeutic mAbs[5] and subunit vaccines.[8] The approach has also been successfully applied to identify expected disulfide bonds in an IgG1 mAb.[9]

In this study, an advanced UPLC/MS^E peptide mapping workflow is demonstrated for the detection and identification of disulfide linkages (including scrambled disulfide linkages) in a recombinant IgG1 mAb. The goal is to develop a viable approach for automatic mapping or monitoring of disulfide bonded peptides in a protein with multiple cysteines.

The significances of the integrated workflow include:

- Enhanced mass resolution and mass accuracy using the SYNAPT® HDMS™ G2 QTof MS System,[10] which is critical for acquiring high-quality MS^E data for large disulfide-bond-linked peptides with high molecular weight.
- Automatic assignment of disulfide linkages by an upgraded BiopharmaLynx (version 1.3) in a randomized mode, which is important for the identification of potential scrambled disulfide bonds in an automatic mode.

To reduce the complexity of protein digest mixture, the mAb was digested with endoproteinase Lys-C. The obtained digests were separated by reversed phase LC with an ACQUITY UPLC® System, followed by online MS^E detection and BiopharmaLynx 1.3 analysis. Digests prepared with and without alkylation protection of free sulfhydryl groups[2] in the sample were used to differentiate native scrambled disulfide linkages in the mAb from those potentially formed during sample preparation stage.

RESULTS AND DISCUSSION

Like all IgG1 mAbs, the Trastuzumab antibody should have nine unique disulfide bonds when formed properly. *In-silico* digestion (by Lys-Cp) shows that eight expected disulfide linked peptides (or unique linkages) are generated after digestion, including two in each of light chain, four in each of heavy chain, one between light chain and heavy chain, and one between the two heavy chains (which has two disulfide bonds connecting with).

To demonstrate that the workflow works properly to identify the formed disulfide linkages, the collected LC/MS^E data were first searched against the mAb sequences with properly linked cysteines to form the 16 expected disulfide bonds in the BiopharmaLynx method setting.

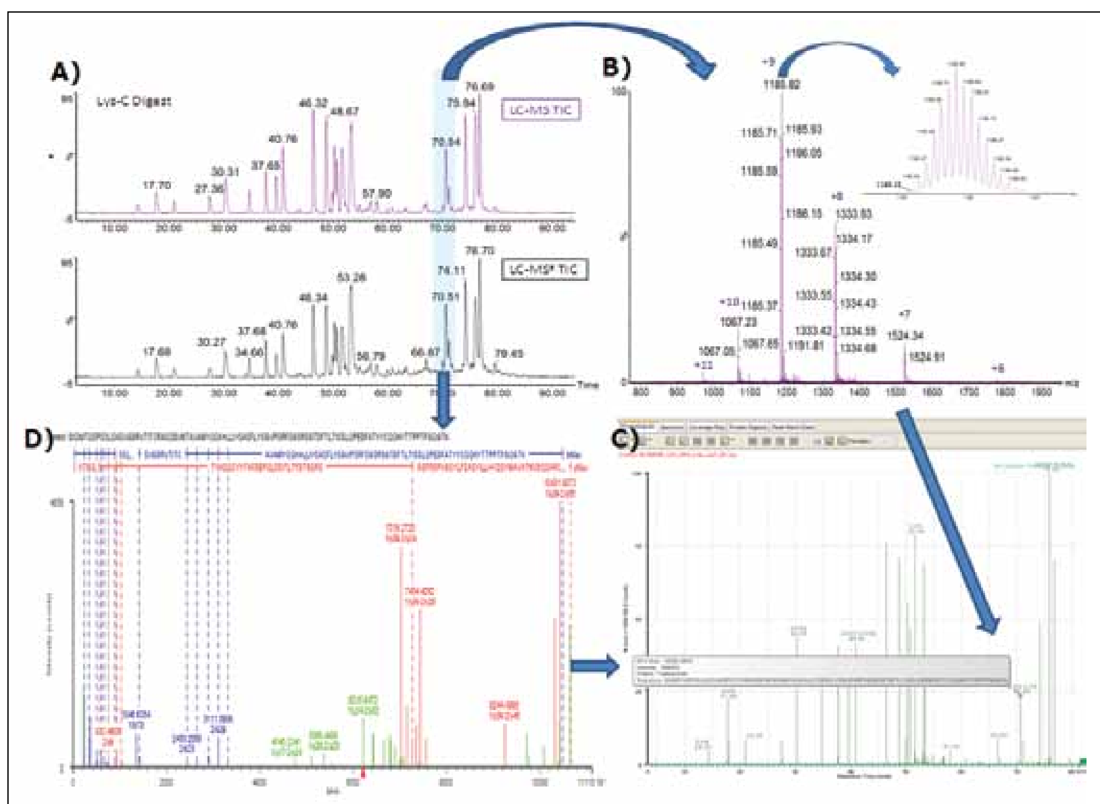

Figure 1. Identification procedure of disulfide linkage 1:K1–1:K4. A) UPLC/MS chromatograms showing the elution time of the linkage; B) MS spectrum; C) BiopharmaLynx interpretation and annotation; D) MS^E spectrum.

Figure 1 shows the determination of the largest disulfide linkages 1:K1–1:K4 (an intra disulfide linkage of light chain, monoisotopic mass 10657.12 Da). The disulfide-bonded peptide was detected and annotated based on the precursor mass MH^+ (Figure 1C) that was obtained from multiply-charged, isotope resolved raw MS data (Figure 1B). The assignment was also confirmed by elevated-energy fragmentation MS^E spectrum (also charge-reduced, isotope-deconvoluted to singly-charged ions, as shown in Figure 1D) upon the retention time-alignment (as highlighted in Figure 1A). The MS^E spectrum not only contains *b/y* ions from the two individual peptides (1:K1 and 1:K4), but also has ions corresponding to disulfide-bonded fragments from both peptides such as 7019.27 Da (1/y39–2/y24) and 10431.19 Da (1/y39–2/y56), providing unequivocal evidences for the disulfide linkage.

It should be stressed that the improved MS instrument resolution (see the inset in Figure 1B) is important to obtain the correct monoisotopic masses for the precursors as well as fragments of the large peptides. A well-resolved isotope pattern for such large peptides greatly facilitates the informatics tool for the identification of large disulfide linkages such as 1:K1–1:K4.

Similarly, BiopharmaLynx identifies other expected disulfide linkages. The mass accuracy of all identified disulfide bonded peptides was within 5.0 ppm. In addition, BiopharmaLynx is also capable of identifying the disulfide-bonded peptides with one of the linked peptides containing miscleavages.

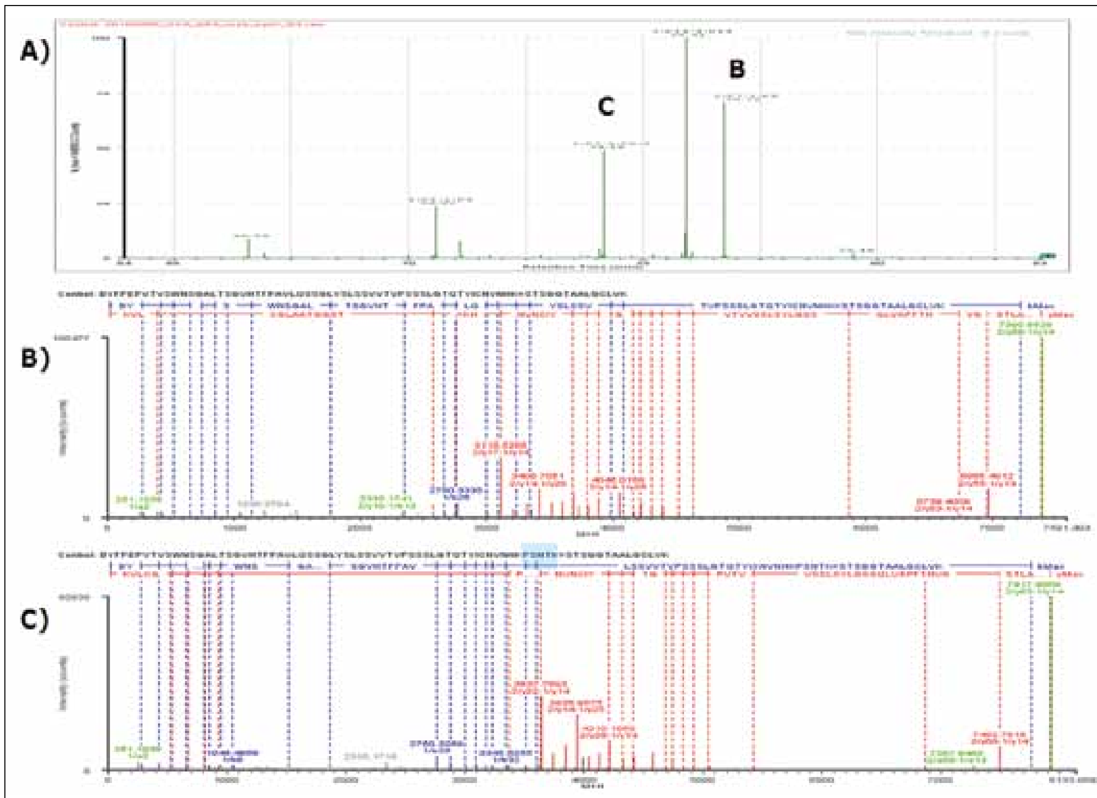

Figure 2. Identification and confirmation of disulfide linkages 2:K7–2:K8 and 2:K7–2:K8-9. A) BiopharmaLynx interpretation and annotation; B) MS^E spectrum of 2:K7-2:k8; C) MS^E spectrum of 2:K7–2:K8-9.

For example, both disulfide linkages 2:K7–2:K8 (without miscleavage) and 2:K7–2:K8-9 (with one miscleavage) were identified (see corresponding peaks B and C in Figure 2A). Their MS^E spectra are plotted in Figures 2B and 2C, respectively, showing confident identification. This capability is especially important because proteases such as Lys-C or trypsin tend to miss the cleavage site when a lysine (K) is followed by a proline (P) residue.

It is observed that high percentage of organic solvent (10% or more acetonitrile) is needed in the sample to help maintain the solubility of large disulfide-bonded peptides during LC/MS analysis. One disadvantage of the use of high organic in sample buffer is that this could result in the elution of the smallest disulfide-bonded peptides 1:K14–2:K13 (SFNRGEC=SCDK) in void volume.

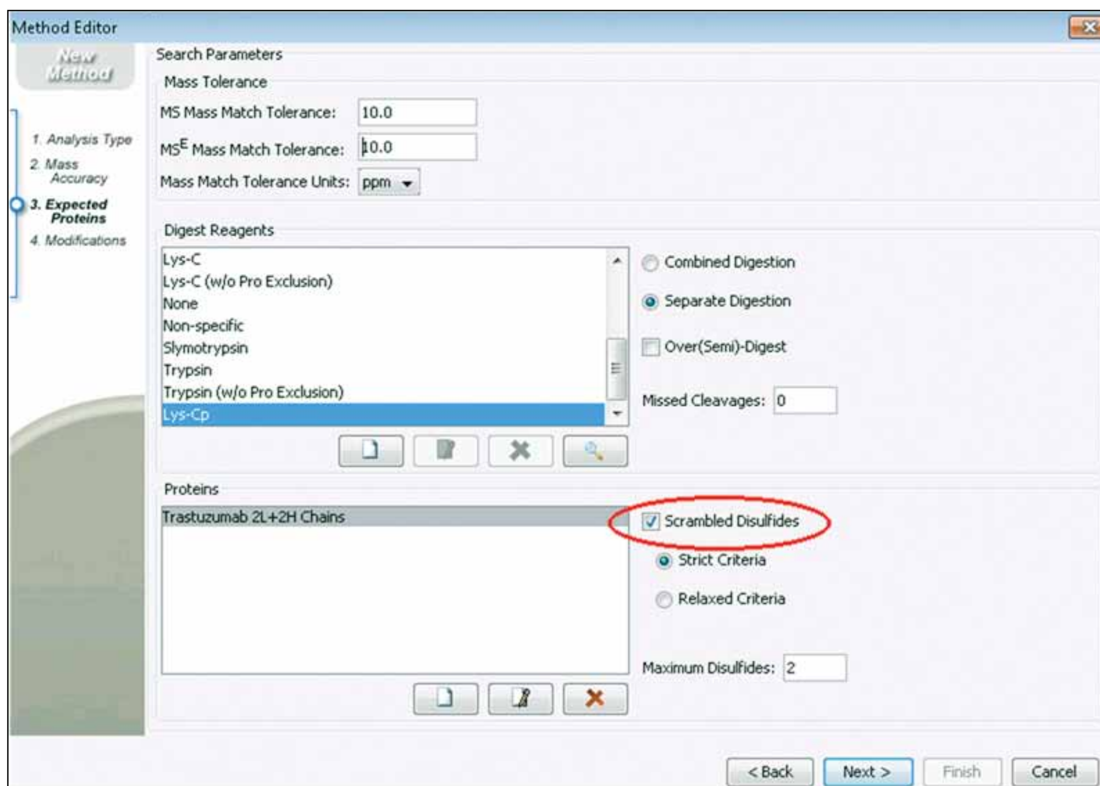

Figure 3. A screen capture showing the Scrambled Disulfides function in BiopharmaLynx 1.3 data processing method setting.

One challenge in disulfide bond mapping is to identify those peptides formed unexpectedly, e.g., due to scrambling. Therefore, the processing method was next created for Scrambled Disulfides (see the highlight part in Figure 3), which allows for detection and identification of all possible disulfide pairing formed between any two cysteines in the mAb. Lys-C digests of Trastuzumab with or without alkylation prior to digestion were analyzed for this investigation.

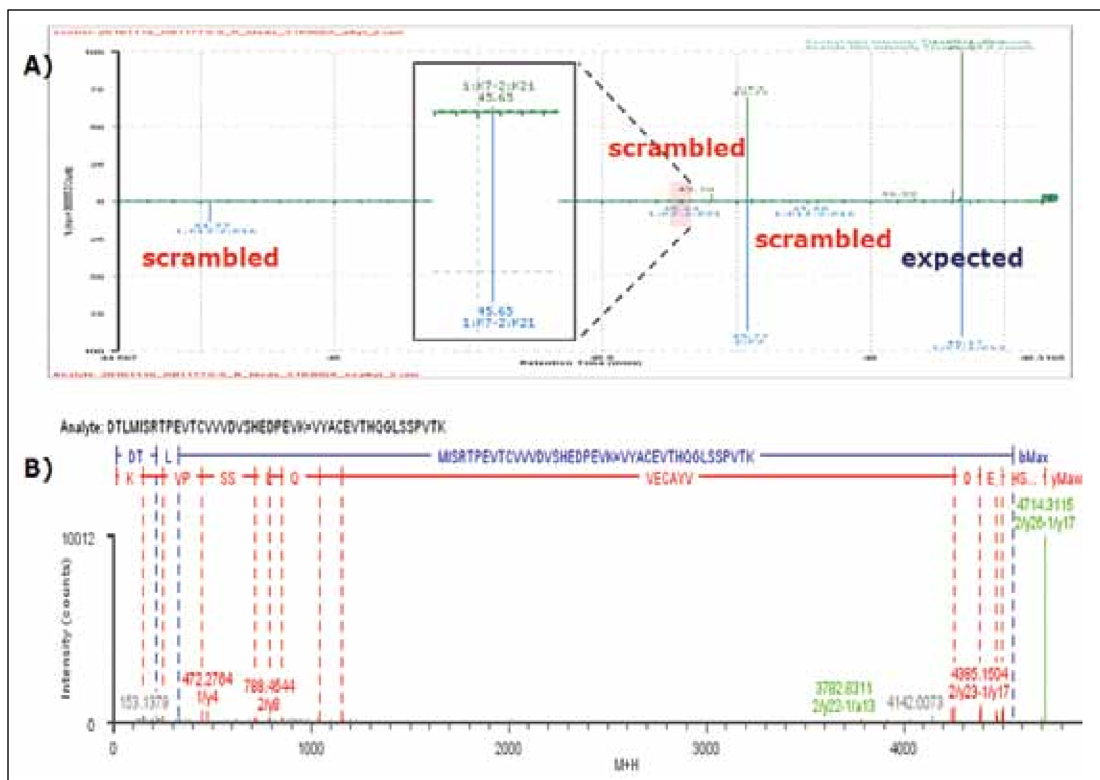

Figure 4. Examples of identified scrambled disulfide linkages with automatic interpretation and annotation. A) BiopharmaLynx interpretation and annotation; B) MS^E spectrum of scrambled disulfide linkage 1:K13–2:K16.

Using this new method, not only the expected disulfide linkages were identified, some minor scrambled disulfide linkages were also detected. Figure 4A illustrates three identified scrambled disulfide linkages (1:K13–2:K16, 1:K7–2:K21, 1:K14–2:K16) in the digest without alkylation. Again, elevated-energy fragmentation (MS^E) data were available for confirmation of the assignments (the MS^E spectrum of 1:K13–2:K16 is plotted in Figure 4B as an example).

The scrambled 1:K13–2:K16 and 1:K14–2:K16 were only identified from the digest without alkylation and not detected in the digest with alkylation, suggesting these were artifacts introduced by sample preparation. The scrambled disulfide linkage 1:K7–2:K21 was identified in both digests (with or without alkylation), however, its abundance is much lower in the alkylated sample compared to the non-alkylated one (see the inset in Figure 4A). As expected, all the identified scrambled disulfide linkages are minor compared to the expected disulfide linkages (as illustrated in Figure 4A).

The successful determination of both expected and scrambled disulfide linkages demonstrated that the integrated workflow is capable of automatically mapping disulfide linkages in a protein. The enhanced function of Scrambled Disulfides requires no manual efforts, and achieves completely automatic data processing for the challenging task of disulfide bond mapping.

CONCLUSION

An integrated workflow, combining high mass resolution UPLC/MS^E with BiopharmaLynx 1.3, was developed for rapid mapping of disulfide linkages in a recombinant IgG1 mAb. The workflow was demonstrated to be capable of simultaneous identification of both expected and scrambled disulfide linked peptides. Assignment of disulfide bond linked peptides is automated, based on accurate MS measurement and confirmed by elevated collision energy MS^E fragmentation data.

The improved mass resolution – 20,000 for resolution mode and 40,000 for high-resolution mode – offered by the SYNAPT G2 HDMS System greatly enhances the MS data quality for large disulfide-linked peptides in enzymatic (e.g., Lys-C) digests. The added Scrambled Disulfides function setting of BiopharmaLynx 1.3 makes it possible for identification of any potential disulfide linkages of a protein in an automated mode.

This integrated approach should be applicable for routine mapping and monitoring of disulfide linkages in mAbs and other therapeutic proteins with multiple disulfide linkages.

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[TECHNICAL NOTE]

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Multi-Enzyme Digestion for Biotherapeutic Peptide Mapping: Examining BiopharmaLynx 1.3 Functionality

Scott J. Berger
Waters Corporation, Milford, MA, USA

TECHNOLOGY BENEFITS

Enabling advanced multi-protease peptide mapping workflows, with the automated data analysis capabilities of the BiopharmaLynx Application Manager, version 1.3, for MassLynx Software.

WATERS SOLUTIONS

BioPharmaLynx™ Application Manager 1.3

MassLynx™ Software

KEY WORDS

Peptide mapping, multiple enzymes, biotherapeutic, coverage

BACKGROUND

The recent availability of high-quality proteolytic digestion enzymes, in addition to trypsin, has facilitated a new generation of biotherapeutic peptide map analyses that can be performed with much greater flexibility.

In particular, multiple protease digestion workflows have enabled greater selectivity for peptide chromatographic retention, MS response, and peptide fragmentation pathways, while simplifying the task of achieving comprehensive sequence and fragmentation coverage for a given biotherapeutic. This technical brief will describe the employment of such workflows, and how they can yield greater biotherapeutic knowledge, often with less analytical development effort.

THE SOLUTION

The most recent release of BiopharmaLynx, version 1.3, features additional flexibility for automating data analysis from higher complexity multi-enzyme peptide map experiments. BiopharmaLynx supports maps generated with the common proteolytic digest reagents (Table 1), and also provides scientists the flexibility to define additional custom digest reagents. BiopharmaLynx 1.3 now extends bioinformatic support for peptide mapping to analyses where multiple digestion enzymes are utilized.

Enzyme	Code	Selectivity	Digest	Exclusions
Arg-C	R	R	C-terminal	R(P)
Asp-N (Asp)	D	D	N-terminal	None
Asp-N (Asp and Glu)	D	D, E	N-terminal	None
CNBr	M	M	C-terminal	None
GluC (V8, NH4OAc pH 4)	V	E	C-terminal	E(P)
GluC (V8, AmBiC pH 7.8)	V	E	C-terminal	E(P)
GluC (V8, PO4 pH 7.8)	V	D, E	C-terminal	D(P), E(P)
Chymotrypsin	C	Y, F, W	C-terminal	Y(P), F(P), W(P)
Lys-C	K	K	C-terminal	K(P)
Lys-C (w/o Pro Exclusion)	K	K	C-terminal	None
None	N	None	None	None
Non-specific	F	All Bonds	C-terminal	None
Slymotrypsin	S	K, R, Y, F, W	C-terminal	K(P), R(P), Y(P), F(P), W(P)
Trypsin	T	K or R	C-terminal	K(P), R(P)
Trypsin (w/o Pro Exclusion)	T	K or R	C-terminal	None
Custom digest reagents	User	User	User	User

Two multi-digest workflows are supported within the peptide mapping method editor (Figure 1): the combined workflow (or "one pot" digest), where multi-protease digestion is carried out in the same sample vial; and the separate workflow (or "or multi-pot" digest), where individual enzyme digests are prepared, quenched, and then mixed prior to LC/MS analysis.

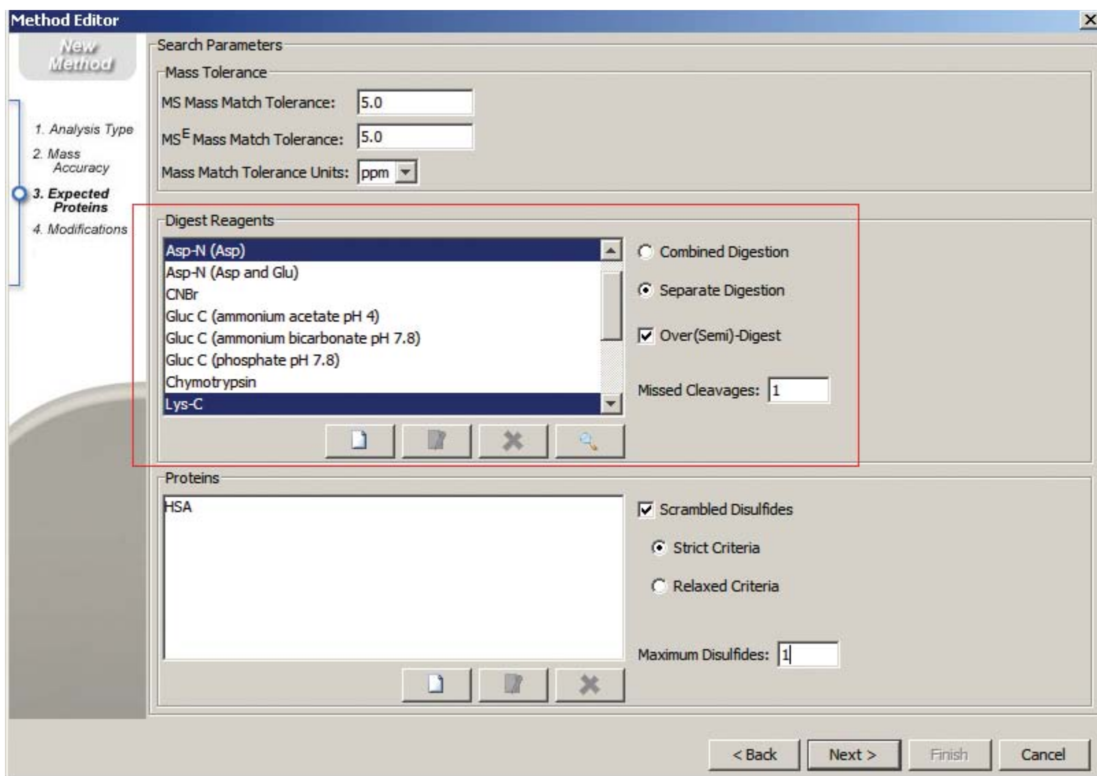

Figure 1. Digest reagent selection within the BiopharmaLynx 1.3 method editor. Users can now select multiple digestion enzyme in addition to overdigestion and underdigestion products for those proteases.

Figure 2 illustrates the digest specificity on a theoretical protein from individual digestion with GluC and LysC. Individually, they each produce three digested peptides, including one very large peptide. The products of the separate multi-enzymatic workflow represent the concatenated list from the individual digests, while the products of the combined multi-enzymatic workflow constitute a unique set of digested peptides, where the large peptides have been further digested by the additional protease.

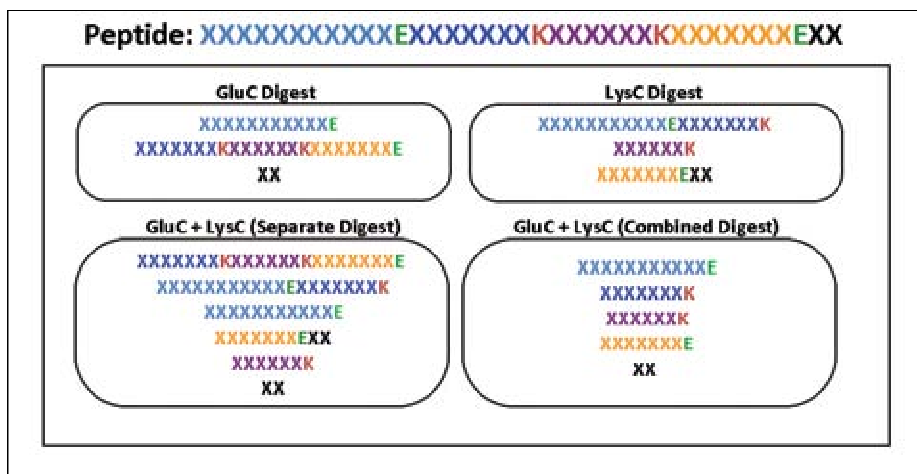

Figure 2. Digestion specificities for single, multi-combined, and multi-separate proteolytic digest workflows.

The following sections will detail the practical utility of these new workflows for biotherapeutic peptide mapping analysis.

Combined multi-digest workflow

The combined workflow generates a set of peptides resulting from the combined specificities of all proteases used in the digestion process. Identified peptides are labeled using multi-letter peptide digest labels derived from the single enzyme digest designators (e.g., DT represents the combined product of AspN(D) and Trypsin (T)).

There are three common reasons to utilize the combined digest multi-enzyme workflow:

- To change the peptide digest population, altering chromatographic properties of the mixture. This will often introduce significant changes in retention time for multiple peptides, and can address peptide coelution, or improve chromatographic quality for one or more components.
- To reduce large peptides obtained from a single enzyme digest to more manageable-sized peptides for fragmentation studies. It can be difficult to generate fragmentation at all peptide bonds when sequencing larger peptides (> 25 AA). Reducing peptide size can often enhance fragment coverage for peptides of interest, and can be particular useful for producing smaller peptides where multiple modifications can be monitored independently.
- Changes to the terminal amino acid residues can significantly alter peptide fragmentation behavior and allow more confirmatory ions from sequence regions not favored for fragmentation of the larger peptide.

Separate multi-digest workflow

The separate workflow assumes that multiple independent digests were produced and that the enzymes were inactivated before the digests were mixed for analysis. BiopharmaLynx searches peptide mapping results for peptides predicted using the digest specificities of each enzyme. Identified peptides are labeled using the single-letter peptide digest nomenclature common to single enzyme digests (e.g., T for Trypsin; K for LysC; D for AspN)

There are three common reasons to utilize the separate digest multi-enzyme workflow:

- To obtain high sequence coverage with minimal method optimization for a given protein. This could prove useful for discovery scientists faced with evaluating large numbers of protein candidates, or during clone selection for a particular candidate.
- To obtain redundant protein coverage from a single peptide map. Modifications can be independently confirmed and quantitated using overlapping peptide sequences from each digest.
- To maximize sequence confirmation from peptide fragmentation using fragmentation selectivity differences between digested peptides covering a common sequence region.

Applying the separate digest workflow to compare digestion enzymes

The new separate digest workflow functionality can also be employed to compare maps prepared with two different enzymes. As this workflow generates concatenated lists of both enzyme digest products, searches against both data sets would return accurate identifications.

Human Serum Albumin (HSA) was individually digested with GluC and LysC, and high quality UPLC®/MS^E maps were generated for both digests. The annotated Total Ion Chromatogram (TIC) traces for both runs (Figure 3) show peptide identifications for fully digested products in both digests.

Without any optimization, it was shown (Figure 4) that 85% coverage was obtained with GluC, 92% coverage with LysC, and 100% sequence coverage from the combined results of both analyses. The absence of "common" coverage reflects the lack of identical peptide fragments generated from both enzyme digests, and the excellent specificity of accurate mass for biotherapeutic peptide mapping studies. From such studies, useful combinations of enzymes can be readily identified to assist in answering global or targeted questions about a biotherapeutic protein.

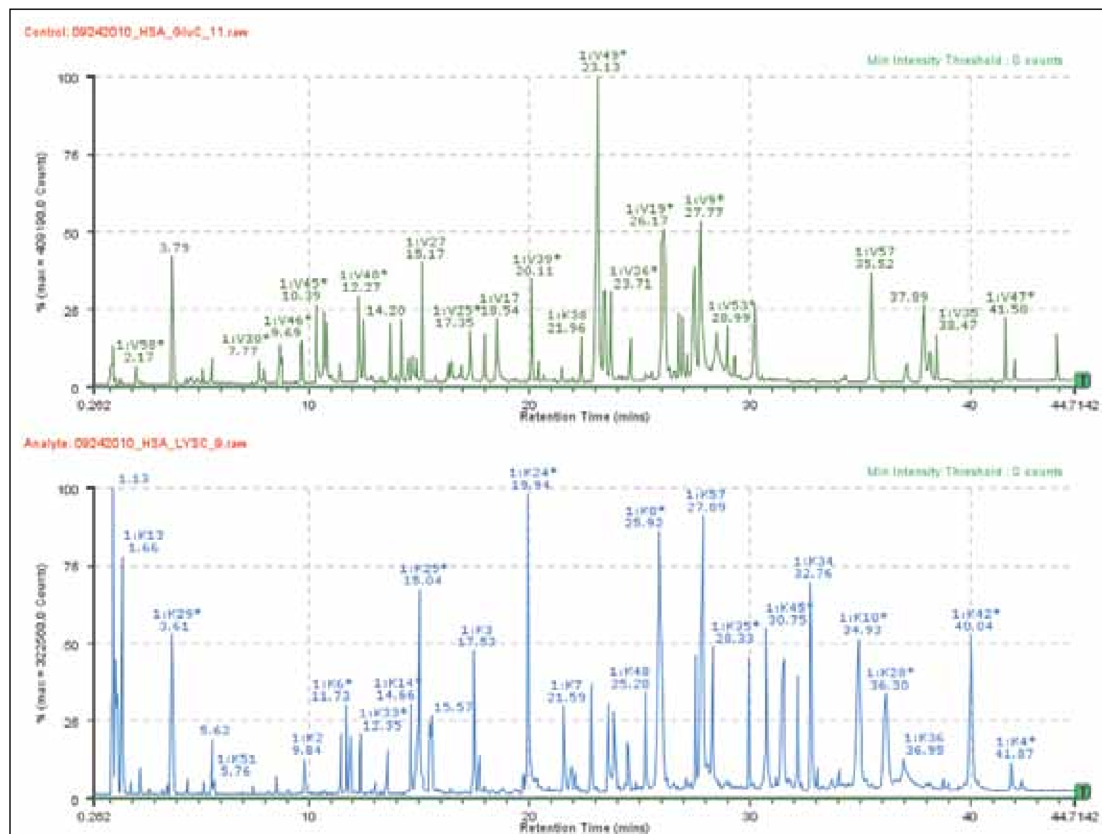

Figure 3. Peptide mapping results (TIC) for Human Serum Albumin (HSA) digested with GluC (top, V labels) and LysC (bottom, K labels).

HSA

Control Coverage (%): 85.0 | Combined Coverage (%): 100.0 | Analyte Coverage (%): 91.8

Control Unique Coverage (%): 85.0 | Common Coverage (%): 0.0 | Analyte Unique Coverage (%): 91.8

1:1 to 50	DAHKSEVAHR	FKDLGEENFK	ALVLIAFAQY	LQQCPFEDHV	KLVNEVTEFA
1:51 to 100	KTCVADESAE	NCDKSLHTLF	GDKLCTVATL	RETYGEMADC	CAKQEPERNE
1:101 to 150	CFLQHKDDNP	NLPRLVRPEV	DVMCTAFHDN	EETFLKKYLY	EIARRHPYFY
1:151 to 200	APELLFFAKR	YKAAFTECCQ	AADKAACLLP	KLDELRDEGK	ASSAKQRLKC
1:201 to 250	ASLQKFGERA	FKAWAVARLS	QRFPKAEFAE	VSKLVTDLTK	VHTECCHGDL
1:251 to 300	LECADDRADL	AKYICENQDS	ISSKLKECCE	KPLLEKSHCI	AEVENDEMPA
1:301 to 350	DLPSLAADFV	ESKDVCKNYA	EAKDVFLGMF	LYEYARRHPD	YSVVLLLRLA
1:351 to 400	KTYETTLEKC	CAAADPHECY	AKVFDEFKPL	VEEPQNLIKQ	NCELFEQLGE
1:401 to 450	YKFQNALLVR	YTKKVPQVST	PTLVEVSRNL	GKVGSKCCKH	PEAKRMPCAE
1:451 to 500	DYLSVVLNQL	CVLHEKTPVS	DRVTKCCTES	LVNRRPCFSA	LEVDETYVPK
1:501 to 550	EFNAETFTFH	ADICTLSEKE	RQIKKQTALV	ELVKHKPKAT	KEQLKAVMDD
1:551 to 585	FAAFVEKCCK	ADDKETCFAE	EGKKLVAASQ	AALGL	

Figure 4. Coverage plot showing GluC (purple) and LysC (orange) coverage for HSA digested with the specified enzyme in a single enzyme digest.

SUMMARY

- BiopharmaLynx 1.3 now supports multi-enzyme LC/MS peptide mapping analysis workflows.
- Such workflows can be employed to alter chromatographic and MS selectivity, to address issues of map coverage, and to enable more comprehensive studies of biotherapeutic variation.
- The separate digest functionality in BiopharmaLynx 1.3 can also be used to rapidly evaluate map coverage and quality for various protein digestion enzymes.

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GLYCAN & GLYCOPEPTIDE ANALYSIS

Glycans impact the efficacy and safety of a biotherapeutic. Identification of the sites of glycan attachment, the glycans that are present, and the relative proportions of each alternative structure are needed to meet pharmaceutical and regulatory requirements. The number, similarity, and diversity of possible structures of these molecules place tremendous demands on the analytical techniques.

Advances in chromatographic approaches utilizing UltraPerformance LC® (UPLC®) Technology lead to better resolution, sensitivity, and speed , providing reliability in the qualitative and quantitative analysis of protein glycosylation.

Oligosaccharides can be obtained from glycoproteins and glycopeptides by either chemical or enzymatic cleavage. Once the oligosaccharides have been liberated from either the glycoprotein or the glycopeptide, an oligosaccharide profile can be obtained. Fluorophores are often used to label oligosaccharides to permit optical detection.

HILIC chromatography, with amide functional groups, is often employed for analyzing released oligosaccharides. Samples are applied in an organic solvent solution to the column and then eluted with increasing concentration of aqueous buffer. This exploits the subtle differences in the hydrophilicity between individual glycans and their affinity for the column matrix. The ability to analyze sialylated, high-mannose, and neutral sugars in a single chromatographic run makes it a useful technique. However, good resolution of the various glycans was often difficult to achieve, particularly for complex mixtures.

COMPREHENSIVELY DESIGNED FOR PERFORMANCE

Waters delivers comprehensive solutions for glycan and released glycan analysis leveraging our extensive expertise in chromatography, mass spectrometry, and informatics for protein therapeutics.

- UPLC® Technology provides improved resolution, sensitivity, and speed for greater reliability in the qualitative and quantitative analysis of protein glycosylation.
- The ACQUITY UPLC® FLR Detector is a high-sensitivity, multichannel fluorescence detector optimized for use with ACQUITY UPLC Systems. Innovative flow cell design, low-noise electronics, and support for high-speed data rates result in a detector that brings the ability to effectively acquire and quantitate high-efficiency separations afforded by UPLC.
- Waters mass spectrometers are ideal for workflows involving released glycans, or for glycopeptides from protein digestion, because of their ability to cope with a wide variety of solvent conditions. Recent generations of ionization sources and detectors have enabled finer detail with higher sensitivity than previously achieved.
- A UPLC/FLR/QTof MS analytical platform, such as the ACQUITY UPLC H-Class Bio with Xevo® G2-S QTof MS, combines Waters technologies into a complete solution for fluorescent-labeled glycan characterization. The chromatographic resolution, reproducibility, and mass spectrometry sensitivity enable glycoprofiling of therapeutic antibodies mandated by regulatory agencies. The system is a robust tool for separation and analysis of minor glycoforms or isomers that are otherwise difficult to assign.

GLYCOBASE DATABASE

Waters and Ireland's National Institute for Bioprocessing Research and Training (NIBRT) have developed a comprehensive solution to complex glycan analysis that combines NIBRT's novel GlycoBase 3+ database with the unique capabilities of ACQUITY UPLC Systems and Glycan Separation Technology chemistries for HILIC-UPLC separations. GlycoBase 3+ is a web-enabled proprietary resource that contains normalized retention data, expressed as Glucose Units, or GU values, for more than 600 2-AB labeled N-linked glycan structures. These values were obtained by systematic analysis of released glycans from a diverse set of glycoproteins using Waters HPLC and UPLC technologies and the NIBRT glycan analytical platform.

GLYCAN SEPARATION TECHNOLOGY (GST) COLUMNS

HILIC is a well-recognized and reliable technique that can effectively separate and quantitate isolated glycans after their derivatization with fluorescent labels. Waters GST Columns are based on ethylene bridge hybrid (BEH) particle and bonding technology for stable and reproducible UPLC analysis of 2-AB labeled glycans. GST columns have been specifically developed and QC tested to provide superior UPLC component resolution in less time for a range of glycan structures.

GLYCAN STANDARDS

Waters Glycan Standard line includes a kit that offers a robust sample preparation strategy for LC with fluorescent detector analysis of 2-AB labeled glycans as well as standards that help to aid in qualifying and benchmarking the ACQUITY UPLC BEH Glycan, 1.7 µm Columns.

RAPID SAMPLE CLEAN-UP FOR N-LINKED GLYCANS

The MassPREP™ Glycoanalysis Kit can be used in the preparation and purification of 2-AB labeled glycans and glycans released from glycoproteins. This kit provides simple and robust sample preparation and includes the MassPREP µElution Plate and RapiGest SF. RapiGest SF can greatly assist in protein deglycosylation of N-Glycans.

ACQUITY UPLC® H-Class System and the MassPREP Glycoanalysis Kit.

[APPLICATION NOTE]

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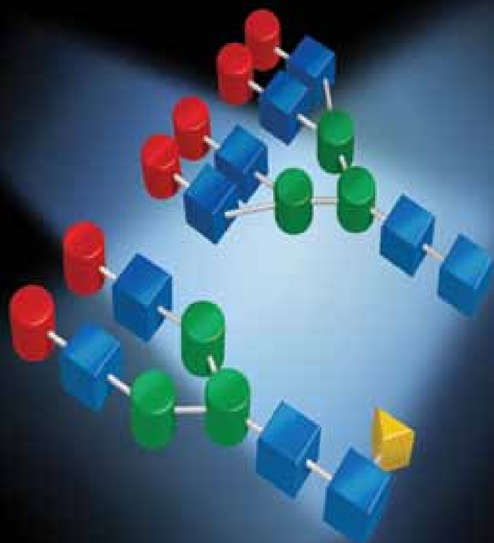
N-Linked Glycans of Glycoproteins: A New Column for Improved Resolution

Beth Gillece-Castro, Kim van Tran, Jonathan E. Turner, Thomas E. Wheat, and Diane M. Diehl
Waters Coropration, Milford, MA, U.S.

INTRODUCTION

Glycosylation is a post-translational modification of proteins that occurs in all eukaryotic cells. The sugar chains on glycoproteins can mediate biological activity, play a role in receptor-mediated recognition, increase solubility, regulate half-life, and exert a stabilizing influence upon conformation. Specific glycan structures are, therefore, associated with safety and efficacy attributes of many protein drugs. Correct glycosylation is essential if a glycoprotein is to achieve and maintain the structure with full biological activity. The measurement of glycans, therefore, is important in biopharmaceutical development projects. The relative amounts of the individual glycan structures are monitored during process development to establish the stability of the growth and purification steps of manufacturing. The same measurements are required in the development of formulations and stability testing.

Waters provides a system solution for gylcan analysis to meet the needs of these applications. The new ACQUITY UPLC® BEH Glycan Separation Technology Column separates the released glycans of biopharmaceuticals as their 2-aminobenzamide (2-AB) derivatives. This column is used with a Waters ACQUITY UPLC instrument with fluorescence detection (FLR) to maximize resolution, sensitivity, and speed.



EXPERIMENTAL

Samples and derivatization

N-linked glycan mixtures were purchased as 2-AB labeled derivatives (ProZyme, San Leandro, CA) or released from glycoproteins with PNGase F. The released glycans were labeled according to the procedure of Bigge et al.[1] Prior to analysis the samples were diluted to produce a 1 pmol/µL solution in 50:50 Buffer A/acetonitrile. Glycans are minimally soluble in higher acetonitrile concentrations, which may lead to sample loss over time.

Chromatographic conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Glycan, 2.1 x 150 mm, 1.7 µm (P/N 186004742)
Column temp.:	60 °C
Sample temp.:	15 °C
Flow rate:	500 µL/min except during aqueous wash
Mobile phase A:	100 mM ammonium formate, pH 4.5
Mobile phase B:	Acetonitrile
Weak needle wash:	90:10 acetonitrile/water (v/v)
Strong needle wash:	10:90 acetonitrile/water (v/v)
Seal wash:	50:50 methanol/water (v/v)
Injection volume:	1.5 µL, Partial Loop; 2 µL, Full Loop
Detection:	Fluorescence (FLR) λex 330 nm λem 420 nm

Alternative needles and mixers are available for peptide mapping. (P/N 205000507, Peptide Needle Kit; P/N 205000403, ACQUITY UPLC High Sensitivity Filter Mixer) The installation of the peptide mapping needle or mixer will not adversely effect glycan analysis. Chromatograms 1 and 2 were collected with an ACQUITY UPLC instrument fitted with the peptide needle and mixer. Chromatograms 3 and 4 were collected with an unmodified ACQUITY UPLC instrument.

Gradient Tables

Human IgG glycans and dextran ladder

(Figures 1 and 2)

Time min	Flow Rate mL/min	%A	%B
Initial	0.5	22.0	78.0
38.5	0.5	44.1	55.9
39.5	0.25*	100	0
44.5	0.25*	100	0
46.5	0.5	22.0	78.0
50	0.5	22.0	78.0

High mannose and sialylated glycans

(Figures 3 and 4)

Time min	Flow Rate mL/min	%A	%B
Initial	0.5	25	75
38.5	0.5	40	60
39.5	0.25*	100	0
44.5	0.25*	100	0
46.5	0.5	25	75
50	0.5	25	75

**Note reduced flow rate during aqueous regeneration*

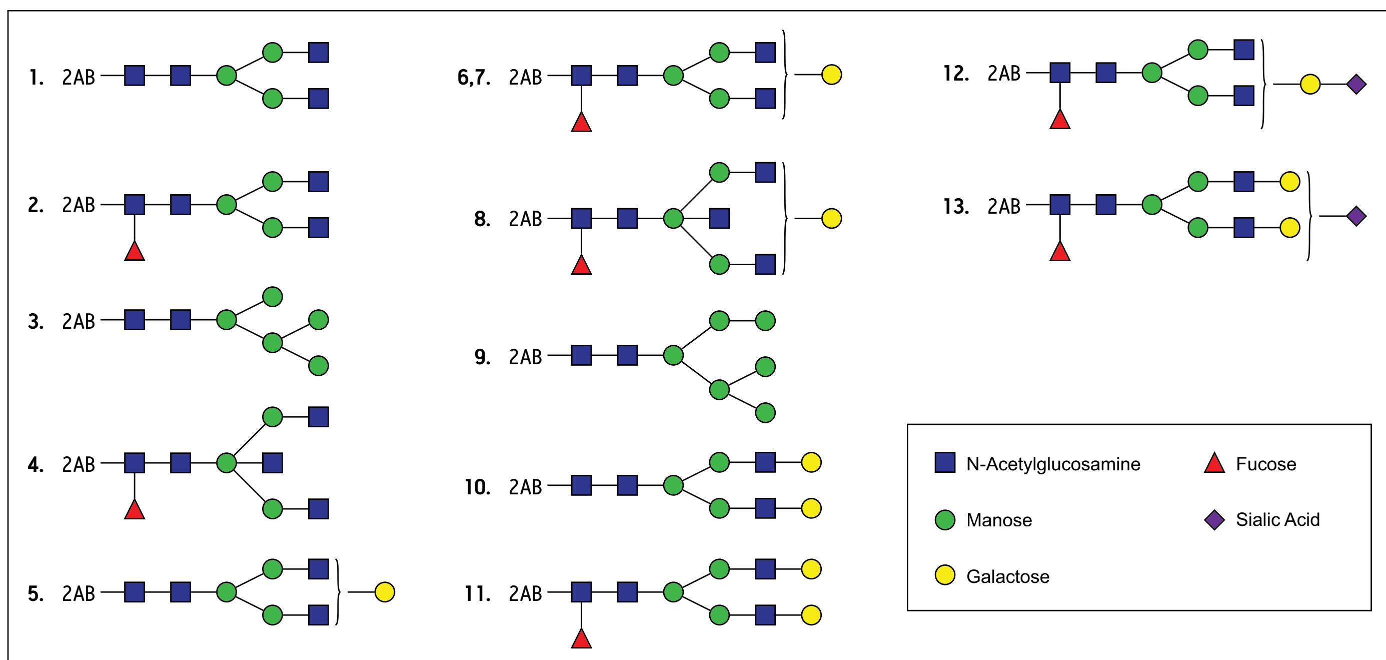


Figure 1. Major structures identified from 2-AB labeled human IgG glycans. By convention the 6-linked core mannose is on top relative to the 3-linked mannose, which is below.

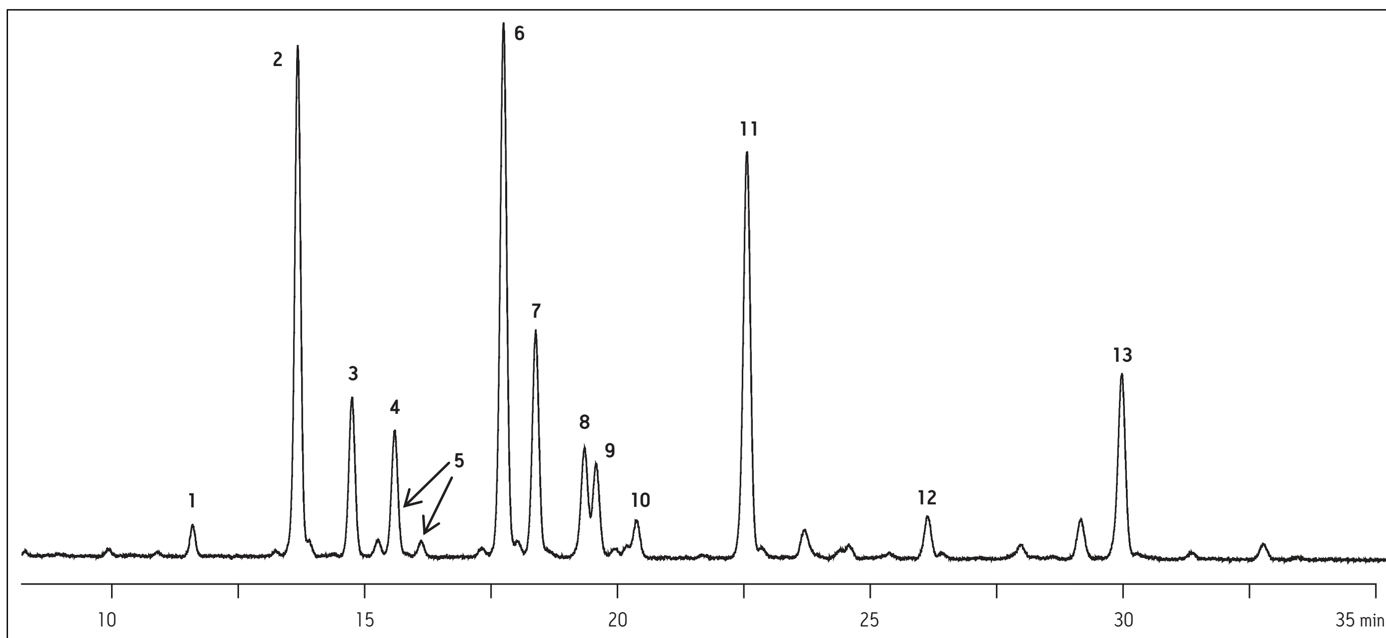


Figure 2. UPLC-FLR Chromatogram of 2-AB labeled human IgG glycans with 2 picomoles loaded onto the column.

RESULTS AND DISCUSSION

The design and development of the UPLC Glycan Separation Technology Column was targeted to the analysis of the glycans that are commonly found or expected on biopharmaceutical proteins. The array of oligosaccharides should, therefore, include high mannose, complex, hybrid, and sialylated glycans as shown in Figure 1. To establish a common detection for all the species that might be observed in a single sample, the glycans were labeled with 2-aminobenzamide. These fluorescent derivatives separate best on an amide-bonded phase. Since the labeled oligosaccharides are very hydrophilic, the preferred mode of separation is Hydrophilic Interaction Chromatography (HILIC). Since the set of glycans includes many closely related structures, it is necessary to use the selected chemistry under conditions that provide the highest possible resolution. The column was, therefore, developed for use on Ultra Performance Liquid Chromatography instruments with sub-2-micron particles. The BEH Technology hybrid material is used as the base for the 1.7-µm particles for maximum physical and chemical stability. The column is optimized for use with the ACQUITY UPLC System with FLR detection to take advantage of the minimum band-spreading. When running the system at the flow rate of 0.5 mL/min, higher than HPLC, a side effect of the small particles is higher back pressure that is accommodated by the ACQUITY UPLC System.

The column was evaluated during the development process with a sample of 2-AB labeled human IgG glycans, and a typical chromatogram is shown in Figure 2. This sample includes high mannose, complex, hybrid, and sialylated glycans as an index of the chemical suitability of the bonded phase. The final selected column composition, as shown in Figure 2, is particularly useful because of the excellent resolution of peak 2 (G0F) and peak 3 (Man5). Numerous isomers are present that are substituted on one of the two or three branches of the glycan. For example, the isomers shown as Peaks 6 and 7 are consistently separated. For quantitative analysis of the mixture, the areas of each of the peaks are compared. Peaks that are well resolved typically produce reproducible and reliable relative quantitation.

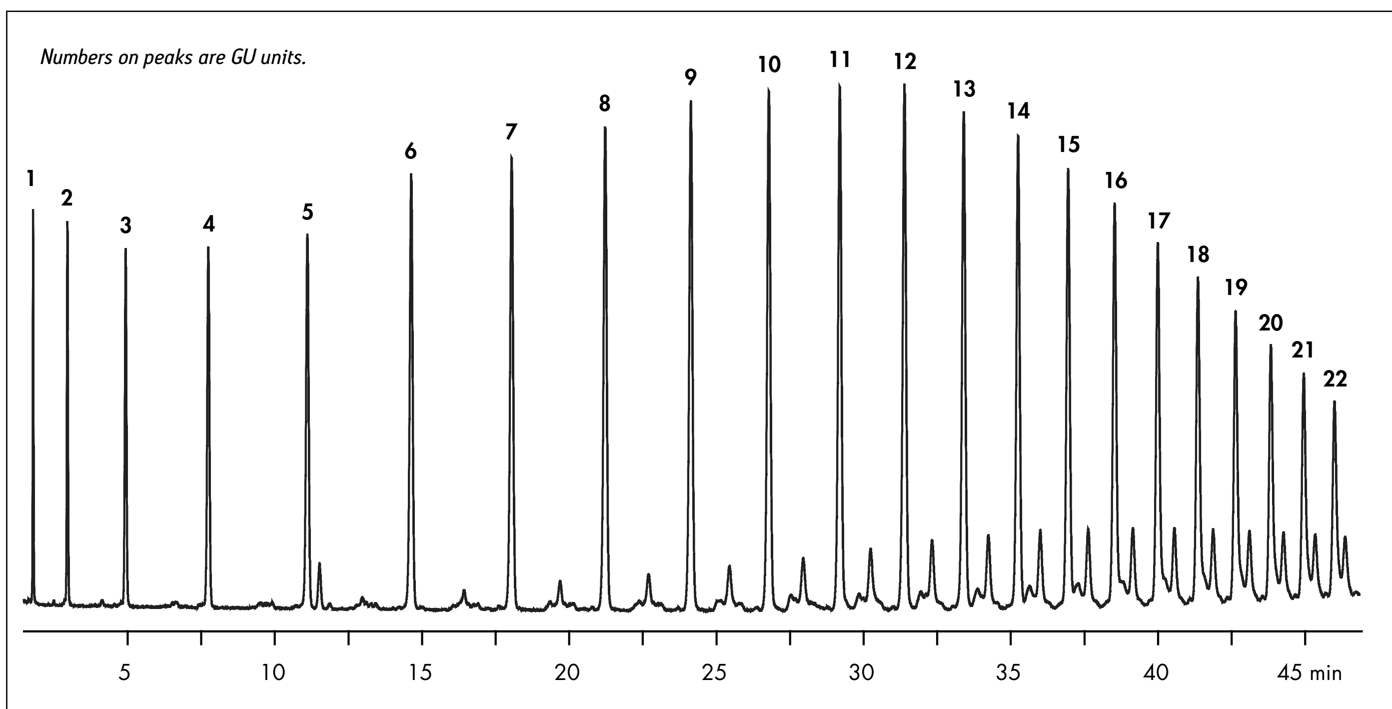


Figure 3. UPLC-FLR chromatogram of 2-AB labeled dextran ladder. Glucose Units (GU) 1 to 22 are eluted with the gradient from 78% to 55.9% acetonitrile.

The column has also been evaluated for the separation of other significant glycans. The homo-polymeric series of glucose oligomers shown in Figure 3 extends from 1 to 22 sugars (Glucose Units, GU) with retention for the smallest species and good resolution for the largest. Measuring retention times relative to the glucose units provides standardization to estimate the effect of individual monosaccharide additions and deletions. It also provides a meaningful reference over column lifetime, and to compensate for any or batch-to-batch reproducibility. GU values can also be useful when characterizing unknown glycan structures.[2]

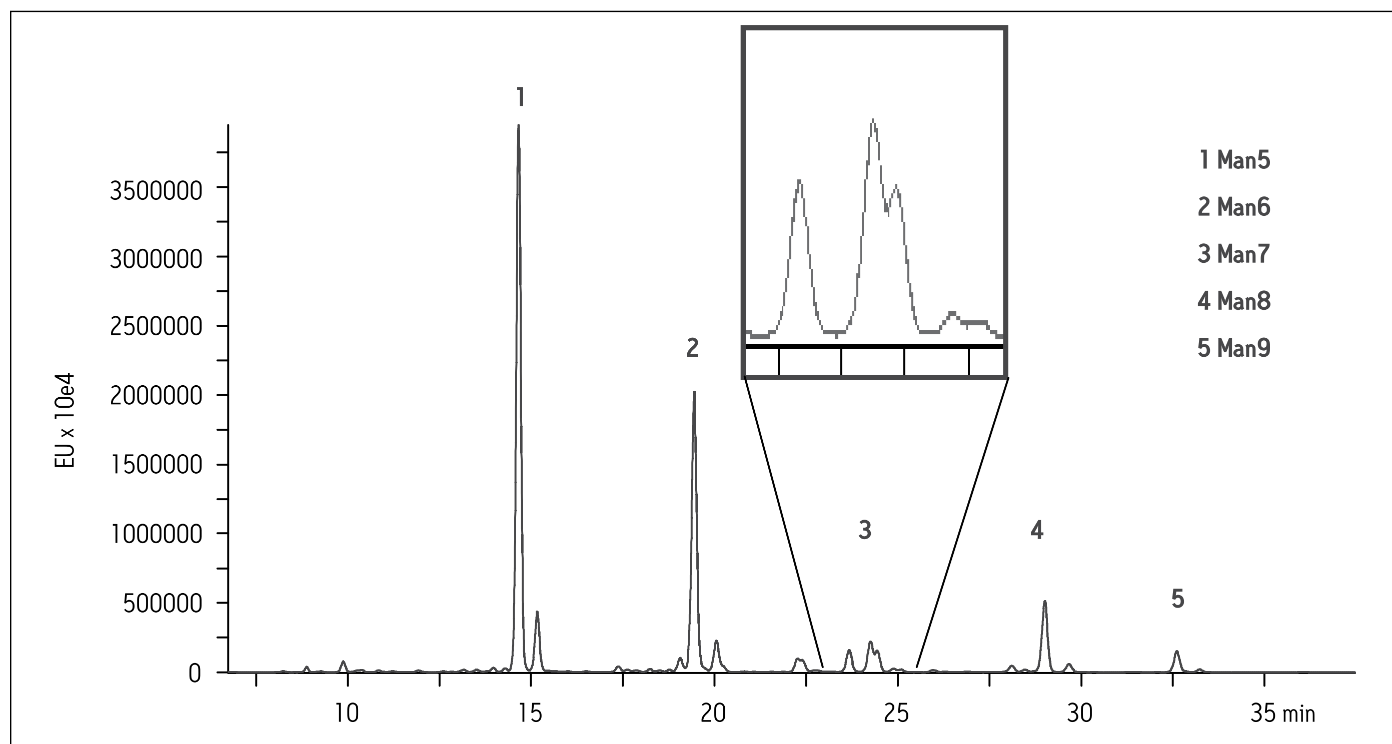


Figure 4. UPLC-FLR chromatogram of 2-AB labeled high mannose glycans from bovine ribonuclease b.

The resolving power of this column was tested with several samples that include neutral and acidic oligosaccharides, branched variants, and linkage isomers. The mixture of high mannose glycans that is present on bovine ribonuclease b contains many such isomers. In particular the Man7 glycan is known to contain three isomers that are substituted on one of the two or three branches of the glycan. A complex of several peaks eluting just before 25 minutes in Figure 4 shows the separation of isomers of the Man7 glycan.

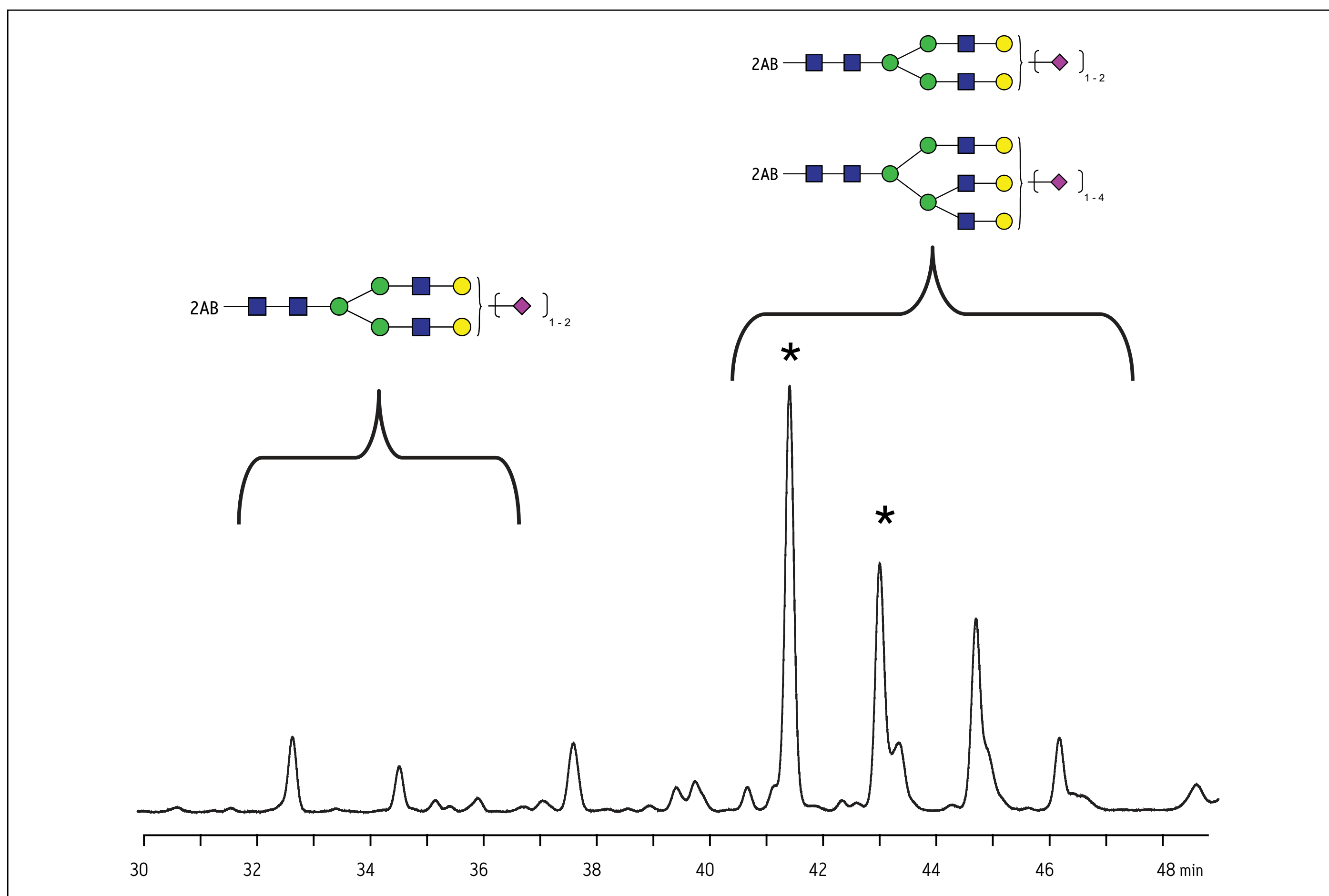


Figure 5. UPLC-FLR Chromatogram of 2-AB labeled bovine fetuin glycans. Highlighted(*) are multiple peaks with a single molecular weight corresponding to disialylated biantennary glycans.

This separation chemistry is also suitable for charged, acidic glycans. Bovine fetuin yields a mixture of mono-, di-, tri-, and tetra-sialylated species that present a challenge to analysis. With larger glycan structures, the number of isomers increases. When sialic acid residues are added to glycan termini they can be attached on the different branches, and the linkage position on the galactose residue may be on the 2, 3, 4, or 6 position. The resolution of these structures is shown in Figure 5. A pair of isomers that are triantennary glycans with two sialic acid residues elute at 41.4 and 43.1 minutes.

CONCLUSION

The Waters UPLC® Glycan Separation technology columns, when operated with the ACQUITY UPLC System and its FLR Detector, provide a highly-resolving, reproducible, rapid method for profiling glycans.

- The resolution, sensitivity, and speed of UPLC profiling outstrips all previous HPLC solutions.
- The N-linked glycans that are released from glycoproteins are labeled with 2-AB.
- Labeled glycans are separated in HILIC mode.
- The same column and mobile phase can be used for neutral and for charged oligosaccharides.
- Positional and linkage isomers can be resolved.
- High resolution leads to unambiguous glycan identification and reliable relative quantitation.
- Increased resolution improves sample throughput and overall productivity.
- Methods will transfer readily between laboratories because of instrument consistency and column batch testing.
- Expands the family of UPLC solutions for the analysis of biopharmaceuticals.

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1. Bigge, J.C., Patel, T.P., Bruce, J.A., Goulding, P.N., Charles, S.M. and Parekh, R.B. (1995) Non-selective and efficient fluorescent labeling of glycans using 2-aminobenzamide and anthranilic acid. Anal. Biochem., 230, 229-238.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Deglycosylation and Sample Cleanup Method for Mass Spectrometry Analysis of N-linked Glycans

Ying Qing Yu, Martin Gilar, Jennifer Kaska, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

OVERVIEW

A rapid method for analysis of glycans is presented. This method describes deglycosylation of glycoproteins aided by an enzyme friendly surfactant (RapiGest™ SF), sample cleanup using a HILIC chromatography performed in a 96-well microtiter plate (MassPREP™ HILIC μElution Plate), and MALDI MS analysis of the resulting glycans (MassPREP MALDI Matrix, DHB).

INTRODUCTION

Glycosylation is one of the most important types of post-translational modification (PTM) in proteins. Due to the high degree of heterogeneity, the characterization of glycans is a challenging task. Mass spectrometry is a primary tool for biopolymer analysis; however, the characterization of (native) glycans is complicated by the time-consuming sample preparation, and their poor MS ionization efficiency. A typical sample preparation method for mass spectrometry involves a chemical or enzymatic cleavage of glycans, followed by salts, surfactants, and protein residues removal. Purified native glycans can be directly analyzed by MALDI-TOF MS.

The efficient sample deglycosylation is a key requirement for a successful and sensitive glycan analysis. Nevertheless, the quantitative glycan release (e.g., using enzymes) is rarely achieved, since the glycosylated sites of the proteins are often obstructed by the protein secondary and tertiary structure.

The goal of this work was to develop a rapid and efficient deglycosylation of N-linked glycoproteins with a glycosidase (PNGase F) aided with the enzyme-friendly surfactant, RapiGest SF. This was followed with a novel micro-scale hydrophilic-interaction chromatography (HILIC) solid-phase extraction (SPE) plate (MassPREP HILIC μElution Plate) for a rapid sample cleanup prior to MALDI MS analysis using highly purified MALDI matrix (MassPREP MALDI Matrix, DHB).

EXPERIMENTAL CONDITIONS

Deglycosylation of N-linked proteins

The glycoproteins were solubilized in 0.1% (w/v) RapiGest SF solution prepared in 50 mM NH_4HCO_3 buffer, pH 7.9. Protein samples (e.g., ovalbumin) were reduced with 10 mM DTT for 45 minutes at 56 °C, and alkylated with 20 mM iodoacetamide in the dark for 1 hour at room temperature. The enzyme PNGase F (2.5–5 units) was added, and the protein solutions were incubated for 2 hours at 37 °C.

LC/MS analysis of the protein deglycosylation

The RP HPLC instrument (CapLC® XE, Waters) was equipped with a microbore RP-HPLC column (Waters Atlantis® dC_{18} Column, 3.5 μm, 1.0 x 100 mm). Time-of-flight (TOF) mass spectrometry analysis was performed using Waters Q-Tof micro.™ Mobile phase A was made of 0.1% formic acid in Milli-Q water. Mobile phase B was made of 0.1% formic acid in 100% acetonitrile. A Linear gradient was run from 0 to 60 % B in 30 minutes (2 % B per min). Separation was carried out with 35 μL/min flow rate; the column temperature was set at 40 °C.

Wash:
200 μL of Milli-Q water

Condition:
200 μL of 90% acetonitrile

Constiute sample:
80-90% acetonitrile
The final volume: 100-750 μL

Load:
Constituted sample

Wash:
200 μL of 90% acetonitrile

Elute:
20-50 μL of 25 mM ammonium citrate
in 25% acetonitrile

Figure 1.
A general instruction for oligosaccharide sample clean up using the MassPREP HILIC μElution plate is illustrated.

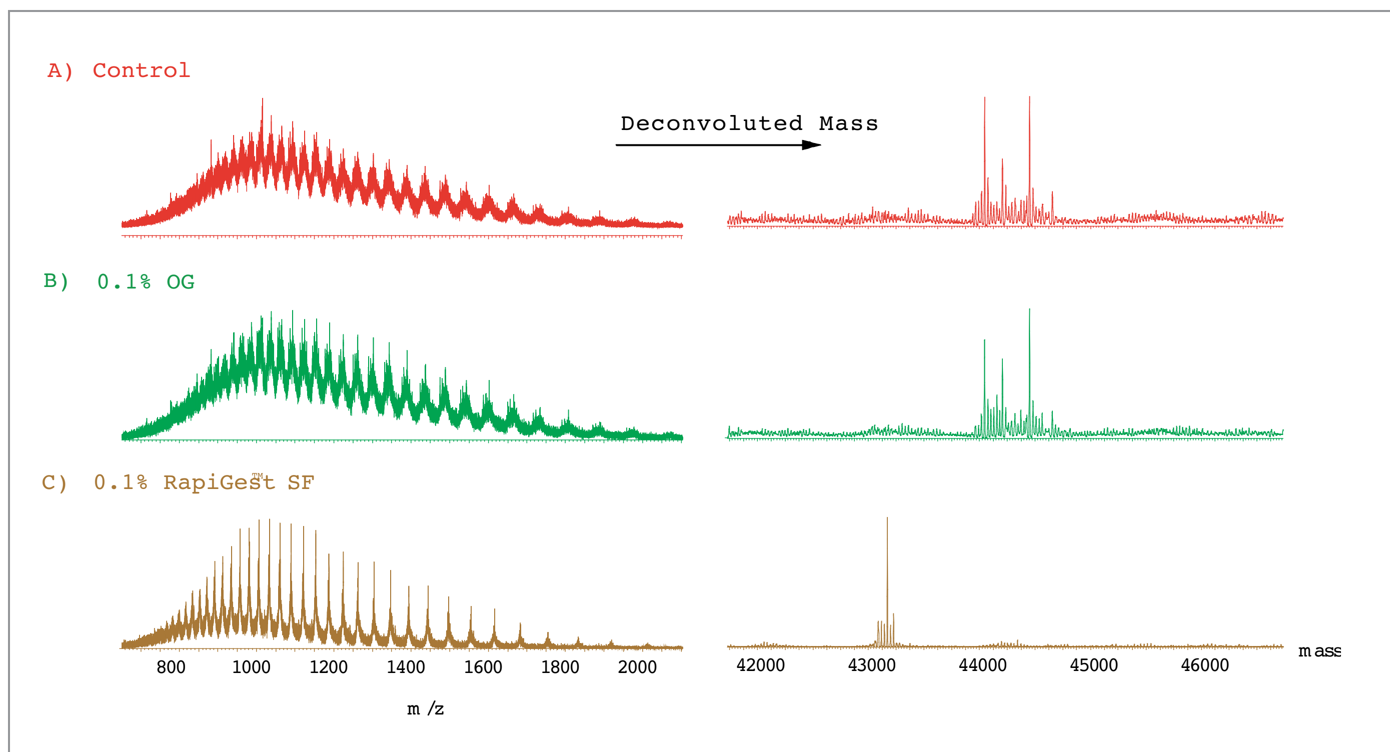

Figure 2. LC/MS spectra of deglycosylated ovalbumin are shown. A) Ovalbumin was solubilzed without the use of denaturant, and was not deglycosylated. B) Ovalbumin was denatured using 0.1% n-octyl-b-glycopyranoside (OG) and deglycosylated. C) Ovalbumin was denatrued in 0.1% RapiGest SF solution and deglycosylated. The MS scans were deconvoluted to the MW of the protein. Complete deglycosylated was observed after 2 hours deglycosylation for the RapiGest SF solubilized ovalbumin.

Glycan cleanup using a 96-well micro-elution HILIC SPE plate

The N-linked glycans released from glycoproteins were extracted using a 96-well, MassPREP HILIC µElution Plate attached to a vacuum manifold. Use of this SPE device involves an initial wash and eluent equilibration of the sample well(s), sample loading, sample well washing to remove undesired products, and final elution of the isolated glycans. Figure 1 shows the optimized MassPREP HILIC µElution Plate SPE protocol for both neutral and sialyated glycans. The entire process requires less than 20 minutes. The HILIC plate performance was evaluated with maltoheptaose standard. Load, wash, and elution SPE fractions were quantitatively analyzed by a HPLC system with Evaporative Light Scattering Detection (ELSD). The mass balance revealed no breakthrough in the load fraction. Most of the material eluted in the first 25 µL elution. Total mass balance was 90%. Recovery was estimated to be approximately 70%.

MALDI Q-Tof MS experiments

Ultra pure MassPREP MALDI Matrix, DHB (2,5-Dihydroxybenzoic Acid) was used for MALDI-Tof analysis. The matrix was reconstituted in 500 µL of pure ethanol to a final concentration of 20 mg/mL. Purified glycan solutions were mixed with DHB matrix in one to one ratio; 1 µl was placed onto a stainless steel MALDI target. Waters Q-Tof Ultima® MALDI was used to determine the molecular weight of the released glycans and performed MS/MS experiments to characterize the structure of the glycans. The typical collision energy used here was 70 to 120 V.

RESULTS

Protein deglycosylation under denaturing conditions

In earlier reports we described the use of a mild and enzyme friendly surfactant, RapiGest SF, for denaturation of the proteins prior to proteolytic enzymatic digestions.[1] It was found that this surfactant improves the speed and completeness of enzymatic proteolysis, most noticeably for globular and membrane proteins.[2] Therefore, we investigated the use of RapiGest SF in conjunction with PNGase F for the enzymatic release of N-linked glycans.

Figure 2 shows the extent of the deglycosylation reaction of chicken ovalbumin solubilized in 0.1% RapiGest SF (Figure 2C) digested with PNGase F for 2 hours in 50 mM ammonium bicarbonate solution. The progress of deglycosylation is apparent in comparison to a control ovalbumin sample (Figure 2A) with no enzyme added. The deglycosylation was also carried out with the addition of 0.1% of non-ionic surfactant, n-octyl-b-glycopyranoside (OG) (Figure 2B).

The LC/MS analysis of samples produced the ESI spectra featuring the multiply charged protein states, which were deconvoluted using Waters MassLynx™ MaxEnt1 Software (deconvoluted MS spectra are shown in the right panel in Figure 2). As expected, no signal corresponding to the MW of deglycosylated protein was found in the control sample (Figure 2A). Interestingly, no distinguishable deglycosylation was also observed in the OG-mediated deglycosylation (Figure 2B). Multiple peaks between 44-45 kDa represent the various N-linked glycoforms of ovalbumin. The reaction in the presence of RapiGest SF shows nearly complete deglycosylation; the protein mass shifted and a prominent peak was detected at approximately 43 Kda which is consistent with the MW of the unmodified protein.

Glycan sample cleanup prior to MALDI-MS analysis

The glycans were extracted using the MassPREP HILIC µElution Plate. In a HILIC mode, the hydrophilic glycans are retained due to a partitioning separation mechanism between the organic mobile phase and a layer of water adsorbed on the surface of sorbent. Since the high concentration of organic solvent is necessary to ensure good retention of glycans, the samples were first diluted with ACN to a final concentration of 80-90%. Some precipitation of glycans may occur if they are present at high concentrations. It is not recommended to centrifuge samples prior to loading to the HILIC micro elution plate. After plate conditioning (sample cleanup section in experimental), glycan samples were loaded by gravity (Figure 1).

MALDI Q-Tof MS/MS of glycans released from ovalbumin

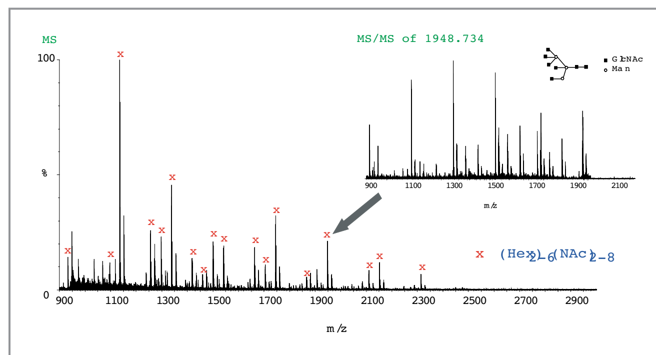

Figure 3. A) MALDI Q-Tof MS spectrum of the oligosaccharides released from ovalbumin. The $(M+Na)^+$ ions corresponding to the glycan species, (Hex)2-6(Nac)2-8, are labeled. B) MS/MS fragmentation was used to elucidate the structure of glycans.

The MALDI Q-Tof MS spectra of underivatized N-linked glycans released from 10 pmol Ovalbumin were obtained (Figure 3). MS/MS fragmentations of selected ions were performed to validate the glycan structures. For example, collision induced dissociation of the complex glycan ion of mass to charge ratio of 1948.734 (M + Na) was shown (Figure 3). This ion is observed in the MS mode with low ion intensity, however, enough fragmentation ions were produced in the MS/MS mode to determine its structure (GlycoSuite database, Proteome Systems, Ltd.).

Surfactant removal using the HILIC µElution Plate

The MassPREP HILIC µElution Plate facilitates the removal of impurities including the surfactants, such as RapiGest SF from the sample. It can be used for surfactant removal in general, for example SDS from peptides/glycopeptides. Figure 4 shows the MALDI MS analysis of the bovine serum albumin (BSA) tryptic digest. No signal was observed for the sample contaminated with 0.1% of SDS, while BSA tryptic peptide signals were observed in high abundance without any ion suppression caused by the presence of SDS.

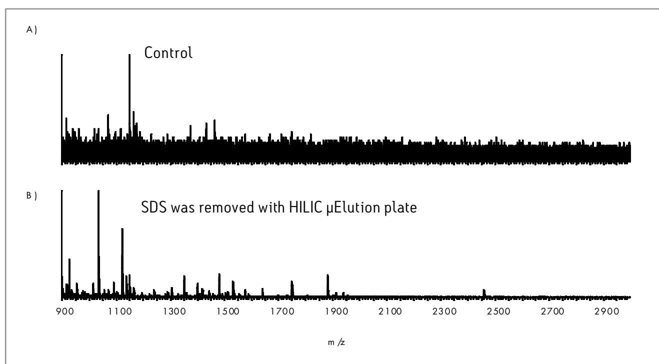

Figure 4. MALDI MS spectra of 5 pmol BSA tryptic peptides. A) Control sample, SDS was not removed by any SPE method. B) MassPREP™ HILIC µElution Plate was used to remove the SDS prior to MALDI-Tof Analysis.

CONCLUSION

We have developed a method suitable for fast and robust analysis of glycans released from glycoproteins. The method utilizes an enzyme friendly surfactant (RapiGest SF) that was shown to greatly accelerate a deglycosylation reaction via glycoprotein denaturation, which makes the glycans more accessible to enzymatic cleavage. A complete deglycosylation of proteins was achieved after 2 hours incubation with PNGase F. The MassPREP HILIC µElution plate was utilized to extract and desalt the glycans prior to their MS analysis using MassPREP MALDI Matrix, DHB. The SPE method is fast and requires minimum sample manipulation.

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

[APPLICATION NOTE]

Rapid Sample Cleanup Method Development for 2-Aminobenzamide Labeled N-Linked Glycans Released from Glycoproteins

Joomi Ahn, Ying Qing Yu, and Martin Gilar
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Recombinant glycoprotein drugs destined for human use have been widely developed in the biopharmaceutical industry in the last decade. Glycosylation of proteins has a direct impact on their biological activities. Since the nature of protein glycosylation is highly dependent on biosynthesis conditions, monitoring and controlling the biomanufacturing processes of glycotherapeutics is required.

New analytical methods for glycan/glycoprotein characterization are highly desirable. Fast and reliable methods for sample cleanup in glycan analysis are an essential part of the biopharmaceutical analytical method toolbox.

Several technologies are currently being used in glycan characterization, such as mass spectrometry (MS), high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), capillary electrophoresis (CE), and liquid chromatography (LC) with fluorescence (FLR) detection.

In LC-FLR, glycans are typically labeled with 2-aminobenzamide (2-AB), which permit highly-sensitive fluorescence detection.[1] This technique allows the quantitation of relative amounts of individual glycans in a heterogeneous complex mixture. In addition, 2-AB labeled glycans provide improved sensitivity in ESI-MS and MALDI-MS for glycan mass profiling analyses.

The preparation of purified 2-AB labeled glycans released from glycoproteins can be time consuming, with multiple steps involving deglycosylation using glycosidases and glycan enrichment followed by 2-AB derivatization (Figure 1).

In this application, we demonstrate how to prepare purified 2-AB labeled N-linked glycans released from glycoproteins such as monoclonal antibodies (IgG1). The goal of this method is to provide a rapid, efficient cleanup protocol for LC-FLR, MALDI-MS, and LC/MS analyses using the Waters® MassPREP™ Glycoanalysis Kit.

The MassPREP Kit provides simple and robust sample purification without compromising sample recovery. This complete sample cleanup solution streamlines glycan method development by reducing time and expenses. This kit includes:

- A Hydrophilic-Interaction Chromatography (HILIC) µElution Plate
- RapiGest™ SF Surfactant to assist protein enzymatic deglycosylation
- MassPREP MALDI matrix, DHB (2,5-dihydroxybenzoic acid) for MALDI-MS analysis

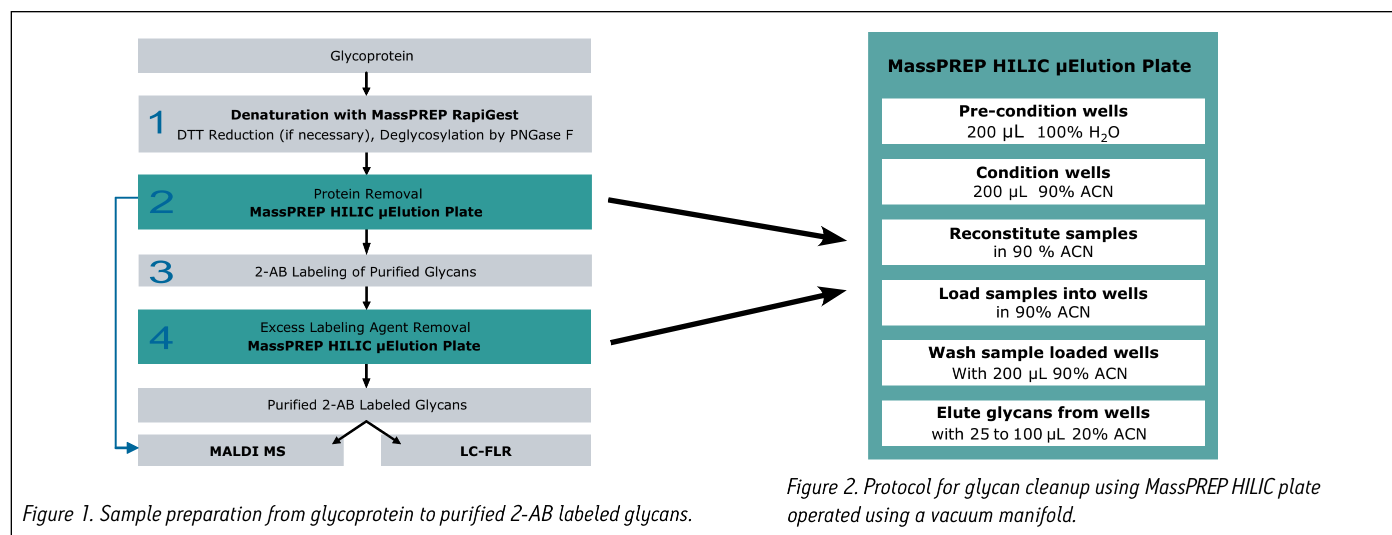

Figure 1. Sample preparation from glycoprotein to purified 2-AB labeled glycans.

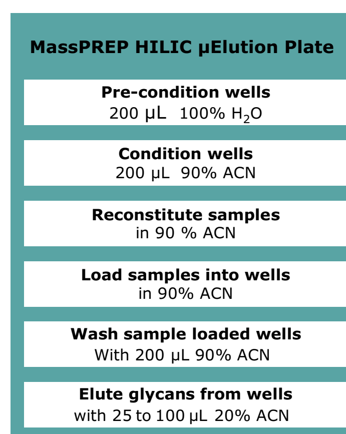

Figure 2. Protocol for glycan cleanup using MassPREP HILIC plate operated using a vacuum manifold.

PRINCIPLES AND EXPERIMENTAL

Figure 1 schematically presents the workflow for glycan sample preparation and the downstream analyses. The following workflow details each step in the protocol.

1. Deglycosylation of N-linked glycans with PNGase F

Monoclonal mouse IgG1 (VICAM, Division of Waters) and ribonuclease B (Sigma) were prepared separately. Each glycoprotein was solubilized in 0.1 % (w/v) RapiGest SF solution prepared in 50 mM NH_4HCO_3 buffer at pH 7.9. RapiGest partially denatures the glycoprotein, which exposes the glycosylated site to enzymes for faster and more complete deglycosylation.[2]

IgG1 was reduced with 20 mM dithiothreitol (DTT) for 45 minutes at 65 °C. DTT reduction was not performed in the ribonuclease B preparation. The enzyme PNGase F (Sigma) was added to the protein solutions and the samples were incubated overnight at 37 °C in sealed vials.

2. Protein removal from glycan samples

Enzymatically released N-linked glycans were extracted from deglycosylated protein solutions using a MassPREP HILIC 96-well µElution solid-phase extraction (SPE) plate.[3]

When loaded in high acetonitrile content, HILIC SPE sorbent retains glycans (hydrophilic compounds). More hydrophobic compounds and surfactants are not retained and washed out from the sorbent. The glycans are eluted with solvents containing predominantly water. The MassPREP HILIC plate was utilized following guidelines in Figure 2.[4] The eluted glycans were then dried.

In some cases when a deglycosylated protein solution containing free glycans is reconstituted in 90% acetonitrile, the proteins present in the sample tend to precipitate and restrict flow in the HILIC SPE plate. The Waters Sep-Pak® tC_{18} µElution 96-well plate effectively removes soluble and insoluble proteins from the glycan sample.

The flow-through eluent from the Sep-Pak tC_{18} protocol, which is shown in Figure 4A, contains free glycans in 10% acetonitrile. The flow-through eluent was dried for use in the labeling process. (Note: the Waters Sep-Pak tC_{18} µElution 96-well plate can be purchased separately from the MassPREP Glycoanalysis Kit.)

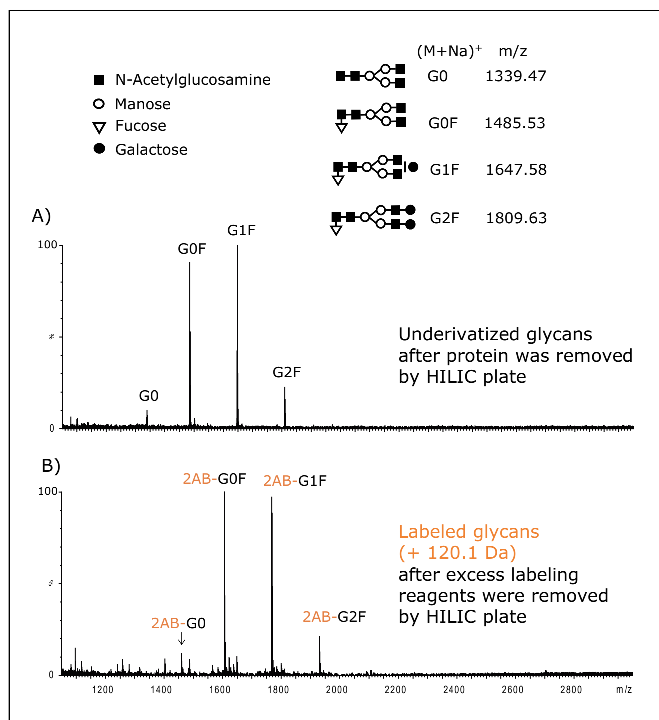

Figure 3. MALDI-TOF MS spectra of: (A) 8 pmol of the unlabeled IgG1 glycans after first HILIC cleanup prior to labeling. (B) 3 pmol of the 2-AB labeled IgG1 glycans after second HILIC cleanup. Each spectrum shows well purified glycans with no inferences from impurities.

3. Labeling glycans with 2-aminobenzamide

Isolated glycans were labeled using the 2-AB labeling kit from Sigma. 150 µL of acetic acid and 300 µL of dimethyl sulfoxide (DMSO) were mixed, and 100 µL of the acetic acid/DMSO mix was added into 5 mg of 2-AB. The entire volume of 2-AB solution was then added into 6 mg of sodium cyanoborohydride. Five to 10 µL of the final labeling solution was added into dried glycans (100 pmol to 50 nmol) and incubated for 3 hours at 65 °C in a sealed vial.

4. Removing excess labeling agents

The 2-AB labeled glycan solution from step 3 was diluted to 90% acetonitrile to reach the HILIC loading condition. The excess labeling reagents were effectively removed from 2-AB labeled glycans using the same HILIC protocol as shown in Figure 2. The purified labeled glycans were then analyzed by MALDI-MS or by LC with FLR detection.

MALDI-TOF MS

Ultra-pure MassPREP Matrix, DHB, was used in the MALDI-TOF MS analysis. The DHB matrix was reconstituted in 500 µL of pure ethanol to a final concentration of 20 mg/mL. The purified 2-AB labeled glycans were mixed with the DHB matrix at a 1:1 ratio. One µL of the glycan/DHB mix was placed onto a stainless steel MALDI target.

MS system:	Waters MALDI micro MX™
Ionization mode:	Reflectron positive
Pulse voltage:	1950 V
Detector voltage:	2350 V
Laser:	250
Laser firing rate:	10 hz
Acquisition range:	800 to 3000 m/z

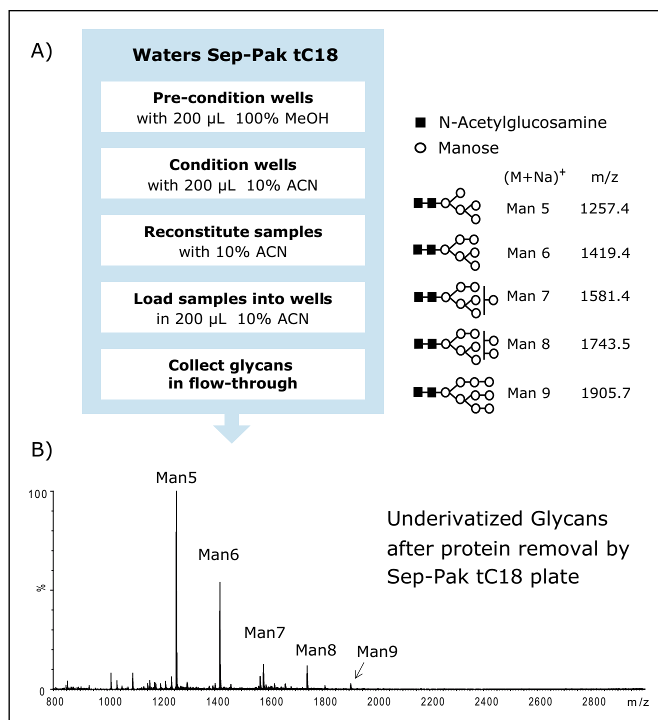

Figure 4. (A) Protocol for protein cleanup of underivatized glycan using Sep-Pak tC_{18}. The flow-through was collected, dried, and analyzed by MALDI-MS. (B) MALDI spectrum of 10 pmol of underivatized glycans released from ribonuclease B following the Sep-Pak tC_{18} protocol. Undesired protein was retained in Sep-Pak tC_{18} plate, and the flow-through sample containing free-glycans was analyzed by MALDI MS.

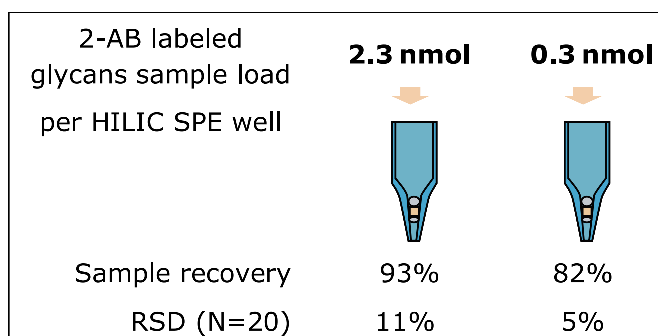

Figure 5. Yields of 2-AB labeled glycans measured at two mass loads in HILIC.

RESULTS AND DISCUSSION

Glycan sample cleanup

Sample cleanup is critical for both underivatized and labeled glycans in MS analyses. For underivatized glycans, the HILIC SPE plate can be used to remove protein, salt, and surfactant prior to MALDI-MS as shown for N-glycans released from IgG1 (Figure 3A). During labeling, an excess 2-AB is required to achieve complete derivatization of glycans.

However, the excess of labeling reagent often needs to be removed from the sample prior to LC-FLR and MS analyses. A suitable protocol using MassPREP HILIC SPE is shown in Figure 1, Step 4. The method successfully recovers 2-AB labeled glycans as shown in Figure 3B.

Because the HILIC sorbent has sufficient affinity for hydrophilic glycans, they can be highly enriched on the SPE plate. With sample loads of several hundred microliters, glycans can be recovered in ~25 to 100 µL of eluent. On the contrary, the cleanup method shown in Figure 4B utilizing the Sep-Pak tC_{18} SPE plate does not allow for sample concentration. Sep-Pak tC_{18} SPE devices can be used for protein removal, as recommended, but the glycans recovered in the sample flow-through are neither concentrated nor desalted.

Recovery of labeled glycans

LC-FLR analysis was used to assess the labeled glycans recovery after HILIC cleanup. The FLR signals of 2-AB labeled glycans were compared to the injections of the sample prior to SPE cleanup. Results are summarized in Figure 5. Approximately 0.3 nmol or 2.3 nmol of 2-AB labeled N-glycans released from ribonuclease B were loaded in an HILIC plate and eluted out with 200 µL of 20% acetonitrile. Excellent SPE recovery was achieved for both mass loads with good reproducibility in 20 parallel experiments (Figure 5).

CONCLUSION

We have shown the use of the MassPREP HILIC μElution plate for extraction of underivatized and 2-AB labeled glycans. The method is fast and reproducible. The key advantages of this method include:

- Removal of the sample background in MS and LC-FLR analyses
- Simple to use with a step-by-step protocol
- Rapid sample processing time
- Applicable to high throughput analyses with 96-well SPE plate
- High glycan recovery
- Convenient kit format

The MassPREP Glycoanalysis Kit is designed for biopharmaceutical applications, minimizing the amount of sample required for the analysis. Because of the format of μElution SPE plate, small volumes of eluents permit shortened evaporation time, eliminating the bottleneck in sample preparation.

Rapid and effective sample preparation helps to improve laboratory productivity and analysis throughput. Increased throughput plays an essential role in reducing the time needed for method development. This, in turn, reduces the amount of materials and laborious processes necessary for validation and qualification of methods developed in biopharmaceutical laboratories.

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3. Waters Care and Use Manual Part Number 715001013.
4. Deglycosylation and sample cleanup method for mass spectrometry analysis of N-Linked glycans. Waters Application Note. 2007; 720001146en.

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

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Structural Analysis of an O-Glycopeptide Derived from Recombinant Erythropoietin by SYNAPT High Definition Mass Spectrometry (HDMS)

Weibin Chen and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Many therapeutic proteins contain carbohydrate moieties (glycans) on the side chain of an amino acid as a result of post-translational modifications (PTM). The glycosylation on a protein drug can play an important role in *in vivo* activity, molecular stability, solubility, serum half-life, and immunogenicity.

The existence of multiple glycans on the same polypeptide backbone creates a mixture of "glycoforms" of a therapeutic protein. It is well acknowledged that certain degree of glycosylation heterogeneity occurs to most natural and recombinant glycoproteins.[1] Therefore, knowledge about the glycosylation structures of the biomolecule is crucial from both a regulatory and business standpoint. The glycosylation heterogeneity must be shown to a regulatory agency to be consistent within specifications and between manufacturing batches. On the other hand, glycan differences between expressed versions of the same recombinant glycoprotein may raise questions on whether the drug is patentable.

Due to the high structural diversity of glycans and the amino acid sequence variations of proteins, glycosylation characterization is a complex task that involves many different analytical techniques. A successful analytical strategy for glycosylation characterization needs to reveal the glycosylation sites of both N- and/or O-linked glycans and to identify the types of carbohydrate structures present at each glycosylation site. To address this site-specific carbohydrate heterogeneity, mass spectrometry has become the method of choice.

Compared to N-glycosylations, site-specific characterization of O-glycosylation by mass spectrometry is inherently more challenging. The analytical difficulties arise from many intrinsic factors associated with this type of glycosylation, such as no consensus amino acid sequence for O-glycan attachment, varied glycan chain length and branching pattern, and lack of common carbohydrate core types at the attachment sites.

Traditional methods that involve the analysis of the released O-glycans after chemical or enzymatic deglycosylation may provide a detailed structural characterization for the glycans derived from a protein, but information on the original attachment sites of the glycans and the micro-heterogeneity of each occupation site is lost. Although this critical information can be obtained by the analysis of the deglycosylated peptides, the method is time consuming and the information is difficult to confirm by an orthogonal analytical method.

Direct analysis of glycopeptides by tandem mass spectrometry (MS/MS) represents a rapid and sensitive method to obtain site-specific characterization on O-glycosylation. It can provide information on glycan structure, glycan attachment site, and peptide sequence within one single experiment. However, direct fragmentation of O-glycopeptides normally generates very complex mass spectra that contain fragment peaks from the intact or truncated glycan chains as well as the deglycosylated peptide backbones (after the complete loss of O-glycan chain). This heterogeneity, together with the superimposition of various charge states, makes the MS/MS spectra interpretation difficult. As a result, the successful application of this approach to the site-specific O-glycosylation is rather limited.

Here we present a study that uses a novel MS system that combines high-efficiency ion-mobility based separations (IMS) with high-performance tandem mass spectrometry, utilizing the Waters® SYNAPT™ High Definition MS™ (HDMS™) System to characterize an O-linked glycopeptide from recombinant Erythropoietin (EPO).

The approach takes advantage of Triwave™ Technology (which utilizes a unique dual-collision-cell design combined with ion-mobility separations) to perform a time-aligned-parallel (TAP) fragmentation (Figure 1) of O-glycopeptides, in which information on the glycan sequence and the glycosylation site is simultaneously obtained.[2]

The results demonstrate the benefit of enabling dual-stage fragmentation where the independently-controlled collision induced dissociation provides an increase in the information obtained to describe site-specific characterization of the glycopeptide.

EXPERIMENTAL

Recombinant erythropoietin (Human, CHO cells) was purchased from EMD Biosciences, Inc (La Jolla, CA). The protein was reconstituted at a concentration of 0.5 mg/mL in 50 mM ammonium biocarbonate (pH 8.0) with 0.1% RapiGest.™ The sample was incu-bated at 60 °C for 30 min, then reduced with 5.0 mM DTT at 37 °C for 30 min, and alkylated with 10mM of iodoacetamide (IAA) in the dark for 45 min. Trypsin digestion was performed overnight at 37 °C (trypsin/ protein ratio, 1:20, w/w). The digest was diluted to 0.15 mg/mL with 0.1% formic acid (FA) prior to injection.

LC conditions

LC system: Waters nanoACQUITY UPLC®
Column: Atlantis® dC_{18} 3 µm, 300 µm x 100 mm
Column temp.: 40 °C
Flow rate: 5 µL/min
Mobile phases: Eluent A: Water with 0.1% FA
Eluent B: ACN with 0.1% FA
Gradient: 1% to 50% B in 30 min
Sample inj. vol.: 5 µL

MS conditions

MS system: Waters SYNAPT HDMS
Ionization mode: ESI positive
Capillary voltage: 3.2 kV
Cone voltage: 33 V
Desolvation temp.: 250 °C
Desolvation gas: 200 L/Hr
Source temp.: 105 °C
Acquisition range: 100 to 2100 m/z
IMS gas: N_2
IMS gas pressure: 0.5 mbar
Pulse height: Variable, 7.5 to 9.5 V

TAP fragmentation

TRAP T-Wave™ Collision Energies: 30 to 50 V

TRANSFER T-Wave CE (alternated): 5V (low) 60 to 100V (high)

RESULTS

The analytical objective is to obtain the glycan sequence and confirm the glycosylation site of an O-glycopeptide from recombinant Erythropoietin using the TAP fragmentation method. To perform TAP fragmentation using the SYNAPT HDMS System, O-glycopeptide of interest (m/z 1061.6, $MH_2$2+) is first selected by the quadrupole ion filter and subsequently transferred into the TRAP cell of the Triwave (Figure 1). The collision energy of the TRAP T-Wave collision cell is optimized for glycan fragmentation using a collision energy of 35V, since more energy is required to achieve efficient fragmentation of the peptide backbone.

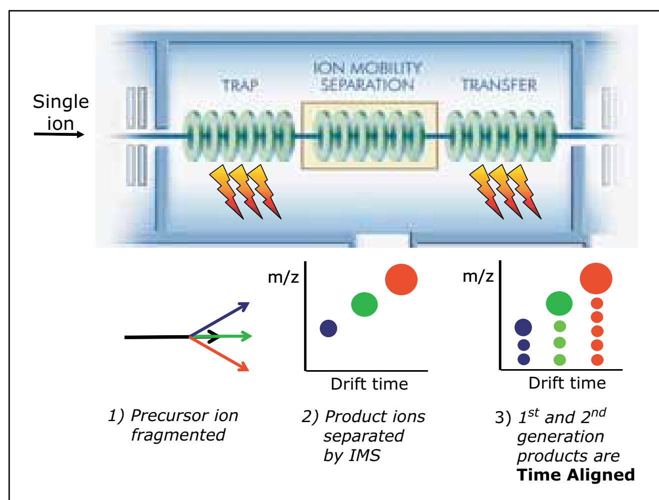

*Figure 1. A schematic illustration of time-aligned-parallel (TAP) fragmentation approach using the Triwave device in the SYNAPT HDMS System. 1). Precursor ion of interest is selected by the quadrupole and fragmented in the TRAP T-Wave. 2). The resulting first-generation product ions are separated according to their mobility in the IMS T-Wave. 3). The first-generation product ions are subjected to an additional stage of fragmentation in the TRANSFER T-Wave. All first-generation product ions are fragmented in the TRANSFER T-Wave, a parallel process; the second-generation product ions are **Time Aligned** to the original first-generation ions.*

The fragment ions generated in the TRAP T-Wave collision cell of SYNAPT HDMS were then transported into the IMS T-Wave where ions were separated into individual packets on the basis of their ion mobility (combination of size, shape, and charge, as well as mass). The ion-mobility-separated fragment ions were subsequently transported through the TRANSFER T-Wave collision cell in which the collision energy was alternated between two settings (high and low).

When the collision energy of the TRANSFER T-Wave is set at a low energy (normally at 5V), no additional fragmentation takes place in the TRANSFER T-Wave. Fragment ions observed in this scan would only represent the fragments generated in the TRAP T-Wave. Thus MS/MS data recorded in this process would contain information only for the deduction of the glycan sequence of the O-glycopeptides.

Figure 2A shows the ion-mobility-separated fragments (m/z versus drift time displayed in DriftScope™ Software) generated from this process, and the summed mass spectrum is shown in Figure 2B. From Figure 2B, it can be seen that fragmentation of the peptide backbone under the chosen conditions is negligible. After mass analysis of the processed MS/MS data, the glycan sequence of NeuNAc-Hex-HexNAc can be easily deduced (Figure 2C). The glycan sequence matches that which is reported about the O-glycan sequence of erythropoietin in literature.[3]

The O-glycopeptide from erythropoietin contains two potential glycosylation sites at positions 6 and 12 from the C-termini of the peptide (Sequence: EAISPPDAASAAPLR). To define which position is the carbohydrate attachment site, we focus on the fragment ion that has m/z value of 834.84 (Figure 2B). Mass analysis on the fragment suggests that this ion comes from a peptide that contains a single HexNAc attached to the glycosylated residue of the O-glycopeptide. The presence of the extra HexNAc serves as an indicator of a glycosylation site because mass spectral peaks covering the attachment site would ordinarily show a mass increase in the MS/MS spectrum compared to the ones generated from un-modified sites.

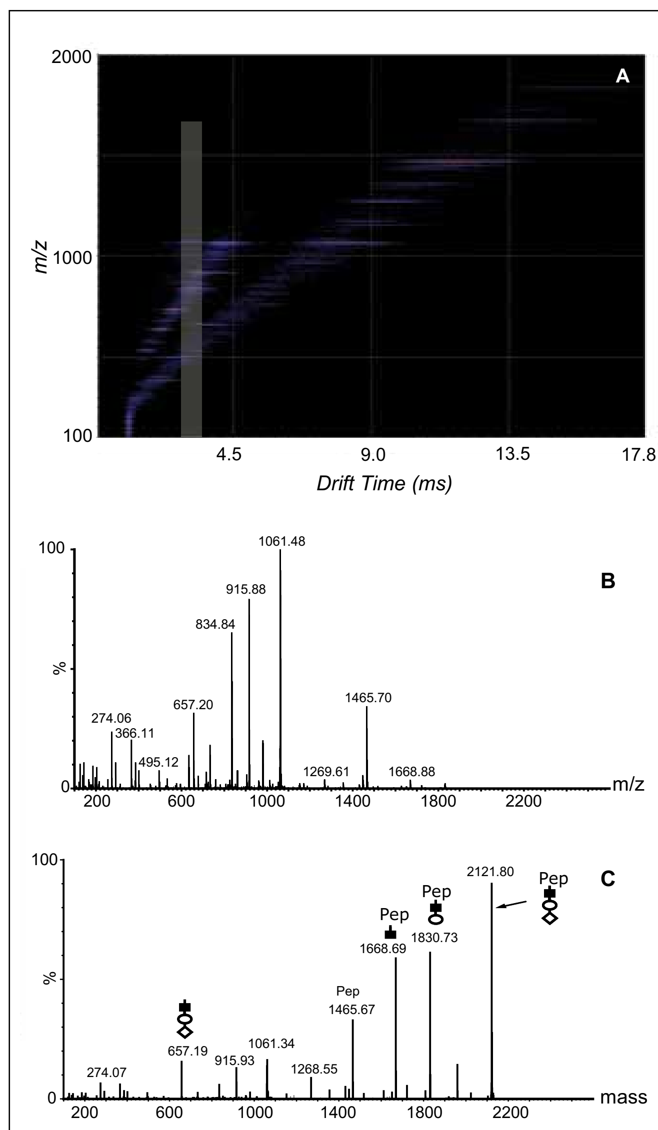

Figure 2. HDMS data (m/z versus drift time, DriftScope Software) and mass spectra for the fragment ions generated in the first-stage fragmentation of the O-glycopeptides. (A). m/z versus drift time for the fragments from the O-glycopeptide. The highlighted region shows the drift-time span for the fragment ion with m/z 834.34. (B). Summed raw ESI-IMS-TOF spectrum of all the fragments from the first-stage fragmentation, during which only the cleavages of the O-glycan occurred. (C). MS/MS spectrum from MaxEnt™ 3 deconvolution of the data in Figure 2B. MaxEnt 3 converts peaks with multiply charge states into the singly charged state for easy sequence deduction. Symbols: (■) HexNAc (❍) Hex (◇) NeuNAc

To obtain the fragmentation of this precursor, the collision energy of the TRANSFER T-Wave is increased to a higher energy (typically 60 to 100V) in the second scan, and the ion-mobility-separated product ions are subjected to a second-generation fragmentation at which peptide backbone cleavage would occur.

Figure 3A shows the corresponding data, in DriftScope Software, obtained from the O-glycopeptide fragments after the collision energy of the TRANSFER T-Wave was raised to 80 V. Figure 3B displays the mass spectrum that is generated by combining all the fragmentation data from drift time 3.45 ms to 3.78 ms.

Since these product ions all share the same drift time as the precursor ion (m/z 834.84, Figure 2A), these fragment ions all come from the same precursor, and the reconstructed spectrum over that range of drift times corresponds to the peptide sequence of the precursor.

In Figure 3B, two series of fragment ion peaks are observed (labeled with red or blue letters). The ion series labeled by red letters correspond to a mass increase of 203 Da when compared to those labeled by blue letters, implying these are the fragments containing the HexNAc at the glycosylation site.

Since the mass difference between y12 (m/z 1355.57) ion and y11 (m/z 1268.67) ion of this ion series is only 87 Da, it can be deduced that the O-glycosylation site is on the serine residue at position 6 not at position 12.

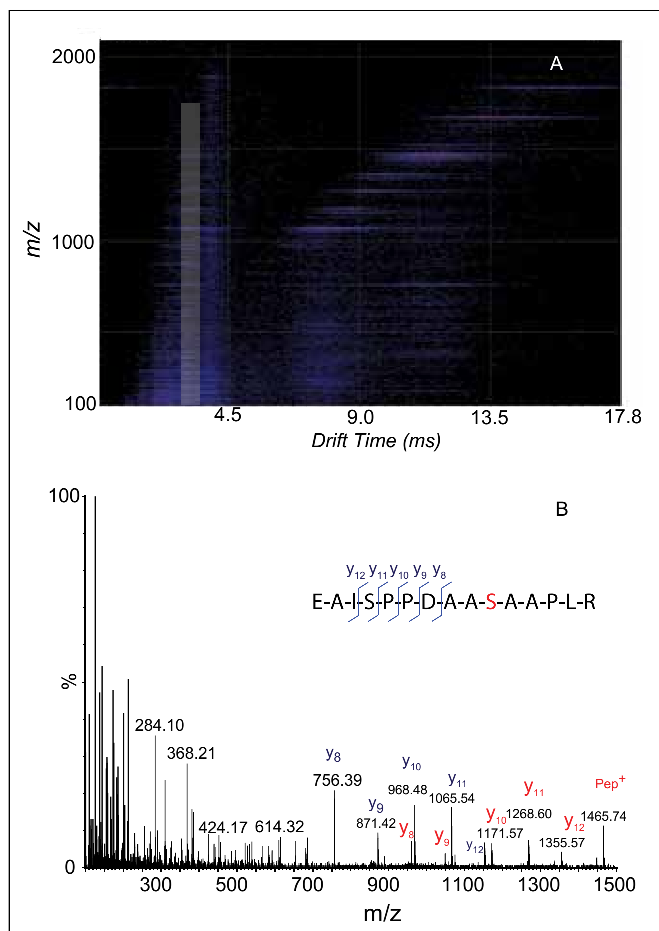

Figure 3. Obtaining the O-glycosylation site by performing second-stage fragmentation on the product ions generated in the TRAP T-Wave. (A) HDMS data (m/z versus drift time, DriftScope Software) of the second generation of fragment ions following CID in the TRANSFER T-Wave (CE of 80V). The boxed highlight region represents the distribution of the second-generation product ions from the precursor m/z 834.84 as shown in DriftScope (note the precursor and fragments share the same drift time). (B) Reconstructed MS spectrum from the boxed region in Figure 3A with sequence ions annotated. The red letters annotate the ions from the peptide with an attached HexNAc at position 6 (serine residue in red), whereas the blue letters label the fragments generated by the peptide without any modification (due to the loss of HexNAc before the fragmentation of peptide backbone occurs).

CONCLUSION

A time-aligned-parallel (TAP) fragmentation approach is applied to site-specific characterization of an O-glycopeptide. Using this method, both the glycosylation site and glycans sequence of the O-glycopeptide from Erythropoietin are obtained in a single experiment.

These results indicate that the SYNAPT HDMS System is a powerful tool for the detailed characterization on glycosylated therapeutic protein, providing critical information:

- to understand the immunogenicity of the drug
- to control the production reproducibility of the drug
- to meet the regulatory approval requirements for the drug

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

[APPLICATION NOTE]

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Separation and Characterization of N-linked Glycopeptides on Hemagglutinins in a Recombinant Influenza Vaccine

Hongwei Xie, Weibin Chen, Martin Gilar, St John Skilton, and Jeffery R. Mazzeo
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Influenza is a viral infection of the respiratory tract. It is one of the leading causes of death in the U.S., killing more than 50,000 people per year.[1] Influenza vaccination is a primary prophylactic method and the principal strategy for reducing morbidity and mortality due to seasonal influenza. Vaccines provide protection by neutralizing antibodies to viral hemagglutinin (HA), a protein that plays a critical role in influenza infection.

Licensed, inactivated vaccines for seasonal influenza usually contain a preset amount of HAs – a mixture of H1, H3, and B, the corresponding HA proteins of the three most common viruses – influenza A subtypes H1N1 and H3N2, and influenza B. These HA proteins are glycoproteins, with multiple N-linked glycosylation motifs and multiple glycoforms for each glycosylation site. Detailed characterization and monitoring of glycosylation in HAs is important for both vaccine development and production because of their role in determining the function of influenza binding onto host cells and therefore infection.

Currently, the methods for characterization of N-linked glycosylations include released free glycan analysis[2-4] and intact mass analysis.[5-7] These methods are useful for analyzing glycoproteins with defined glycosylation sites, such as monoclonal antibodies. Glycan profiling can be performed at the intact protein level (intact mass analysis) or as carbohydrates (free glycan profiling). The glycosylation site can usually be determined by peptide mapping[8] after the glycan moieties are enzymatically removed (mostly by PNGase F) because the mass of asparagine (N) residues with glycosylation increase by 1 Da upon deglycosylation.

However, it is a challenge for these methods to characterize glycoproteins with multiple glycosylation sites, such as HAs, because it is difficult to distinguish between glycan moieties that link to different sites in a protein. Furthermore, complex samples like vaccines have multiple N sites with an -NXS/T- motif. The determination of N-linked glycosylation sites by peptide mapping may be extremely challenging because modifications of N sites with a 1 Da mass increase could be due to either glycosylation or deamidation.

Using improved resolution of UPLC,® four major N-linked glycoforms of a tryptic peptide prepared from a monoclonal mouse IgG1 antibody tryptic digestion were analyzed by LC/UV-MS.[9] The experiment demonstrated that glycosylations could be detected and quantified. Both glycosylated site and glycan moiety could be characterized simultaneously.

In previous studies,[10-11] we demonstrated that tryptic peptide mapping with UPLC/MS^E was capable of separating and characterizing site-specific modifications such as N-deamidation and M-oxidation in an unbiased manner.

In this application note, we demonstrate that UPLC/MS^E is capable of separating and characterizing N-linked glycosylations of HA proteins in a recombinant influenza vaccine candidate expressed from insect cell-baculovirus expression vector system (BEVS). Glycopeptides and glycoforms are separated by an ACQUITY UPLC® System at the peptide level, and are detected online by a SYNAPT™ MS System. The UPLC/MS^E data are processed by BiopharmaLynx™ Software to report N-linked glycosylation information. The method offers a way to improve the characterization as well as reduce the amount of time spent on data processing. Furthermore, having a general technique that can be applied to such problems opens this type of analysis to non-experts and could benefit an organization by streamlining their work.

EXPERIMENTAL

A tryptic digest was prepared for the influenza vaccine candidate sample containing HA proteins H1, H3, and B that were expressed from insect cell-BEVS system. The peptide mixture contained N-linked glycopeptides together with other peptides from the proteins. The preparation procedure included:

1. Protein denaturing by heating at 80 °C for 10 min in the presence of 0.05% RapiGest™ SF and at pH 7.4
2. Reduction with dithiothereitol for 30 min at 56 °C
3. Alkylation with iodoacetamide for 30 min at room temperature in the dark
4. Digestion with trypsin for 4 hrs at 37 °C and pH 7.4
5. Quenching and de-activitation of trypsin by adding formic acid (FA, at 0.1%)

The digest was diluted to 0.2 µg/µL with 0.1% FA in 5% acetonitrile (ACN) for UPLC/MS^E analysis.

UPLC/MS^E experiments were performed using a SYNAPT MS System coupled with an ACQUITY UPLC System. The UPLC system was configured with a 2.1 x 150 mm, BEH300 C18 1.7-µm Peptide Separation Technology column. About 4 µg of the peptide mixture in a 20-µL volume was injected, and eluted using a 120-min gradient (1 to 40% ACN in 0.1% FA) at a flow rate of 0.2 mL/min and column temperature of 60 °C. Four injections were repeated.

MS^E data were acquired in ESI positive ion mode, with collision cell energy alternating between low energy (5 V) to collect peptide precursor (MS) data and elevated energy (ramping from 20 to 40 V) to obtain peptide fragmentation (MS^E) data. The scan time was 0.5 sec (1 sec total duty cycle). Capillary voltage of 3.0 kV, source temperature of 100 °C, cone voltage of 37 V, and cone gas flow of 10 L/h were maintained during the analyses. The system was tuned for a minimum resolution of 10,000 (V-mode) and calibrated using a 100 fmol/µL Glu1-fibrinopeptide B (GFP) infusion. Sampling of the lock spray channel (100 fmol/µL GFP in 50:50 ACN/water containing 0.1% FA) was performed every 1 min to ensure high mass accuracy.

The collected data were processed by BiopharmaLynx, v. 1.2, an application manager for MassLynx™ Software, using a strict tryptic cleavage rule, and setting cysteine carbamidomethylation as a fixed modification and N-linked glycosylations as variable modifications. Additional BiopharmaLynx method settings were detailed in a previous publication.[12]

RESULTS AND DISCUSSION

It has been reported[13] that glycoproteins expressed from insect cell lines present two major types of N-linked glycans: paucimannosidic structures (Man(1-3)GlcNAc2 or Man(1-3)GlcNAc[Fuc]GlcNAc) and oligomannose structures (Man(5-9)GlcNAc2), where Man represents mannose, GlcNAc is N-acetylglucosamine, and Fuc is fucose. All 11 possible glycosylation forms were entered as variable N-linked glycosylation modifications for BiopharmaLynx data processing. The number of N sites available for N-linked glycosylation (with -NXS/T-motif) are 9, 12, and 10 in HA proteins H1, H3, and B, respectively. After BiopharmaLynx processing of the collected UPLC/MS^E data, we were able to identify 7, 4, and 6 N sites with glycosylation for H1, H3, and B, respectively. Each N-linked glycosylation site may have multiple attached glycoforms. The following are three typical examples (one from each HA protein in the vaccine sample) selected to demonstrate how UPLC/MS^E can separate and characterize glycopeptides and glycoforms.

Figure 1 shows the separation and identification of unmodified and glycosylated tryptic peptide T8 in HA protein B (named B_T8 and B_T8*, respectively; in the following text, all glycosylated peptides are marked with an asterisk (*) to signify a corresponding modified form).

B_T8* eluted earlier than B_T8 because the attached sugar group increases hydrophilicity (Figure 1A). The tryptic peptide sequence was confirmed by MS^E spectra (Figures 1B and 1C). The glycosylation of B_T8* was indicated by a series of characteristic sugar ions at m/z 138.05, m/z 204.09, m/z 366.14, and m/z 528.12 in the low m/z range and confirmed by ion y28. The 1038.36 mass difference of y28 before and after glycosylation corresponds to a glycan moiety (-Man3NAcGlc[Fuc]NAcGlc). The fragment ions with sugar groups at high m/z range give further structural information about the attached glycan moiety. Based on the MS signal intensities processed by BiopharmaLynx and extracted ion chromatographic areas of precursor masses, the relative concentration of B_T8* is 20%.

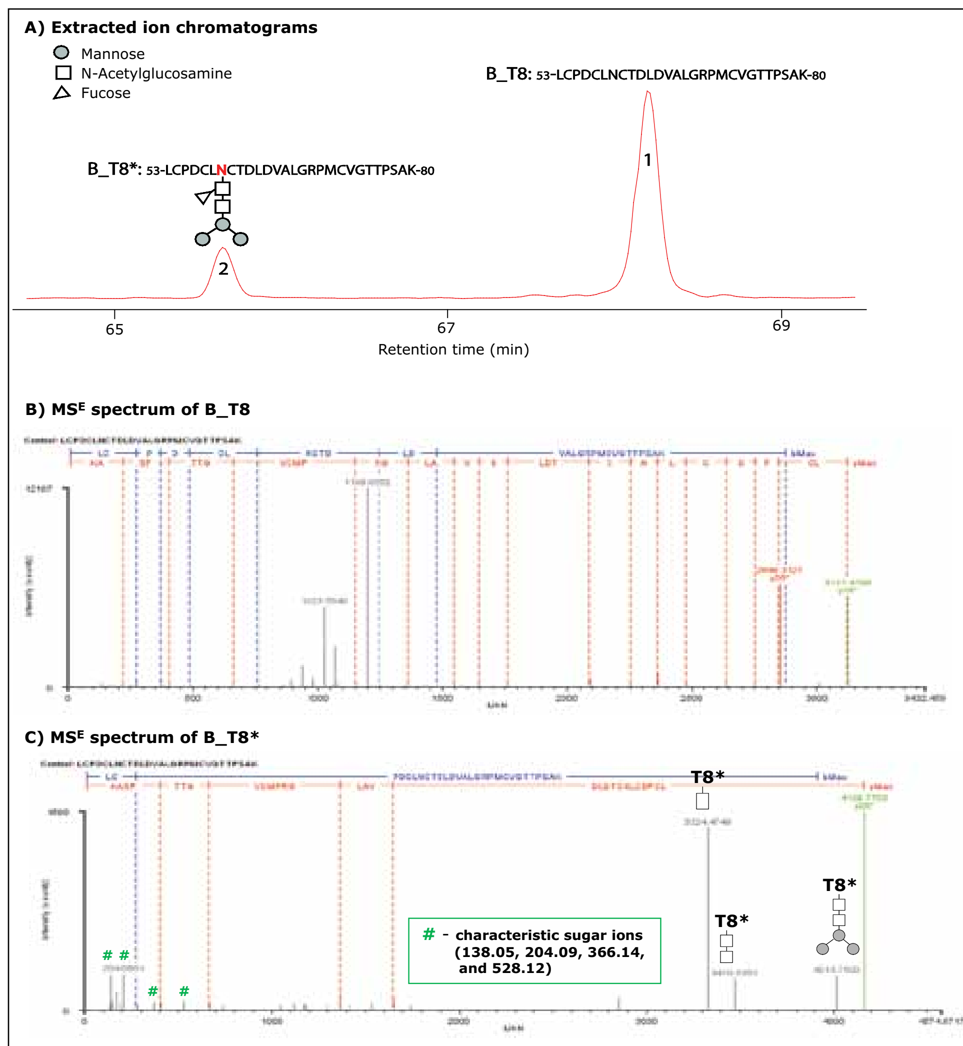

Figure 1. Separation and characterization of unmodified and glycosylated tryptic peptide T8 in HA protein B.
A) Extracted ion chromatograms of precursors
B) MS^E spectrum of B_T8 (The unassigned ions m/z 1199.65 and m/z 1023.55 are fragments of coeluted Twin-20 that was added during vaccine formulation)
*C) MS^E spectrum of B_T8**

Unlike B_T8*, most identified glycopeptides were fully glycosylated with multiple glycoforms. For example, peptide T24 of HA protein H1. No unmodified H1_T24 was identified, but four glycoforms of H1_T24* were chromatographically resolved and identified (Figure 2A). The glycoform elution order correlated to the size of glycan moiety. The heavier the glycan moiety, the earlier the glycoform eluted for this glycosylated peptide. The peptide has two N sites. MS^E spectra confirmed that the glycosylation only occurred on N286 which has the -NSS- motif, and that no glycans attached to N293 with the -NVH- motif. This is consistent with the rule that N-linked glycosylations only occur on N sites with -NXS/T- motif. The MS^E spectrum of H1_T24*-Man3NAcGlc2 is shown in Figure 2B. Again, the characteristic sugar ions in the low m/z range and the fragment ions with sugar groups in the high m/z range further confirm the glycosylation and provide structural information of the glycan moiety.

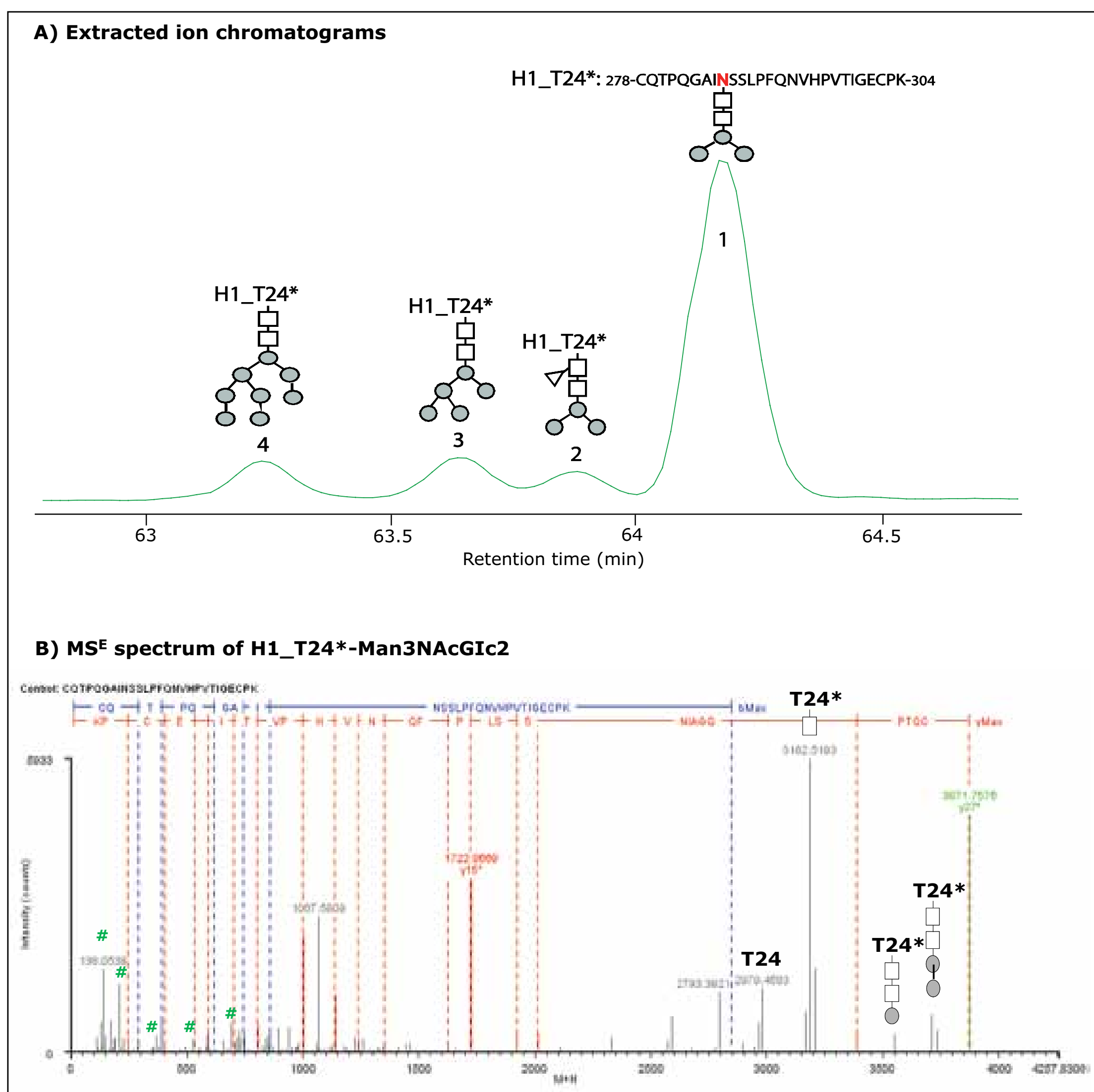

Figure 2. Separation and characterization of glycoforms of glycopeptide T24 in HA protein H1.*
A) Extracted ion chromatograms of precursors
B) MS^E spectrum of H1_T24-Man3NAcGlc2.*
 # – characteristic sugar ions

However, not all identified glycoforms were chromatographically resolved. For example, five glycoforms were identified on tryptic peptide T21 from HA protein H3 (see Figure 3A), but these glycoforms were eluted in one LC peak at 91.65 min. Their retention times show small differences (~ 2 seconds) in the extracted ion chromatograms (data not shown). This evidence and the example described above indicate that the chromatographic behavior of glycoforms is related to the nature of the peptide sequence to which the glycan is attached. The peptide sequence of H3_T21 and glycosylations could be confirmed by MS^E spectra. An example MS^E spectrum of H3_T21*-Man9NAcGlc2 was plotted in Figure 3B.

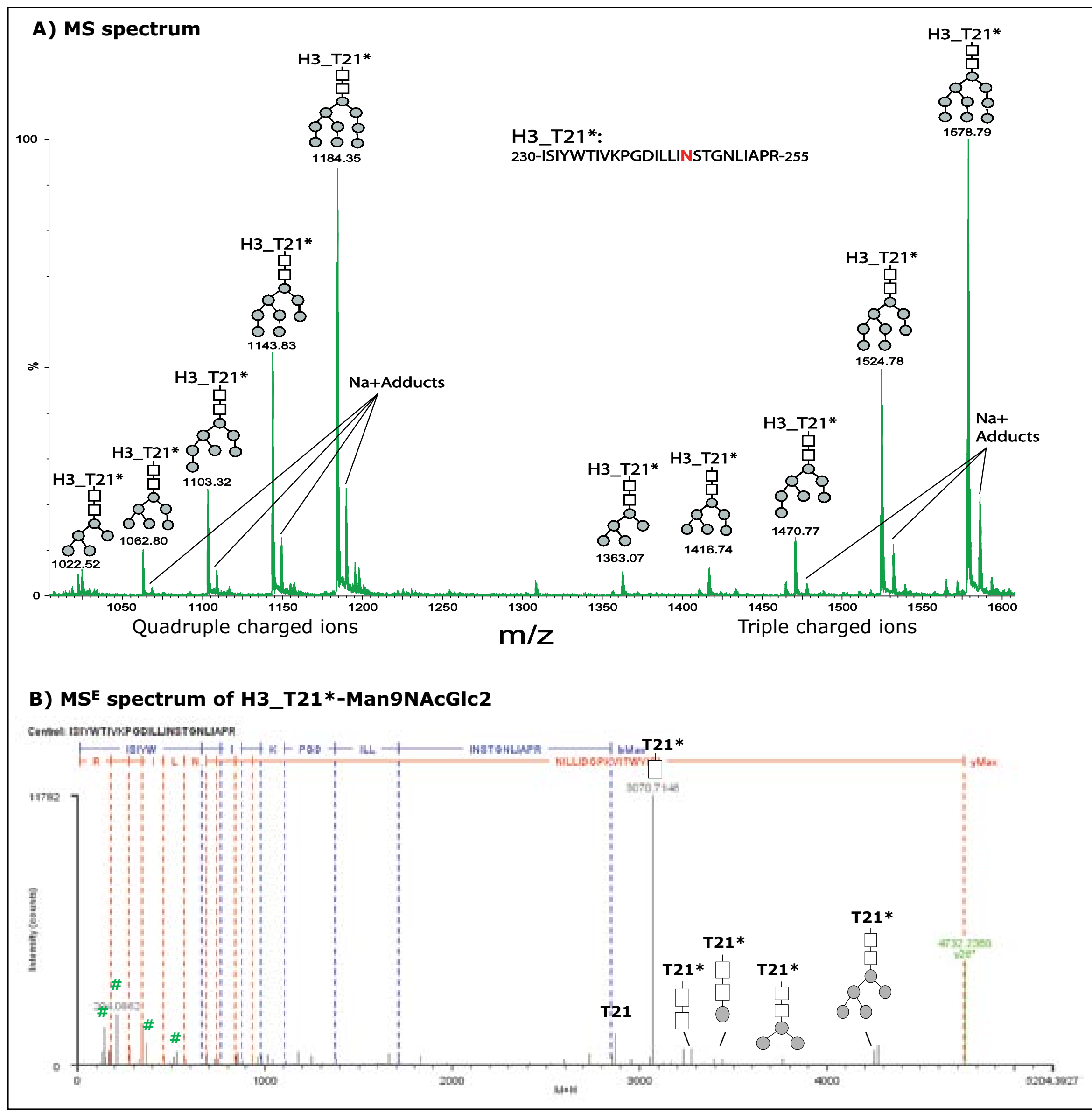

Figure 3. Characterization of glycoforms of glycopeptide T21 in HA protein H3.*
A) MS spectrum
B) MS^E spectrum of H3_T21-Man9NAcGlc2.*
 # – characteristic sugar ions

CONCLUSIONS

The results presented here demonstrate that UPLC/MS^E can separate and characterize multiple glycopeptides and multiple glycoforms on hemagglutinin glycoproteins in an influenza vaccine sample. Because HA proteins have multiple glycosylation sites and multiple glycoforms, characterization of glycosylations in influenza vaccine samples would be a challenging task for traditional methods such as glycan analysis and intact mass analysis.

Unlike traditional glycosylation characterization methods, in this work the glycosylation sites were unambiguously identified utilizing the MS^E technique. MS^E also provided useful structural information of glycan moieties from the glycan fragments. In addition, the methods do not involve any additional enrichment or purification procedure, and the data acquired can be automatically processed by BiopharmaLynx.

Further optimization could make this method applicable not only to the characterization of glycosylations in complex samples such as an influenza vaccine, but also as a rapid and routine method for characterizing glycoproteins.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Trastuzumab Glycan Batch-to-Batch Profiling using a UPLC/FLR/Mass Spectrometry Platform

Ying Qing Yu, Joomi Ahn, and Martin Gilar
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

Used together, UPLC with detection by FLR and time-of-flight MS comprise a powerful solution for producing required analytical data for batch-to-batch glycan profiling of a recombinant mAb, Trastuzumab. The chromatographic resolution, reproducibility, and mass spectrometry sensitivity enable glycoprofiling of therapeutic antibodies mandated by regulatory agencies. This UPLC/FLR/MS system represents a robust tool for separation and analysis of minor glycoforms or isomers that are otherwise difficult to assign.

WATERS SOLUTIONS

ACQUITY UPLC®

ACQUITY UPLC® FLR Detector

Xevo™ QTof MS

KEY WORDS

Trastuzumab, rmAb, glycosylation

INTRODUCTION

Glycosylation plays a vital role in the safety and efficacy of many therapeutic proteins such as recombinant monoclonal antibody (rmAb). Glycosylation of rmAb occurs at the Fc region on the heavy chain (Figure 1). Several studies have shown the correlation between glycosylation variations caused by cell-line selection and changes in culture-medium parameters.[1] These variations can have a profound effect on the biological activities of the rmAb drugs, which leads to changes in drug potency in the final product. Regulatory agencies require monitoring of batch-to-batch rmAb drug-production quality, and mandate detailed assessment of the protein glycosylation's micro-heterogeneity and consistency.

In this study, we applied a robust, sensitive, and reproducible analytical platform that comprises a UltraPerformance LC® (UPLC®), a fluorescence (FLR) detector, and a Xevo™ QTof Mass Spectrometer (MS) for batch-to-batch glycan profiling of an rmAb, Trastuzumab.

Trastuzumab is a therapeutic rmAb (IgG1 subclass) that is widely used for breast cancer treatment. N-linked glycans were released from three batches of Trastuzumab enzymatically, and labeled with a fluorescent tag, 2-aminobenzamide (2-AB). An ACQUITY UPLC HILIC Column was used to separate the released and labeled glycans; the LC was interfaced with the Xevo QTof MS via electrospray ionization. Peak areas from the FLR detector were utilized for glycan quantitation; MS was used for peak assignment using an accurate molecular weight of corresponding glycans.

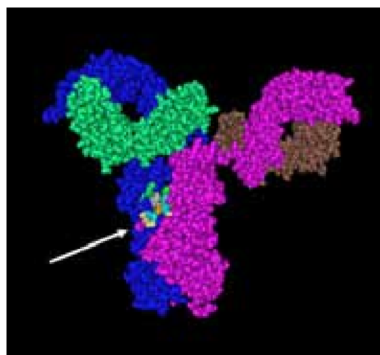

Figure 1. A crystal structure of Trastuzumab. The arrow points to the oligosaccharides that are located in the Fc region of the heavy chain.

EXPERIMENTAL

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Glycan Column 2.1 x 150 mm, 1.7 µm
Column temp.:	40 °C
Flow rate:	400 µL/min
Mobile phase A:	100 mM ammonium formate, pH 4.5
Mobile phase B:	Acetonitrile
Gradient:	72% to 62% B in 45 min
Weak wash:	75% acetonitrile
Strong wash:	20% acetonitrile
Injection:	5.0 µL partial loop

FLR conditions

FLR:	Waters ACQUITY UPLC Fluorescence Detector
Excitation:	330 nm
Emission:	420 nm
Data Rate:	1 pts/s
PMT Gain:	1.00
Time Constant:	Normal

MS conditions

MS System:	Waters Xevo QTof MS
Ionization Mode:	ESI +
Capillary Voltage:	3200 V
Cone Voltage:	35 V
Desolvation Temp.:	350 °C
Desolvation Gas:	800 L/Hr
Source Temp.:	120 °C
Acquisition Range:	800 to 2000 *m/z*
Collision Energies:	6 V
Lock Mass:	Cesium iodide, CSI, (1 µg/µL in 50% isopropanol)

MassPREP HILIC µElution plate

Pre-condition wells
200 µL 100% H_2O

Condition wells
200 µL 90% ACN (x2)

Reconstitute samples
in 90 % ACN

Load samples into wells
in 90% ACN

Wash sample loaded wells
With 500 µL 90% ACN (x2)

Elute glycans from wells
with 100 µL 1mM
Ammonium Tris-Citrate in 10% ACN (x3)

Figure 2. Released Glycan Extraction Protocol. (MassPREP™ HILIC µElution plate was operated using a vacuum manifold.)

METHOD

- Protein solubilization: 20 µL of Trastuzumab (21 µg/µL) was solubilized with 172 µL, 0.1% RapiGest™ in 50 mM ammonium bicarbonate.
- Reduction/alkylation: 1 µL Dithiothreitol (DTT, 1M) was added to the sample to a final concentration of 5 mM. The sample was heated at 60 °C for 30 min, and cooled down to room temperature; 2 µL iodoacetamide (IAM, 1M) was added to alkylate the free cysteine (final concentration was 10 mM); incubation time was 30 min in the dark at room temperature.
- Deglycosylation: PNGase F (New England Biolab, 50,000 unit/mL, 5 µL) was added to the sample for overnight incubation. The final protein concentration was about 2.1 µg/µL.
- Extraction of released glycans: 50 µL of the deglycosylated protein sample was reconstituted with 450 µL pure acetonitrile prior to HILIC µElution-plate extraction. For details see the extraction protocol.
- FLR labeling using 2-AB: The labeling was performed using a modified protocol.2 50 µL (instead of 5 µL) of a 2-AB/DMSO/acetic acid/sodium cyanoborohydrate mixture of defined composition was used for the labeling.
- 2-AB Glycan extraction: The same protocol was used as for extraction on unlabeled glycans. The eluted glycans were lyophilized and reconstituted in 40 µL of 50% acetonitrile in water prior to UPLC/FLR/MS analysis.

RESULTS AND DISCUSSION

UPLC/FLR detection sensitivity

The rmAb N-linked glycans present in the sample are biantennary and high mannose type. They exhibit considerable heterogeneity and wide dynamic range. Identification and quantification of low-abundant glycans requires sensitive fluorescence detectors. About 5 pmol of sample is typically injected on the column in order to detect minor glycans. The limit of detection for FLR was estimated to lie between 1 to 5 femtomoles.

UPLC resolution of glycans

UPLC HILIC separation provides significantly greater resolution compared to conventional HPLC methods.[3] UPLC better resolves isomeric glycans, such as G1 and G1F isomers, makes the data interpretation less ambiguous, and improves quality of quantitation (peak integration).

UPLC/FLR injection-to-injection reproducibility

The main purpose of LC/FLR glycans analysis is its relative quantitation. Injection-to-injection variability of UPLC/FLR system was evaluated as shown in Figure 3. The variation (RSD) in peak areas of three injections of the same sample was less than 2% even for minor peaks.

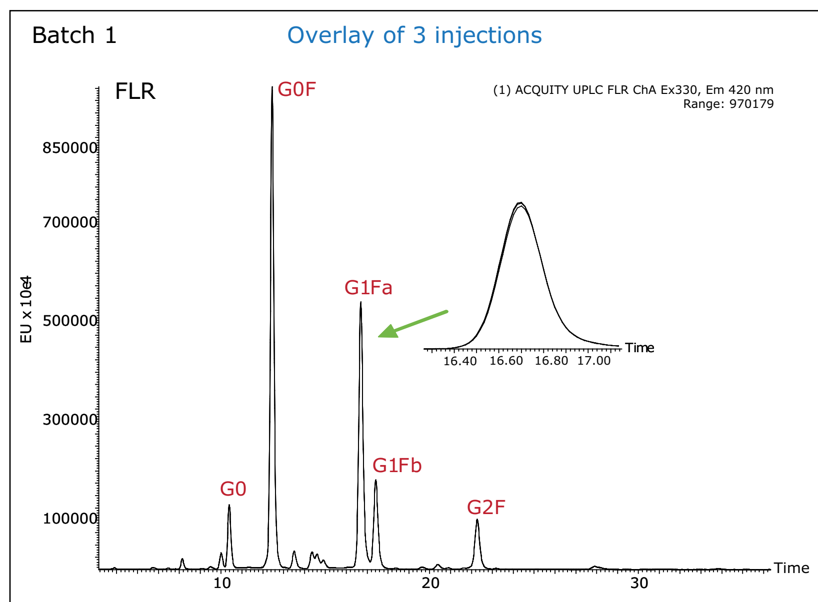

Figure 3. UPLC/FLR chromatograms of triplicate injections of 2-AB labeled glycans released from the same Trastuzumab batch. Overlay of three injections showed perfect alignment of chromatograms. The peak area response variation was less than 2% (RSD). The most abundant glycans were labeled at the peak top.

Glycan mass profiling using Xevo QTof MS

While FLR data are useful for glycan quantitation, MS provides information in addition to chromatographic retention times. Accurate mass data permit the assignment of glycans present in mAb with high confidence (Figures 4A, B). Proposed structures for the 2-AB labeled Trastuzumab glycans are shown in Figure 5.

An example of FLR and MS data for G2F peak along with MS spectrum is shown in Figure 3B. MS/MS fragmentation was used to elucidate glycan structure (data not shown). The sensitivity of Xevo QTof MS was sufficient to assign even the minor components. For example, the two sialylated glycans, G2FS1 and G2FS2 with a low fluorescence signal, did not show peaks in the MS base peak ion (BPI) chromatogram. However, the extracted ion chromatograms (XIC) of the doubly charged ions of G2FS1 and G2FS2 glycans clearly confirm the presumed identities (Figure 6).

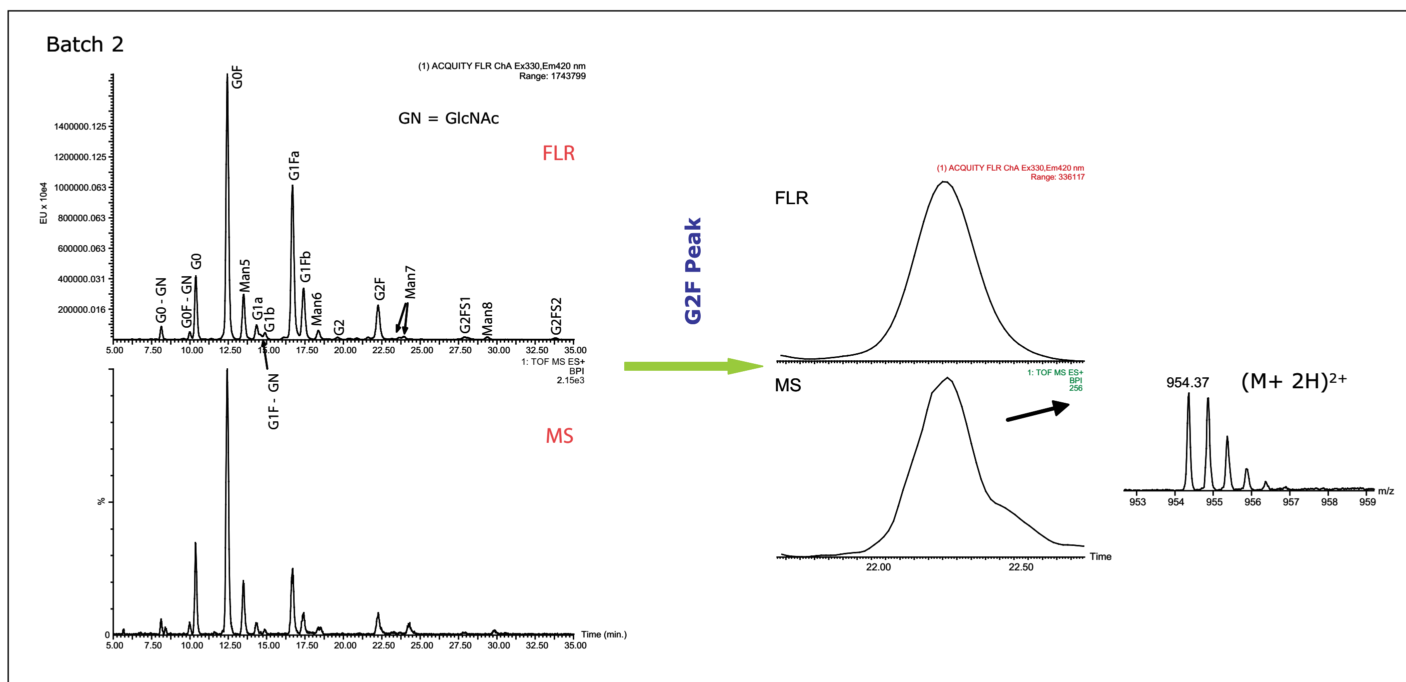

Figure 4A (left). UPLC/FLR/MS analysis of 2-AB labeled glycans from Trastuzumab (Batch 2). The top chromatogram is the FLR chromatogram; the bottom is the MS chromatogram. The glycan identified were confirmed by their accurate mass. Glycan structures are listed in Figure 5.

Figure 4B (right). 2-AB labeled glycan assignment was made by aligning the FLR chromatogram peak with the BPI MS peak. The summed BPI MS scans for G2F peak are shown on the right. The mass error was 20 ppm.

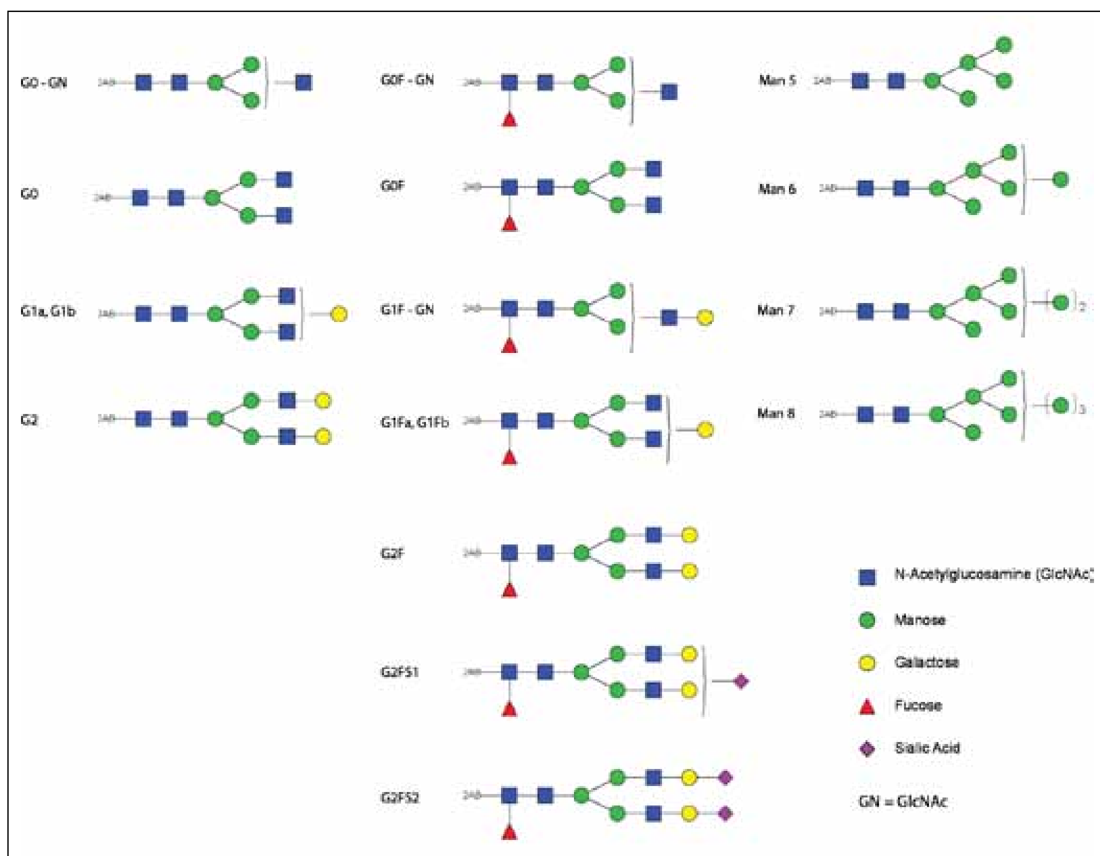

Figure 5. Proposed structures for the 2-AB labeled Trastuzumab glycans. The left panel lists the non-fucosylated complex type, the middle panel shows the fucosylated type, and the right panel shows the high mannose type.

Batch-to-batch glycoprofiling comparison

Three different Trastuzumab batches were analyzed (Batch 1 through 3). In order to accurately compare the glycan profiles, the robustness of sample preparation (sample preparation and extraction step using HILIC μElution plate), its variability was also evaluated. Figure 7 shows the overlay of the FLR chromatograms of glycans released from these three Trastuzumab batches, while the graph in Figure 8 compares both the relative glycan abundance and the sample preparation variability. The narrow error bars in confirm that glycan relative quantitation is highly repeatable. The differences between batches of glycan profiles were significantly greater than variability introduced by sample preparation.

Among other differences, we observed significantly higher GOF content in Batch 3 than the other two batches. Man6, Man7, and Man8 were observed only in Batch 2.

Xevo QTof MS sensitivity was sufficient to confirm the glycan identity for peaks at 0.3% relative intensity (FLR data) of overall glycan content. Table 1 summarizes identified glycans with their relative abundance (%), standard deviation, and %RSD of the integrated FLR peaks (N = 3).

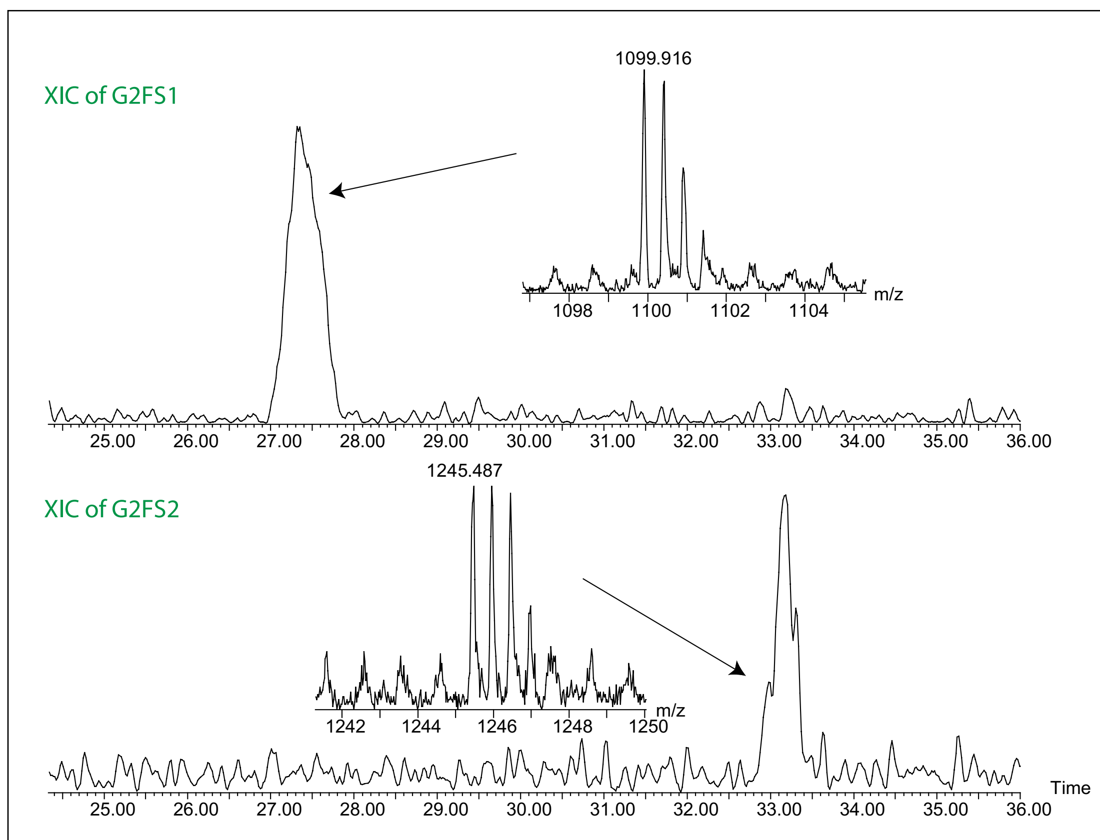

Figure 6. Extracted ion chromatogram (XIC) for G2FS1 and G2FS2. The extracted mass is the doubly charged ions $(M+2H)^{2+}$ for both components.

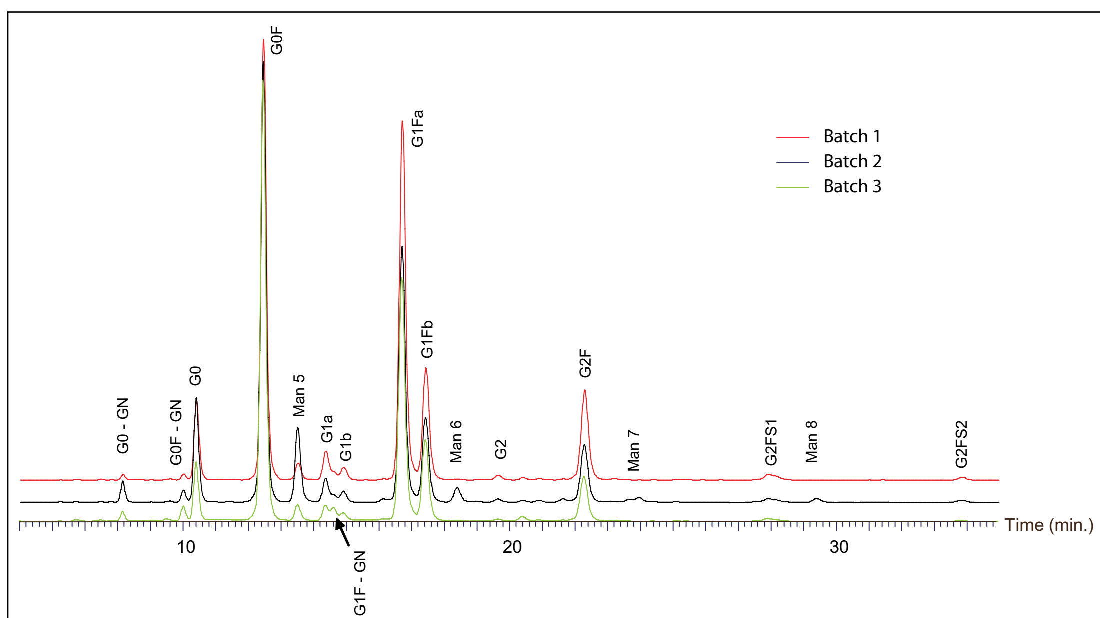

Figure 7. UPLC/FLR chromatograms of 2-AB labeled glycans released from three Trastuzumab batches.

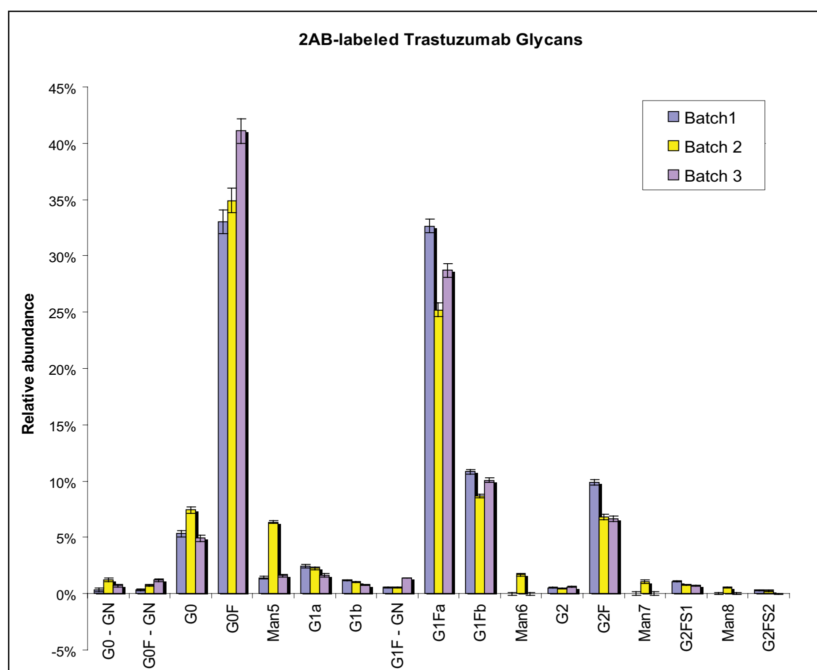

Figure 8. Relative abundance of 2-AB labeled glycans from three batches of Trastuzumab. Each relative abundance value has error bars based on triplicate analyses (three aliquots of deglycosylated Trastuzumab samples have undergone μElution extraction, FLR labeling, and additional μElution SPE cleanup). Error bars were calculated from sample preparation replicates. For details see Table 1.

	Batch 1		Batch 2		Batch 3	
	Rela. Conc. (%)	RSD (%)	Rela. Conc. (%)	RSD (%)	Rela. Conc. (%)	RSD (%)
G0 - GN	0.34 ± 0.01	3.78	1.22 ± 0.14	11.50	0.71 ± 0.01	1.69
G0F - GN	0.36 ± 0.04	11.96	0.74 ± 0.09	11.94	1.19 ± 0.16	13.00
G0	5.35 ± 0.22	4.14	7.43 ± 0.28	3.86	4.95 ± 0.35	7.10
G0F	33.03 ± 0.85	2.58	34.90 ± 1.1	3.11	41.09 ± 1.49	3.63
Man5	1.41 ± 0.05	3.80	6.35 ± 0.11	1.75	1.61 ± 0.02	1.03
G1a	2.45 ± 0.04	1.70	2.25 ± 0.15	6.62	1.61 ± 0.15	9.00
G1b	1.20 ± 0.06	5.04	1.06 ± 0.03	2.98	0.77 ± 0.02	2.81
G1F - GN	0.55 ± 0.003	0.49	0.55 ± 0.02	3.16	1.38 ± 0.04	2.64
G1Fa	32.65 ± 0.59	1.80	25.22 ± 0.62	2.44	28.72 ± 0.77	2.68
G1Fb	10.83 ± 0.30	2.75	8.68 ± 0.2	2.25	10.06 ± 0.44	4.37
Man6	—	—	1.68 ± 0.13	7.89	—	—
G2	0.54 ± 0.04	7.55	0.42 ± 0.04	10.03	0.59 ± 0.03	5.10
G2F	9.89 ± 0.31	3.10	6.82 ± 0.23	3.44	6.63 ± 0.47	7.14
Man7	—	—	1.08 ± 0.17	16.11	—	—
G2FS1	1.09 ± 0.08	7.62	0.79 ± 0.02	2.59	0.71 ± 0.03	3.55
Man8	—	—	0.55 ± 0.07	12.90	—	—
G2FS2	0.31 ± 0.04	14.31	0.26 ± 0.05	18.20	—	—

Table 1. Summary of the relative abundance of the identified 2-AB labeled glycans (N=3).

CONCLUSIONS

- UPLC HILIC/FLR analysis provides sensitive and accurate methods for quantification of glycans.
- Injection repeatability is well below 1% for major peaks and better than 2% RSD for minor components. Sample cleanup introduces only minor variability into the quantitative glycoprofiling.
- UPLC HILIC/FLR/MS analysis revealed significant differences in glycan profiles between three batches of Trastuzumab.

Used together, ACQUITY UPLC with detection by FLR and Xevo QTof MS comprise a powerful system for producing required analytical data. The chromatographic resolution, reproducibility, and mass spectrometry sensitivity enable glycoprofiling of therapeutic antibodies mandated by regulatory agencies. The UPLC/FLR/MS system represents a robust tool for separation and analysis of minor glycoforms or isomers that are otherwise difficult to assign.

This UPLC/FLR/MS platform improves the overall quality of the rmAb-carbohydrate characterization assay and the batch-to-batch consistency test, which are components of drug release tests. The proposed method enables a routine and robust rmAb glycan analysis and may become a tool of choice for biopharmaceutical rmAb characterization.

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

Efficient Glycan Separations using the ACQUITY UPLC H-Class Bio System

GOAL

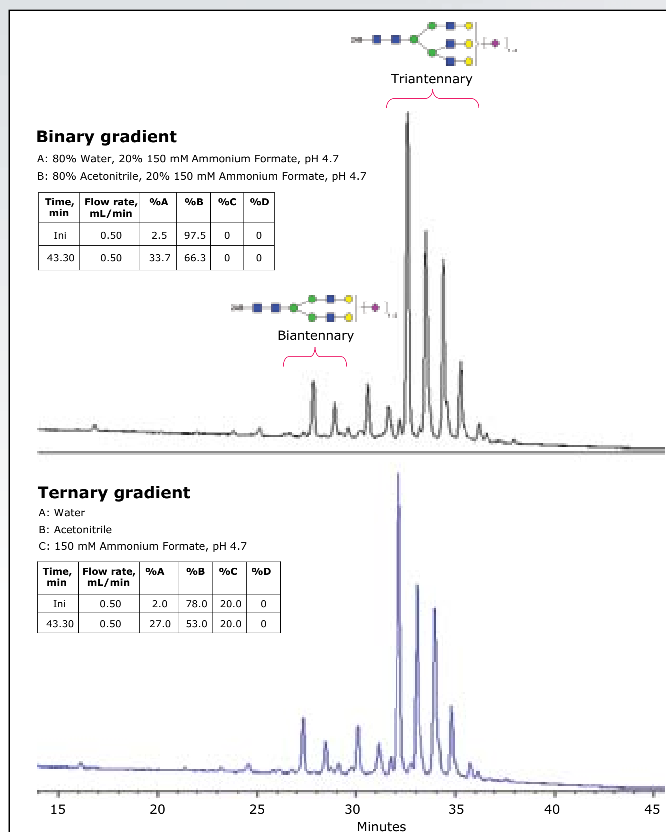
To demonstrate the successful characterization of the *N*-glycans released from fetuin using the ACQUITY UPLC® H-Class Bio System in HILIC mode along with the flexibility of the novel solvent mixing technology, Auto•Blend Plus.™

BACKGROUND

The high complexity of glycan structure is related to its synthesis and its biological role. A complete glycosylation profile is required for various regulatory purposes. Due to the biological significance of glycoproteins, glycan analysis needs rapid, efficient, sensitive, and reproducible methods. The analysis of glycans involves efficient separations of their constituents, including *N*- and *O*-glycans.

Depending on the nature of the glycan and whether mass spectrometry detection is to follow its chromatographic separation, mobile phase formulations require adjustments. A large degree of flexibility is desired when selecting among different modifiers and ionic strengths during optimization of gradient conditions. Optimizing glycan separation conditions using a binary solvent system can be a very time-consuming process since many mobile phase compositions are used in search for the required selectivity and resolution. The parameters available for adjustment include identity and concentration of the organic component of the mobile phase as well as the pH and ionic strength.

Robust analyses of the glycans of glycoproteins can be readily developed with the ACQUITY UPLC H-Class Bio System featuring Auto•Blend Plus Technology and Glycan Separation Technology Columns.



Time, min	Flow rate, mL/min	%A	%B	%C	%D
Ini	0.50	2.5	97.5	0	0
43.30	0.50	33.7	66.3	0	0

Time, min	Flow rate, mL/min	%A	%B	%C	%D
Ini	0.50	2.0	78.0	20.0	0
43.30	0.50	27.0	53.0	20.0	0

2-AB labeled glycans released from fetuin were separated using a standard binary HILIC method. The same separation was obtained in a ternary, Auto•Blend Plus mode where mobile phase preparation is easier and less error-prone. The ternary method also provides an easier protocol for optimizing the method for a particular sample.

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These experiments are time-consuming and tedious, and errors can occur during preparation of the numerous mobile phases through the method development process. The quaternary-based ACQUITY UPLC H-Class Bio System, with Auto•Blend Plus Technology, allows automatic programming of the solvent composition from up to four stock solvents, saving both preparation and analysis time while increasing reproducibility.

THE SOLUTION

Waters Glycan Separation Technology Column chemistry, used with sub-2-µm UPLC Technology and fluorescence (FLR) detection, provides an efficient separation of protein glycans utilizing hydrophilic interaction liquid chromatography (HILIC). In HILIC mode, the oligosaccharides are retained by polar interaction and the elution is realized by the aqueous gradient.

To demonstrate the additional flexibility provided by the ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology, this configuration was used for the characterization of the *N*-glycans isolated from fetuin and labeled with a 2-aminobenzamide (2-AB) fluorescent tag. These analytes have been separated using a high-efficiency separation with a binary gradient. This method was transferred to ternary gradient conditions. Selectivity and resolution for glycans of fetuin were identical with a ternary gradient that was generated using Auto•Blend Plus Software.

Solvent C was selected as a modifier and its concentration remained constant throughout the gradient. 150 mM ammonium formate, pH 4.7, was used as solvent C buffer, whereas pure solvents were chosen for solvent A (water) and solvent B (acetonitrile). This system configuration provides an easy approach for optimization of gradient conditions. Changing the proportion used from solvent C is all that is required for the user to introduce modifications of mobile phase composition that affect selectivity and resolution. By using Auto•Blend Plus, we can program the system to test different modifier compositions, ionic strengths, and pH to achieve the best separation conditions commonly explored during glycan analyses.

The ACQUITY UPLC H-Class Bio System provides robust, highly resolving, reproducible, and rapid separations using ternary gradient conditions as developed with Auto•Blend Plus Technology.

SUMMARY

The ACQUITY UPLC H-Class Bio System was successfully applied to the separation of protein glycans. By allowing the biochemist to work with stock solutions to manipulate gradient compositions, Auto•Blend Plus Technology offers a large degree of freedom to control selectivity, optimize the gradient, and manage the modifier composition independently from the rest of the solvents. This approach saves a significant amount of time for the laboratories performing glycan characterization. The high separation efficiency and reliability of ACQUITY UPLC H-Class Bio System with Auto•Blend Plus Technology simplifies method development and provides overall laboratory productivity and efficiency.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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[APPLICATION NOTE]

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Analysis of Glycopeptide Glycoforms in Monoclonal Antibody Tryptic Digest using a UPLC HILIC Column

Martin Gilar, Ying-Qing Yu, Joomi Ahn, and Hongwei Xie
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

In this application note, we present a UPLC HILIC/TUV/MS method for the separation of glycopeptides that is complementary to HILIC/FLR separation of *N*-linked glycans released from the protein. With this method, information about glycan heterogeneity and site occupancy is preserved and the same tryptic digest used for peptide mapping can be used. Since it does not require glycan release and labeling, complexity of sample preparation is reduced. This method is useful in the development and quality control of new protein-based therapies.

WATERS SOLUTIONS

ACQUITY UPLC

ACQUITY UPLC BEH Glycan Column

SYNAPT MS

KEY WORDS

Glycoprotein characterization, glycosylation, HILIC, glycan heterogeneity, site occupancy

INTRODUCTION

Glycosylation of proteins affects their tertiary structure and potentially therapeutic efficacy. Therefore, the glycosylation of therapeutic proteins such as monoclonal antibodies (mAb) needs to be closely monitored.

Reversed phase liquid chromatography (RP-LC) is a primary method chosen for protein characterization via peptide mapping. Peptide mapping applications require efficient columns to resolve complex peptide mixtures into unique peptides. Modified peptides, such as oxidized or deamidated ones, can also be separated from the unmodified peptides.[1] UltraPerformance Liquid Chromatography® (UPLC®) technology provides the resolving power needed for these challenging separations.[2]

It has been reported that RP-UPLC is able to resolve glycosylated peptides into their glycoforms.[3] However, the complete resolution of glycopeptide micro-heterogeneity (same peptide sequence, various glycoforms) remains difficult. This is because retention in RP-LC is mainly due to peptide hydrophobicity, and is less affected by the presence of hydrophilic glycans. The separation is further complicated by the presence of non-glycosylated peptides in the sample that often elute in the vicinity of the glycopeptides of interest.

Several separation methods are available for glycan analysis, including capillary electrophoresis (CE), high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and hydrophilic interaction chromatography with fluorescent detection of labeled glycans (HILIC/FLR). While those methods are useful, the confirmation of glycan identity relies on their retention time, available standards, and use of specific exoglycosidase enzymes.[4] Fraction collection of resolved glycans is often combined with a matrix assisted laser desorption/ionization (MALDI) MS method for confirmation of mass of glycans and their MS/MS structure identification. Because of the advantages of on-line MS, the off-line MALDI method is being recently replaced with LC/MS glycan analysis.

Two LC/MS methods currently under development are MS analysis of the intact proteins and LC/FLR/MS analysis of the glycan released from a glycoprotein. In the first case, the mass spectrum (after deconvolution) provides information about the protein molecular weight and its heterogeneity due to glycosylation.[5] For mAbs, where the glycosylation nature is well understood, the intact mass information can be translated into the relative quantitation of glycoforms.[6]

EXPERIMENTAL

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Glycan Column, 2.1 x 150 mm, 1.7 µm amide sorbent
Column temp.:	40 °C
Flow rate:	0.2 mL/min
Mobile phase A:	10 mM ammonium formate, pH 4.5
Mobile phase B:	10 mM ammonium formate, in 90:10 acetonitrile/water
Gradient:	90 to 55 % B in 45 min (81 to 49.5% acetonitrile in 45 min)
Detection:	ACQUITY UPLC TUV, 280 nm
MS system:	SYNAPT™ MS system
MS acquisition:	ESI positive ion V-mode, collision cell energy 5 V, 0.3 sec acquisition cycle, capillary voltage 3.0 kV, cone voltage 37 V, source temp. 100 °C, desolvation temp. 250 °C, cone gas 10 L/h, desolvation gas 550 L/h
Sample:	Humanized mAb tryptic digest

Though useful as fast screening, the intact protein MS method may fail to detect minor glycoforms.

The second method for glycoprotein characterization utilizes specific enzymes (PNGase F) to release *N*-linked glycans from the protein. Glycans are typically enriched, labeled with fluorescent dye, and analyzed in HILIC mode. Highly efficient UPLC HILIC columns have been shown to facilitate an excellent glycan separation and relative quantification.[7]

HILIC separation of glycans is considered to be a reliable method. However, for proteins with multiple *N*-linked glycosylation sites, released glycans of the same type elute in chromatogram as cumulative peaks. Therefore, the information about the occupancy of different *N*-linked sites is lost. This is also the case for CE and HPAEC-PAD methods. While this does not present a problem for proteins with a single glycosylation site, such as monoclonal antibodies, it precludes full characterization of proteins with multiple glycosylation sites.

In this application note we propose an orthogonal method, UPLC HILIC/TUV/MS, in which the information about glycan heterogeneity and site occupancy is preserved. This method is complementary to UPLC HILIC/FLR analysis of the released glycans and the RP peptide map. The same tryptic digest used for the peptide map can be used in the method. The ACQUITY UPLC® System with a UPLC BEH Glycan Column is used for UPLC/FLR analysis of released glycans and the proposed method[7] for the separation of glycopeptides.[3]

RESULTS AND DISCUSSION

Figure 1 illustrates the retention differences between peptides and glycopeptides in HILIC chromatography mode. The glycopeptides are highlighted with a blue box. The presence of highly polar glycan moiety greatly improves the retention of glycopeptide(s) of interest, and therefore they are well resolved from the remaining non-glycosylated tryptic peptides generated by the tryptic digest of the mAb. This specific retention behavior has been confirmed with other glycoprotein digests and appears to be a generic behavior of all glycopeptides, including the O-linked ones (data not shown).

Further inspection of Figure 1 reveals that the glycopeptide EEQY**N**STYR with glycans attached at asparagine position is resolved into seven distinct peaks. The pattern and intensity resembles the separation obtained for glycans released with PNGase F, labeled with 2-AB dye, and analyzed in HILIC mode using the same column (Figure 2). Glycan structures identified in this study are shown in Figure 3.

The expanded view of the glycoprotein separation highlighted in Figure 1 is presented in Figure 4. Seven main peaks are clearly seen in chromatogram.

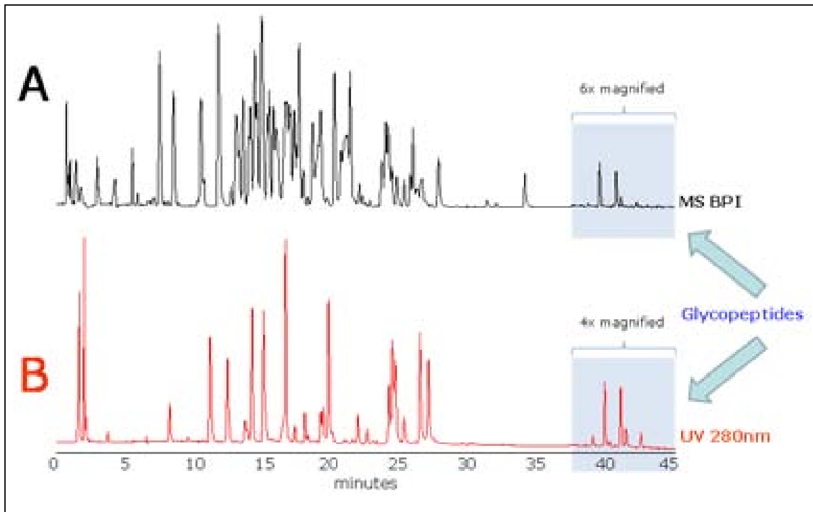

Figure 1. Separation of humanized mAb tryptic digest on 2.1 x 150 mm UPLC BEH Glycan Column in HILIC mode. A) MS BPI chromatogram. B) TUV at 280 nm. Glycopeptides are more retentive and therefore well resolved from other peptides.

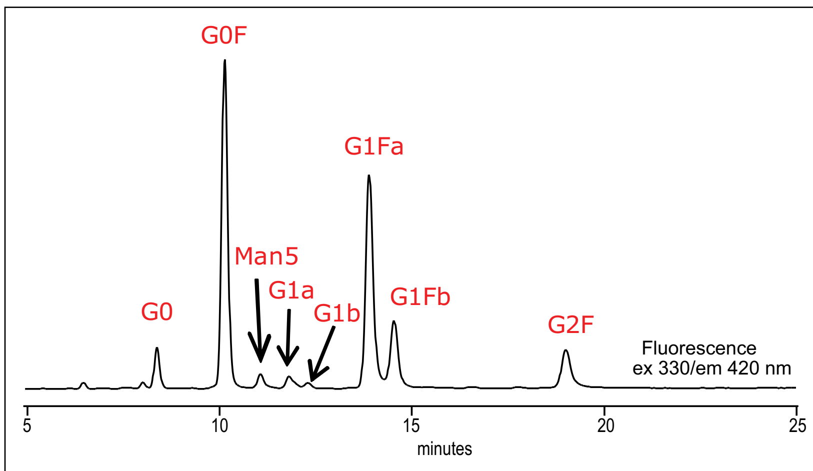

Figure 2. UPLC HILIC/FLR analysis of glycans released from mAb using a 2.1 x 150 mm UPLC BEH Glycan Column with fluorescence detector. Mobile phase A: 100 mM ammonium formate, pH 4.5; B: acetonitrile; gradient 72 to 66% B in 27 minutes. For experimental details see reference 7.

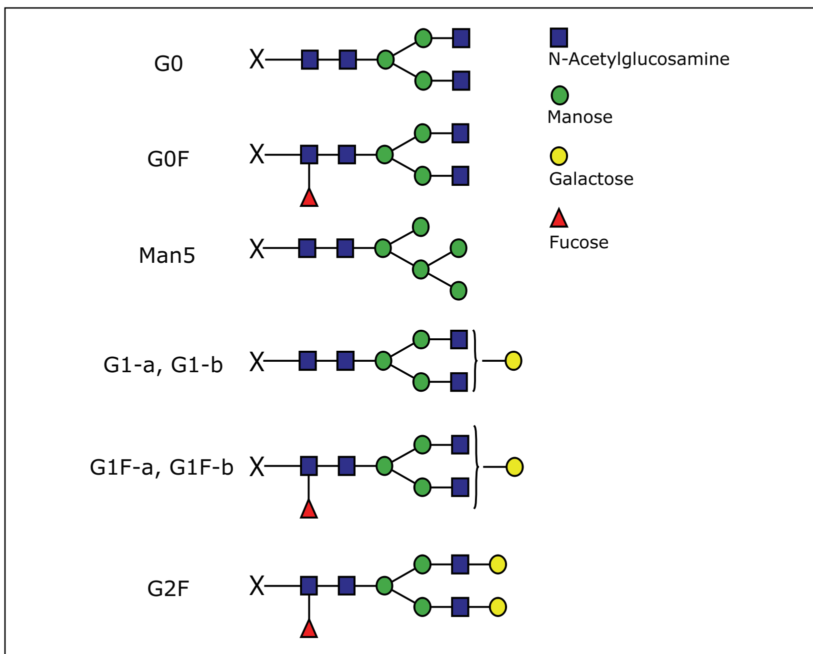

Figure 3. Structures of glycans identified in this study. For the UPLC HILIC/FLR analysis shown in Figure 2, the X corresponds to

*2-AB label. For glycopeptide analysis (Figures 1 and 4) the X represent EEQY**N**STYR peptide with glycosylated asparagine.*

Since the EEQY**N**STYR peptide contains two aromatic amino acids, it absorbs UV light at 280 nm and serves as "tag," providing equimolar response for all glycopeptides. Therefore, the relative quantitation of glycan micro-heterogeneity could be measured at 280 nm with little UV background interferences from the mobile phase.

Peak identity was confirmed by accurate MS data as shown in Figure 4C. When extracting XIC (extracted ion chromatograms) for expected glycan species in mAb, we detected the presence of G0, G0F, G1 G1F, and G2F glycans, and also Man5 variant that is coeluting with the dominant G1Fa peak (Figure 4C).

Relative quantitation based on UV 280 nm and XIC data was performed for three repetitive analyses. The results are listed in Table 1. Interestingly, the similar pattern in Figures 4B and 4C and similar quantitation results in Table 1 suggest that the glycopeptide variants have rather uniform MS responses (at least for neutral glycans observed in this study, see Table 1). MS also enables quantitation of glycopeptides that are not resolved and cannot be quantified by UV.

Rel. quantitation of glycopep. %			Rel. quantitation of glycopep. %		
	UV 280 nm	RSD%		XIC MS	RSD%
	6.3 ± 0.3	4.6		6.1 ± 0.1	1.4
	35.7 ± 0.2	0.5		38.3 ± 0.8	2.1
	3.1 ± 0.1	4.4		2.4 ± 0.2	10.3
	0.8 ± 0.1	14.4		1.1 ± 0.1	9.5
	—	—		1.1 ± 0.0	2.8
	34.4.± 0.1	0.2		31.2 ± 0.6	1.9
	11.2 ± 0.0	0.2		11.6 ± 0.2	1.6
	8.5 ± 0.2	2.0		8.2 ± 0.3	4.0

Table 1. Relative quantitation of glycopeptides using integration of peaks at UV 280 nm chromatogram and extracted ion chromatograms (XIC) in MS (see Figure 3). Relative standard deviation values are calculated from three replicate experiments.

There are several benefits of this proposed orthogonal method compared to HILIC/FL analysis of released glycans in terms of easier sample preparation, use of the same sample as for peptide maps in RP, and information regarding the site occupancy of glycosylation site(s). In comparison with released glycan using HILIC with fluorescence detection is more sensitive than UV. Tens of pmole of protein digest or more needs to be injected on column for the proposed HILIC/UV glycopetide method, while HILIC/FLR requires only injection of sub pmol amounts of sample. Also, the resolution of released glycans in HILIC mode appears to be better than for glycopetides (compare Figures 2 and 4).

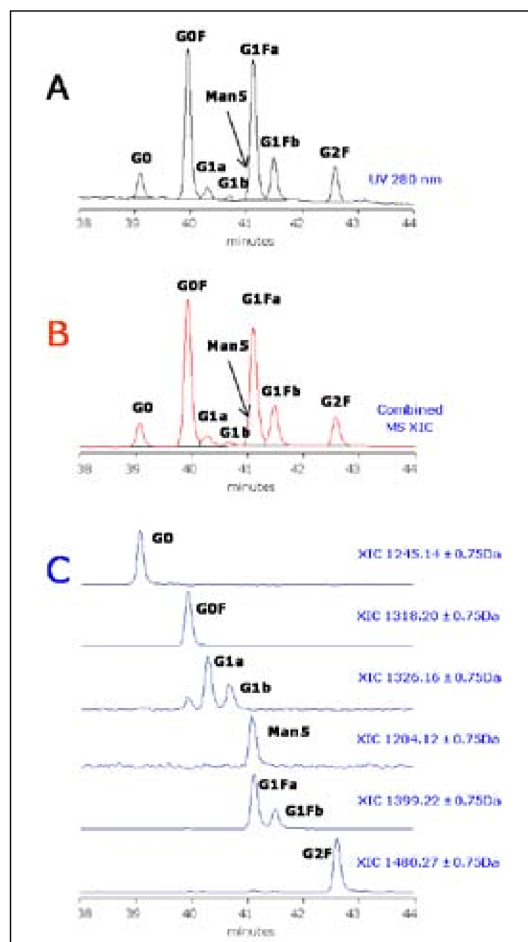

Figure 4. Separation of mAb tryptic digest on 2.1 x 150 mm UPLC BEH glycan column in HILIC mode. A) UV 280 nm chromatogram; B) combined XIC MS chromatograms for all glycopeptides; C) XIC traces for glycopeptides revealing positional isomers of G1 and G1F glycans.

CONCLUSIONS

This application note describes a novel UPLC HILIC/TUV/MS method for characterization of protein glycosylation. Benefits of the new method include:

- The ACQUITY UPLC BEH HILIC Glycan Column facilitates good resolution of glycopeptides from non-glycosylated peptides.
- Peptide glycoforms are well resolved for mAb related glycans.
- The method eliminates the need for glycan fluorescent labeling and SPE purification. It is complementary to established glycan characterization methods.
- Mobile phase permits sensitive MS detection.
- MS detection is helpful for site occupancy analysis (glycan micro-heterogeneity) of glycoproteins with multiple glycosylation sites (complex glycopeptides).
- The HILIC method complements RP peptide mapping, which does not provide sufficient information on glycan heterogeneity.

We believe that the proposed method is suitable for characterization of glycoproteins and in particular for monoclonal antibodies, which represent the main class of biotherapeutics. This method can speed up the development and quality control of new therapies, as well as improve safety and efficacy of protein drugs.

Preliminary results (not shown) suggest that the proposed method is useful also for characterization of *O*-linked glycans. Because of the lack of specific and efficient enzymes for their release, characterization of *O*-linked glycans in form of glycopeptides is a promising alternative that will be further investigated.

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Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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UPLC-FLR Method Development of 2-AB Labeled Glycan Separation in Hydrophilic Interaction Chromatography (HILIC)

Joomi Ahn, Ying Qing Yu, and Martin Gilar
Waters Corporation, Milford, U.S.

APPLICATION BENEFITS

The ACQUITY UPLC® System used with Glycan Separation Technology Columns delivers the ability to achieve highly-resolved glycan separations in less time. This provides significant benefits to high-throughput glycosylation monitoring and profiling in biopharmaceutical drug development.

WATERS SOLUTIONS

ACQUITY UPLC
ACQUITY UPLC FLR Detector
Glycan Separation Technology Column

KEY WORDS

Recombinant mAb, glycosylation, HILIC, optimizing gradient conditions, flow rate, buffer concentration, mobile phase pH, column temperature

INTRODUCTION

Monoclonal antibody (mAb) drug development has been the most active area in the biopharmaceutical industry in recent years. One of the important aspects in recombinant mAb development is to profile glycosylation patterns. Since the glycans play key functions in biological activities, the glycosylation variances during protein production affect the pharmaceutical properties such as efficacy and elimination rate. One of many analytical approaches for glycan analysis is hydrophilic interaction chromatography (HILIC) with fluorescence detection. It provides high sensitivity, good reproducibility, and the ability to separate complex glycan mixtures.

The Waters ACQUITY UPLC System with fluorescence detection (FLR) combined with a Glycan Separation Technology (GST) Column provides superior resolution compared to HPLC systems. These glycan columns, packed with 1.7-µm amide sorbent, efficiently separate the fluorescent-labeled glycans in HILIC mode. Highly-resolved glycan separations, especially for positional isomers and coeluting minor peaks, now can be more accurately measured in UPLC/FLR.

In this application note, a general guideline for researchers to optimize HILIC gradient conditions is shown. The focus is to develop a UPLC method with optimal resolution for 2-aminobenzamide (2-AB) labeled N-linked glycans released from human IgG.

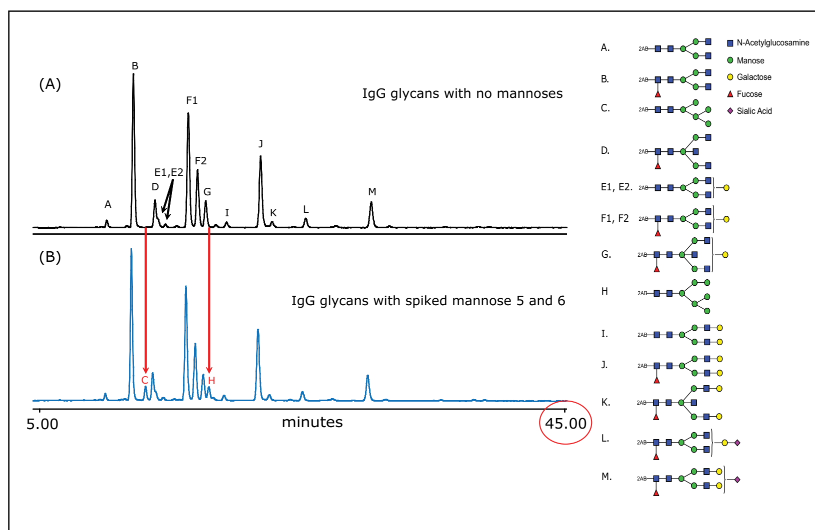


Figure 1. (A) 2-AB labeled glycans released from Human IgG, (B). The same IgG glycans with spiked mannose 5 and mannose 6.

EXPERIMENTAL

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Glycan Column 2.1 x 150 mm, 1.7 µm
Column temp.:	40 °C*
Flow rate:	400 µL/min*
Mobile phase A:	100 mM ammonium formate, pH 4.5*
Mobile phase B:	Acetonitrile
Gradient:	72 to 62% B in 45 min*
Weak wash:	72% acetonitrile
Strong wash:	20% acetonitrile
Injection:	1.0 µL partial loop

(*All other conditions are indicated in figure legends.)

MS conditions

Detector:	ACQUITY UPLC Fluorescence Detector
Excitation:	330 nm
Emission:	420 nm
Data rate:	5 pts/sec
PMT gain:	1.00
Time constant:	Normal

Samples

2-AB labeled N-linked glycans released from human IgG (ProZyme, San Leandro, CA, U.S.) were used. In addition, 2-AB labeled mannose 5 and mannose 6 were spiked into the sample to make a more chromatographically challenging sample.

We demonstrate the capabilities of glycan columns to separate fluorescent-labeled glycans in HILIC mode, including the positional isomers and coeluting minor peaks previously unresolved by HPLC.

RESULTS AND DISCUSSION

2-AB labeled glycan separation was performed using the HILIC method. For method optimization, the focused gradient with shallow gradient slope was used to effectively separate the region where the interested glycans were closely eluting. Other chromatographic settings such as flow rate, buffer concentrations, pH, and column temperature were also varied to maximize the peak resolution in the same region. The optimized HILIC condition used in Figure 1 was established based on the examination of various settings, as illustrated in Figures 2 through 6.

The complex mixture of 2-AB labeled glycans released from IgG was separated with superior resolution, as shown in Figure 1. All glycans were resolved including isomers E1/E2 and F1/F2. The gradient shown in all figures was run in 45 minutes and the entire run was completed in 1 hour using a 2.1 x 150 mm column. When the glycan HILIC method is transferred from a 3.0-µm HPLC to a 1.7-µm UPLC column, improved resolution in shorter run time can be achieved with UPLC system.

We demonstrate in Figure 1B that mannose 5 (peak C) and mannose 6 (peak H) can be successfully separated from their neighboring peaks, which often coelute.

Initial acetonitrile concentration in HILIC gradient

The impact of initial gradient strength on the separation is illustrated in Figure 2. The best separation was achieved at gradient condition 72% to 62% B. The resolutions in highlighted regions were changed positively and/or negatively. For example, the boxed region from A to B shows improved resolution; however the second boxed region from B to C shows the decreased resolution.

The gradient slope was set to 10% acetonitrile change in 45 min in all experiments in Figure 2 (0.22% B/min) to eliminate the gradient slope effect as a variable.

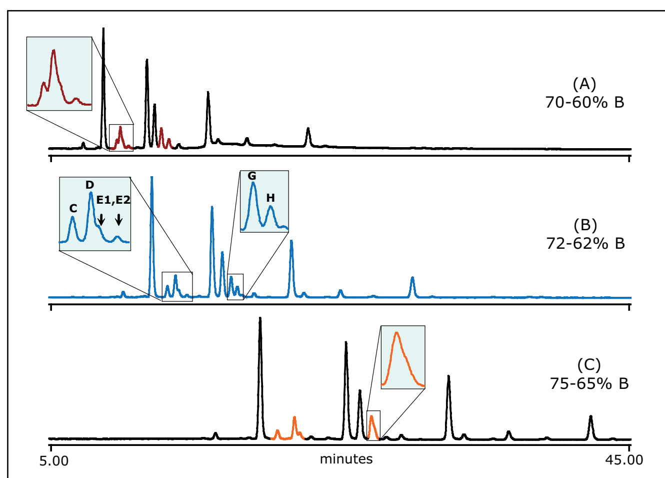


Figure 2. The impact of initial gradient strength on the separation of 2-AB labeled glycans. The flow rate was set at 0.4 mL/min with column temperature at 40 °C. Mobile phase A and B were 100 mM ammonium formate pH 4.5 and 100% acetonitrile respectively (see Figure 1 for peak denotation).

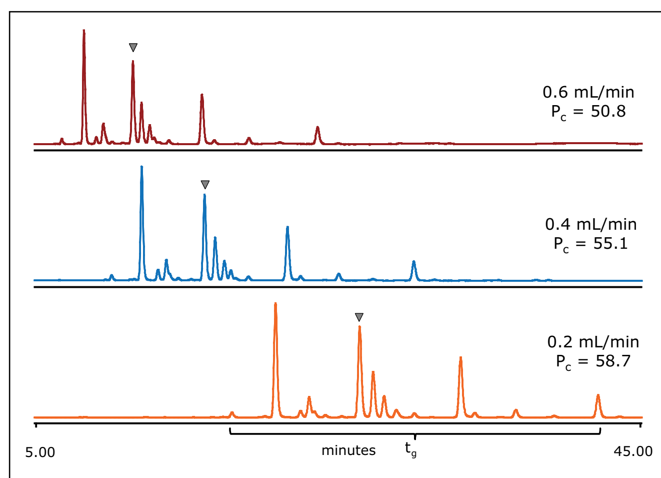
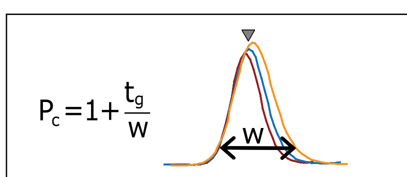


Figure 3. The impact of flow rate. The gradient was run in 72% to 62% B in 45 min at 40 °C. Mobile phase A and B were 100 mM ammonium formate (pH 4.5) and 100% acetonitrile respectively.

Flow rate

The separations in Figure 3 show the effect of the flow rate. Changing the flow rate influences the peak capacity as well as the resolution due to the contribution of the gradient slope change. When the flow rate increases from 0.2 to 0.6 mL/min the peak widths (w) become narrower; however, the effective separation window (tg) is simultaneously reduced (see Figure 3). Therefore, the impact of flow rate on peak capacity (defined as $P_c = 1+t_g/w$) was moderate.



The flow rate 0.4 mL/min was chosen because it showed minor improvements in resolution. These gradient conditions were selected for further separation as described in the next section.

Buffer concentration

The buffer concentration affects the resolution and the selectivity, as shown in Figure 4. When elevating the ammonium formate concentration, the retention time of charged sialylated glycans is affected more significantly than the retention of neutral glycans. With mobile phase containing 250 mM ammonium formate we observed complete resolution of E1 from D, which was very difficult to achieve by varying other chromatographic conditions.

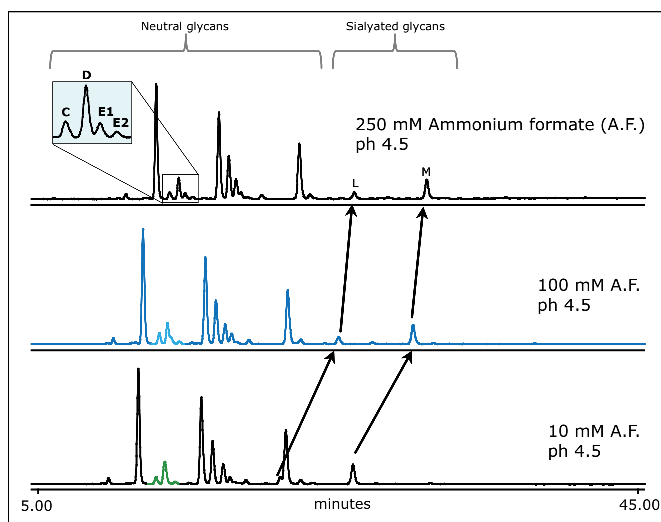


Figure 4. The impact of the buffer concentrations on the sialylated glycans. The column was run in gradient 72% to 62% B for 45 min at 0.4 mL/min. The column temperature was set at 40 °C (see Figure 1 for peak denotation).

Mobile phase pH

As shown in Figure 5, there is no significant change in separation between pH 4.5 and pH 7.5 in mobile phase A (100 mM ammonium formate). The mobile phase pH was adjusted by titrating ammonium with formic acid. However, the low pH in mobile phase will contribute to the retentivity shift of sialylated glycans (figure not shown).

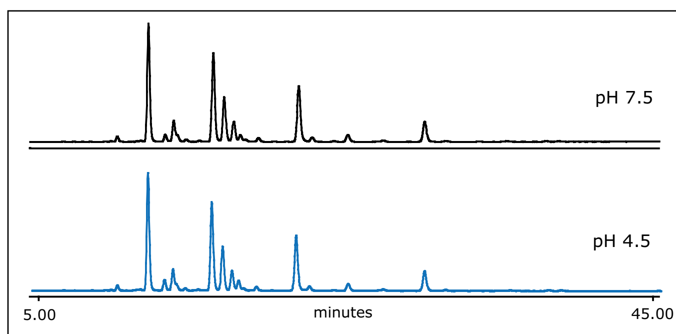


Figure 5. No significant retention change in two different pH conditions at 100 mM ammonium formate. The gradient was run in gradient 72% to 62% B for 45 min at 0.4 mL/min. The column temperature was 40 °C

Column temperature

The column temperature affects the analyte retentively; all glycans shifted to earlier retention times with increased column temperatures as expected by chromatographic theory. The changes in highlighted regions marked in red and purple were also observed as a result of column temperature. Since the resolution is important for separating complex glycan mixture, the column temperature can be another parameter to be optimized (Figure 6).

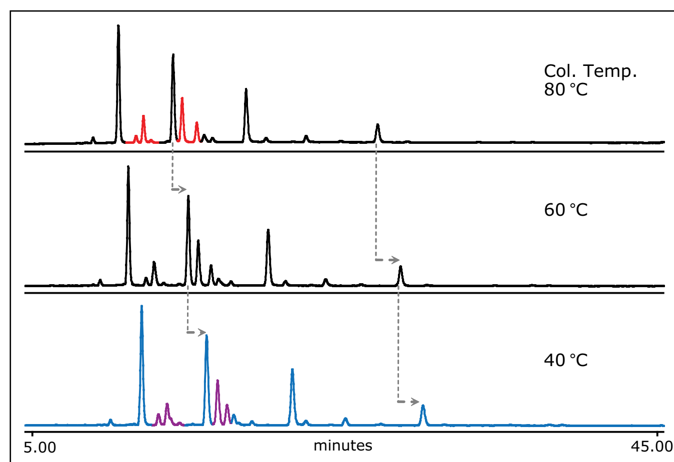


Figure 6. The retention shift due to the column temperature change.

CONCLUSIONS

- High quality of resolution and selectivity of the Glycan Separation Technology Column is demonstrated for 2-AB labeled glycans released from IgG. The impact of chromatographic settings in glycan separations is described as a guideline of HILIC method optimization. This guideline helps save the time and effort of method development to identify and quantify the glycans with confidence.
- Analysis time was significantly reduced with the ACQUITY UPLC System. Typically, a 2- to 3-hour HPLC gradient was needed to resolve 2-AB labeled IgG glycans to the full extent; our optimization method showed that a 1-hour UPLC run time was sufficient to separate the complex glycoforms.
- This system solution provides the capability to achieve highly-resolved glycan separation in less time. This adds value to high-throughput glycosylation monitoring and profiling in biopharmaceutical drug development.

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[APPLICATION NOTE]

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UPLC/FLR/QTof MS Analysis of Procainamide-Labeled N-Glycans

Vera Ivleva, Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- An alternative glycan fluorescent labeling reagent is demonstrated for fast and efficient UPLC separation that is compatible with MS ionization
- Significant increase in MS detection sensitivity for both neutral and acidic *N*-glycans

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC FLR Detector

ACQUITY UPLC BEH Glycan Column

Xevo® QTof MS

MassLynx™ Software

KEY WORDS

UPLC, fluorescent detection, IgG glycans, 2-aminobenamide (2AB), procainamide, fluorescent tag, MS/MS structure elucidation

INTRODUCTION

Characterization of the protein glycosylation profile is of the great importance and is required for various regulatory purposes and production of biopharmaceutical drugs. The released glycan pool is of great complexity and structural heterogeneity, which requires an efficient method of separation and a highly sensitive detection method.

UltraPerformance LC® (UPLC®) in hydrophilic interaction chromatography (HILIC) separation mode is becoming a routine and widely recognized technique for rapid, efficient, sensitive, and reproducible analysis of 2-aminobenzamide (2-AB) labeled glycans. Even though 2-AB is now the most common glycan labeling reagent, its use is limited by low MS sensitivity. Recent reports on LC/FLR/MS of glycans tagged with procaimanide[1] demonstrate this alternative reagent would be advantageous for improving MS ionization efficiency without compromising LC separation.

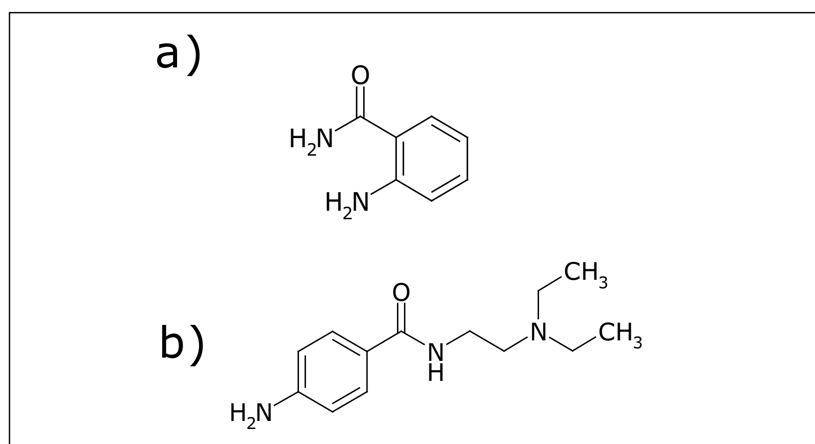

Figure 1. a) 2-aminobenzamide (2-AB), b) procainamide fluorescent tags. Monoisotopic mass increase compared to native glycan are 120.069 Da and 219.173 Da, respectively.

EXPERIMENTAL

Sample

Human IgG N-linked glycan standard library (ProZyme, PN GKLB-005) was fluorescently labeled with either 2-aminobenzamide (2-AB) or procainamide (4-amino-*N*-(2-diethylaminoethyl) benzamide) reagents (Fig. 1) without prior purification. Fluorescent labeling was performed in solution of 100 µL of glacial acetic acid: DMSO (3:7, v/v) mixture with 11 mg procainamide or 5 mg 2-AB, following by addition of 6 mg of sodium cyanoborohydrate. This labeling reagent was added to 2 µg of human IgG library standards and heated for 4 hours at 65 °C. All samples were reconstituted in acetonitrile/water (1:1) before the injection.

LC conditions

LC system:	Waters ACQUITY UPLC
Detection:	ACQUITY UPLC Fluorescent Detector, 1 points/s sampling rate. λ_{ex} 330 nm, λ_{em} 420 nm for 2-AB-labeled glycans; λ_{ex} 308 nm, λ_{em} 359 nm for procainamide-labeled glycans
Column:	Waters Glycan Separation Technology ACQUITY UPLC® BEH 1.7-µm, 2.1 x 150 mm (PN 186004742)
Column temp.:	60 °C
Sample temp.:	8 °C
Wash:	Weak wash 75% acetonitrile; strong wash 20% acetonitrile
Mobile phase A:	50 mM ammonium formate, prepared by titrating ammonium formate solution with formic acid to pH 4.5
Mobile phase B:	100% acetonitrile
Gradient:	72% to 55% B in 45 min, 0.4 mL/min flow rate

MS conditions

MS system:	Waters Xevo QTof, positive ion mode
Voltages:	Capillary 3.2 kV, sampling cone 30 V, extraction cone 4 V
Temp.:	Source temp. 100 °C, desolvation temp. 350 °C
Desolvation gas flow:	800 L/hr
LockMass calibration:	CsI 1 mg/mL (water/acetonitrile, 1:1), 5 µL/min flow rate
MS survey data:	E_{col} ≈20 V to 55 V ramp

Data management

MassLynx v. 4.1 Software for control and data acquisition

RESULTS AND DISCUSSION

Derivatization of glycans with 2-AB fluorescent reagent through reductive amination is a routine procedure for the majority of LC/FLR/MS analyses due to the high stability of labeled glycans and their compatibility with MS methods. The sensitivity of fluorescent detection is also suitable for relative quantitation. The sample preparation conditions are well established for efficient labeling, including release of oligosaccharides and tagging. Commercial labeling kits are also available.

Besides 2-AB, other reagents can potentially be used as fluorescent tags. Labeling with an alternative reagent may improve LC separation and FLR-MS detection. An example of such aromatic amine compound is procainamide, which was reported to have both such advantages and can potentially be used as preferred labeling reagent over 2-AB due to increased MS response.[1] Procainamide and 2-AB labeling of IgG glycan standards library was performed in parallel for the same amount of standards mixture.

Qualitatively, FLR response of procainamide-labeled glycans is comparable to that of 2-AB-labeled glycans. The sensitivity of FLR was optimized by tuning λ_{ex}= 308 nm, λ_{em}= 359 nm. Procainamide chromatograms show similar extent of peak separation within the same gradient. The most noticeable difference is that procainamide labeled IgG glycans are more retained under the same chromatographic conditions (Figure 2). Overall chromatographic selectivity is similar between the two labeling reagents.

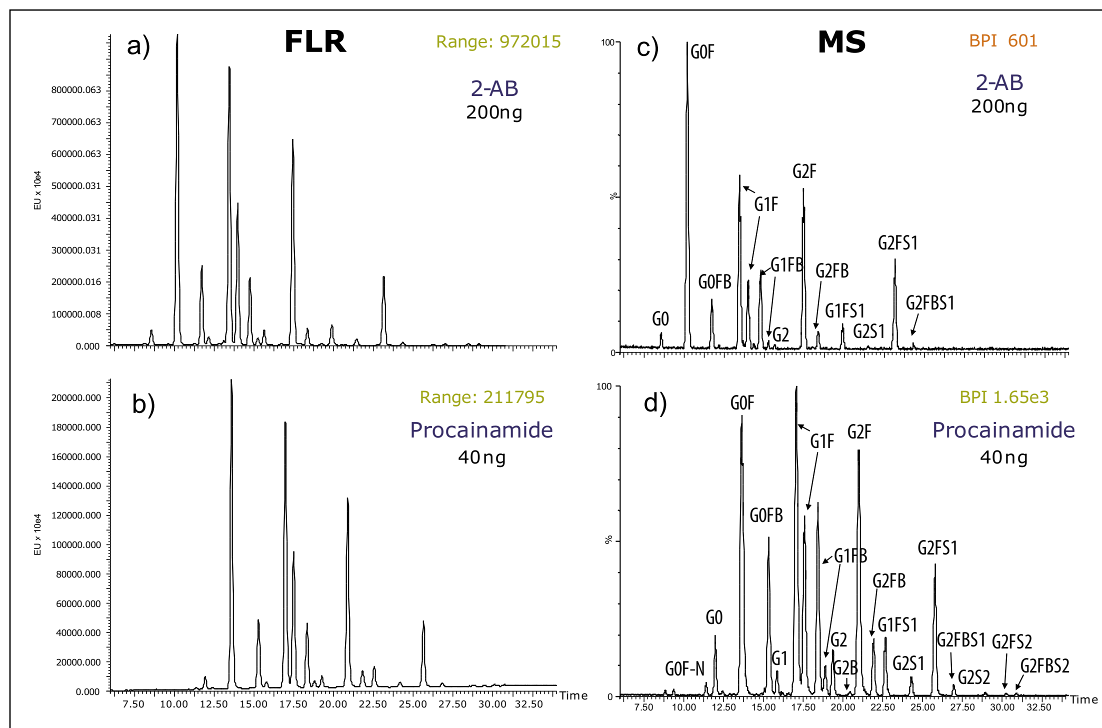

Figure 2. FLR/LC/MS chromatograms of 2-AB-labeled and procainamide-labeled IgG glycans (Left panel shows the FLR data channel, the right panel shows the BPI data cannel).

As described in previous study,[1] the advantage of using procainamide over 2-AB is the increased MS ionization efficiency. MS response of 2-AB-labeled G0F, G2F, and G1F is ~15 times lower than that of procainamide-labeled glycan standards (Figure 2 (c, d)). Be aware that MS signal becomes strongly non-linear at high sample concentrations, adversely affecting the shape of MS chromatographic peaks. This should be taken in consideration while doing method transfer from 2-AB labeling to procainamide: the injected sample amount of the latter should not exceed 40 ng of released IgG glycans. The relative ratio of the major MS peaks is similar between procainamide and 2-AB except for intensity spikes of G1F and G1FB.

Procainamide derivatives reveal more minor peaks compare to 2-AB due to its higher MS-ionization efficiencies. Multiple sialylated glycans were detected, they were not observed in the 2-AB glycan MS chromatogram, namely G2S2, G2FS2, and G2FBS2. Also, G0F-N, Man5, G1, and G2B neutral glycans were observed in a procainamide-derivative form but not as 2-AB-derivative (Figure 2(d)). The enhanced MS sensitivity due to procainamide derivatization also improves MS/MS spectra quality. The chromatographic resolution was adequate to analyze each individual peak in the glycan mixture with varying degree of galactosylation and fucosylation in a 45 min gradient.

MS/MS was performed on doubly protonated precursor ions of the five most intense chromatographic peaks (G0F to G2FS1) in order to optimize collision energy (E_{col}). For the same species, procainamide-labeled IgG glycans need about 10 to 20% higher E_{col} (~ 25-40 V depend on the size of the glycan) compared to E_{col} required for fragmenting 2-AB-labeled glycans. No qualitative difference in MS/MS pattern was observed for these two labeling reagents. Figure 3 demonstrates survey spectrum of G0FB component. The entire glycan sequence can be deduced from the series of characteristic ion fragments, which are produced predominantly by glycosidic bond cleavage, so that the method can be potentially applied for the analysis of unknown glycans.

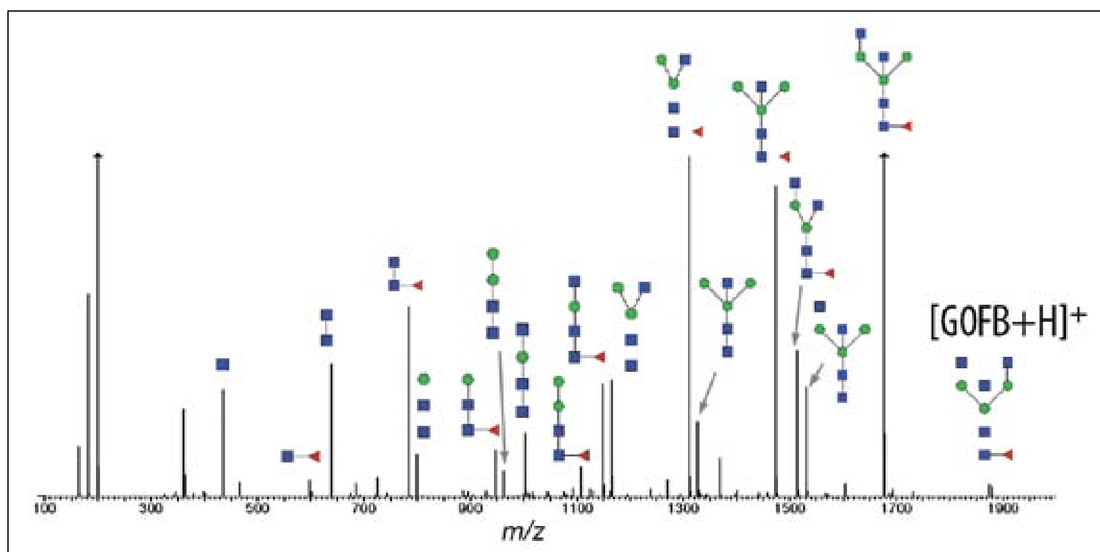

Figure 3. MaxEnt-3 deconvoluted MS/MS spectrum of procainamide-labeled glycan.

CONCLUSIONS

Procainamide labeling proved to be a great alternative to traditional 2-AB labeling technique. It follows the same reduction-amination labeling procedure like 2-AB, but it has shown to be a better alternative for fluorescent tagging.

Parallel comparison of two derivatization techniques demonstrated that procainamide labeling of glycan mixture is suitable for UPLC/FLR/MS/MS analysis and it shows excellent chromatographic peak resolution and MS sensitivity.

The main reason to choose procainamide labeling over traditional 2-AB method is that the former greatly improves the identification of very minor glycans, enhances MS ionization efficiency, allows MS/MS fragmentation for low level species, and therefore, is suitable for variety of de-novo study. Minimal method transfer is required except for sample dilution and slight gradient adjustment if it is needed. It is suitable for both neutral and acidic glycans, since there are no losses of sialylated species upon ESI ionization.

The procainamide derivatization method will be well received by FDA as a part of complete glycoprotein characterization.

Reference

1. S Klapoetke, J Zhang, S Bechto, X. Gu, and X Ding. *J. Pharm. Biomed. Analysis* 2010

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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[APPLICATION NOTE]

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Analysis of N-Linked Glycans from Coagulation Factor IX, Recombinant and Plasma Derived, using HILIC UPLC/FLR/QTof MS

Ying Qing Yu
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

HILIC UPLC/FLR/QTof MS is a powerful glycan characterization tool for characterization of complex glycans samples. Conventional HPLC method lacks the resolution power and sensitivity. Fractionation for MS analysis step is eliminated since the ACQUITY UPLC System is directly interfaced into a Xevo QTof MS. SimGlycan Software is part of the system solution, which interprets the collision fragmentation data, and offers a mean to elucidate glycan structures.

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC Fluorescence Detector

ACQUITY UPLC BEH Glycan Column

Xevo® QTof MS,

SimGlycan (Premier Biosoft)

KEY WORDS

Glycans, recombinant Factor IX protein (rFIX), plasma derived Factor IX protein (pd-FIX), 2-aminobenamide (2AB), sialic acid, structure elucidation

INTRODUCTION

Glycosylation of therapeutic protein drugs is of particular importance because it plays a vital role in the clinical performance of these drugs. In this work, we study the N-linked glycans from two Coagulation Factor IX biologics that are used for Hemophilia B treatment; one is recombinant (rFIX, BeneFIX) and the other one is derived from human plasma (pd-FIX, Mononine). Both Factor IX proteins are heavily glycosylated (Figure 1).[1] Previous findings on their glycoforms were done primary using orthogonal HPLC separation techniques, typically via Ion Exchange chromatography (IEX) and hydrophilic interaction (HILIC) modes, due to the complex nature of the Factor IX glycans. For mass profiling and structure characterization, mass spectrometry (MS) was typically used offline for LC fractions.

Waters has developed a HILIC UPLC®/FLR/QTof MS analytical platform for fluorescent-labeled glycan characterization. Significant improvements can be made such as peak resolution, speed, sensitivity, and the ability to identify and quantify even the minor glycans. An ACQUITY UPLC System is directly interfaced to a Xevo QTof MS, eliminating the need for fractionation. Comparative analysis of rFIX and pd-FIX glycans using this platform is demonstrated.

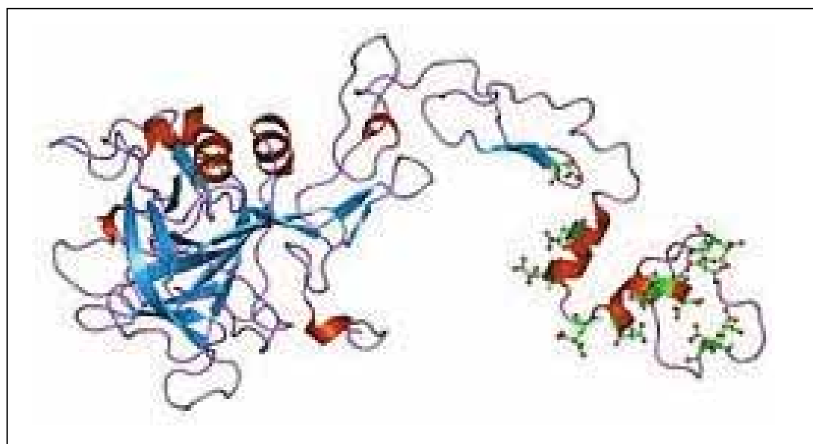


Figure 1. Coagulation factor IX structure.

EXPERIMENTAL

Sample

Figure 2 shows the basic glycan analysis workflow. FIX proteins are reduced and alkylated using DTT and IAM, followed by PNGase F enzymatic digestion overnight to release the glycans. The glycans are extracted using HILIC SPE device and labeled with 2-aminobenamide (2AB) dye, and the excess dye was removed by HILIC SPE again.[2]

LC Conditions

LC System:	ACQUITY UPLC
Detection:	ACQUITY UPLC FLR
Column:	ACQUITY UPLC BEH Glycan Column (2.1 x 150 mm)
Column temp.	60 °C
Sample temp.	10 °C
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	100 mM Ammonium Formate (~ pH 4.3)
Mobile phase B:	100% Acetonitrile (Fisher Optima)

Gradient table

Time (min)	Flow rate	%A	%B	Curve
Initial	0.400	39.0	70.0	
60.00	0.400	50.0	50.0	6
60.10	0.250	95.0	5.0	6
63.00	0.250	95.0	5.0	6
64.00	0.300	30.0	70.0	6
65.00	0.400	30.0	70.0	6
72.00	0.400	30.0	70.0	6

MS conditions

MS system:	Xevo QTOF MS
Ionization mode:	ESI positive
Capillary voltage:	3200 V
Cone voltage:	30 V
Desolvation temp.:	350 °C
Desolvation gas:	800 L/Hr
Source temp.:	100 °C
Acquisition range:	700 to 2000 m/z
Collision energies:	4 V
Lockmass:	Cesium Iodide (CSI): 1 µg/µL in 50% MeCN

Data Management

MassLynx 4.1
SimGlycan v. 2.9 (Premier Biosoft)

RESULTS AND DISCUSSION

rFIX and pd-FIX has very different glycans profiles (Figure 3, 4). Glycans released from rFIX are mostly fucosylated bi-, tri-, and tetra-antennary complex type glycans. Additional lactosamine units were observed in some glycans. Man5 and a core N-glycans with an addition of fucose were also identified (Table 1). Glycans from pd-FIX are show more heterogeneity, especially for the larger glycans.

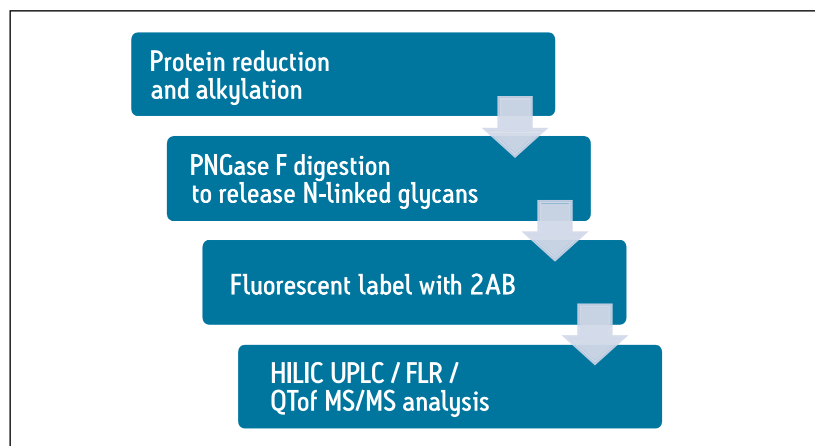


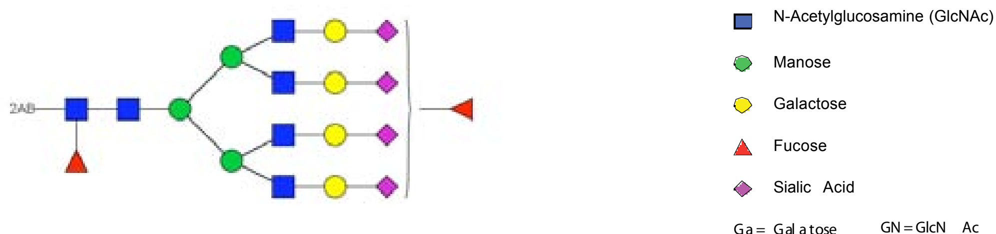
Figure 2. Glycan analysis workflow.

Only the complex type glycans with polylactosamine units were observed from pd-FIX; the level of fucosylation is from 0 to 2 (Table 1). Two sulfated bi-antennary glycans were observed, which was not reported in the literature; these sulfated glycans were well resolved from their non-sulfated counterpart (Figure 4). The possibility of phosphorylation was excluded by alkaline phosphatase reaction (data not shown). In addition, MS/MS fragmentation was used to further confirm their identification (see Figure 5).

Peak No.	Glycan	rFIX (BeneFIX)	pd-FIX (Mononine)	Observed MW	Theoretical MW
1	Man3 + 1F	+	—	1176.456	1176.455
2	G0F - GN	+	—	1379.534	1379.534
3	G0F	+	—	1582.612	1582.674
4	Man5	+	—	1354.511	1354.502
5	1A/1F - GN	+	—	1541.587	1541.587
6	2A/1F	+	—	1906.710	1906.719
7	2A/1F + GN	+	—	2109.760	2109.798
8	2A/1F/1S	+	—	2197.816	2197.814
9	3A/1F	+	—	2271.840	2271.850
10	2A/2S	—	+	2342.880	2342.851
11	2A/2S/SO3	—	+	2422.814	2422.808
12	2A/1F/2S	+	+	2488.912	2488.909
13	3A/1F/1S	+	—	2562.924	2563.945
14	2A/1F/2S/SO3	—	+	2568.868	2568.866
15	4A/1F	+	—	2636.962	2637.982
16	3A/1F/2S	+	—	2854.042	2854.042
17	4A/1F/1S	+	—	2928.066	2928.07
18	3A/3S	—	+	2999.112	2999.079
19	3A/1F/3S	+	+	3145.112	3145.137
20	4A/1F/2S	+	—	3219.134	3219.174
21	3A/2F/3S	—	+	3291.188	3291.195
22	4A/1F/3S	+	—	3510.224	3510.269
23	4A/4S	—	+	3655.277	3655.306
24	4A/1F/4S	+	+	3801.358	3801.365
25	4A/1F/3S +(GN+Ga)	+	—	3875.401	3875.402
26	4A/2F/4S	—	+	3947.373	3947.423
27	4A/1F/4S +(GN+Ga)	+	—	4166.418	4166.497
28	4A/1F/3S +2(GN+Ga)	+	—	4240.533	4240.533
29	4A/1F/4S +2(GN+Ga)	+	—	4531.617	4531.629
30	4A/2F/2S + 2(GN+Ga)	—	+	4677.610	4677.687
31	4A/3F/4S + 2(GN+Ga)	—	+	4823.694	4823.745
32	4A/1F/4S + 3(GN+Ga)	+	—	4896.783	4896.761

Table 1. A complete list of 2AB labeled glycans observed from rFIX and pd-FIX. Complex type glycan with various degree of sialylation along with Man5 and Man3/1F were observed. The average mass error is less than 5 ppm.

Example of Glycan Nomenclature: 4A/2F/4S



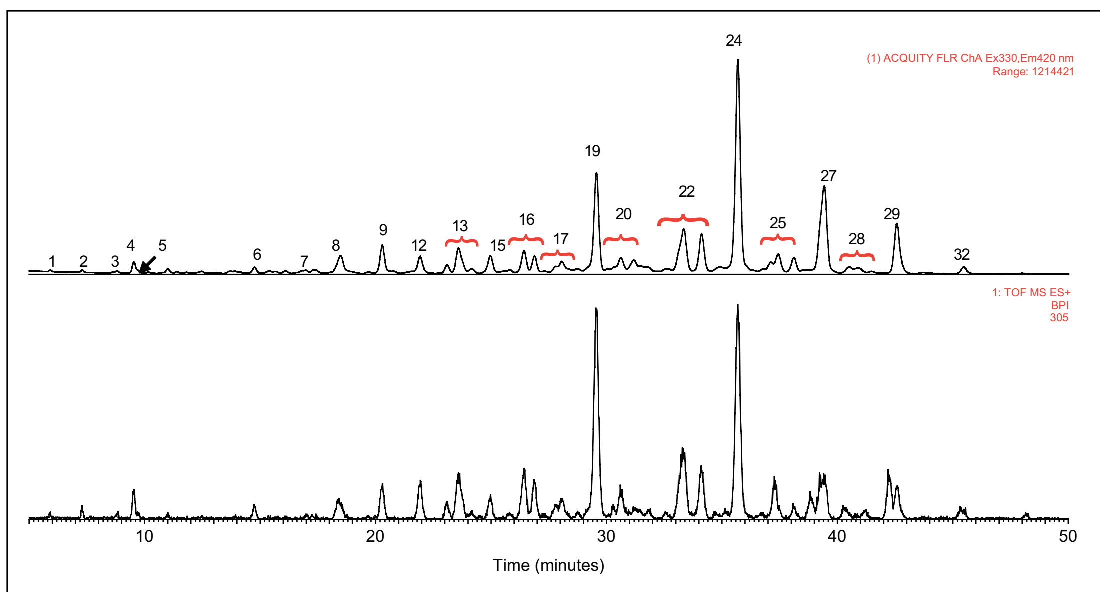


Figure 3. HILIC UPLC/FLR/QTof MS of 2AB labeled rFIX (BeneFIX) glycans. Low abundant glycans in the low mass region were observed and confirmed by MS analysis. In addition, isomeric glycans (sialic acid positional isomers) were well resolved. The peak number corresponds to the glycan listed in Table 1.

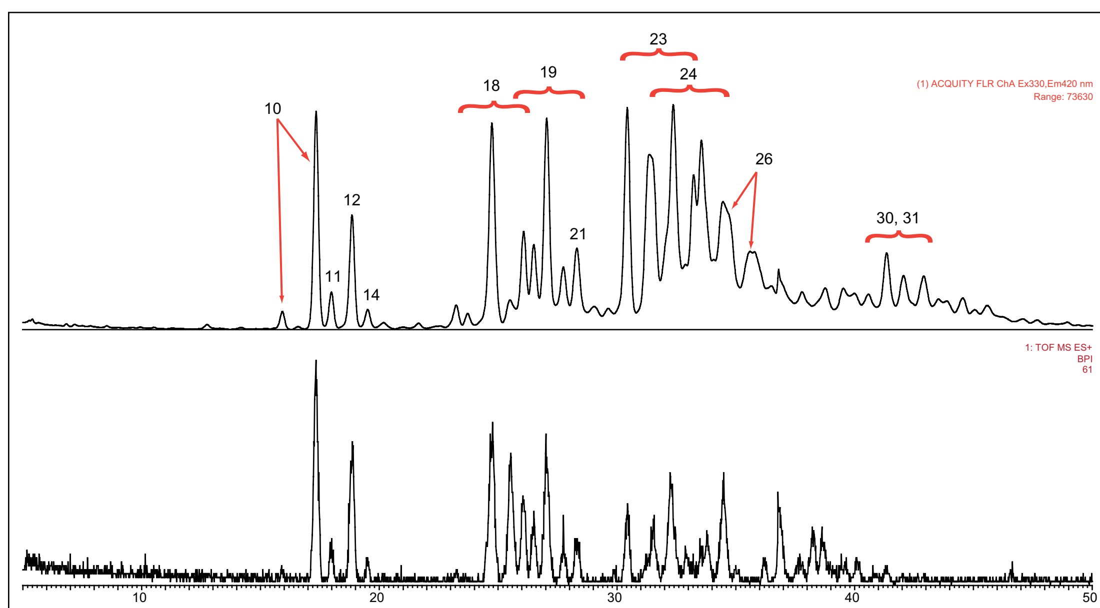


Figure 4. HILIC UPLC/FLR/MS of 2AB labeled pd-FIX (Mononine) glycans. More glycans were identified, such as sulfated glycans and multiply fucosylated glycans. The possibility of phosphorylation was excluded by alkaline phosphatase reaction (data not shown). In addition, MSMS fragmentation was used to further confirm their identification (see Figure 5 A-C).

The glycan profile from both rFIX and pd-FIX is very complex. HPLC-based methods lack the resolution needed to identify and quantify various glycan forms. The ACQUITY UPLC System coupled with the ACQUITY UPLC BEH Glycans Column alone is able to achieve baseline separation of glycans that are different in mass and degree of sialylation; terminal sialic acid isomers are also well separated.

The accurate mass measurement from the Xevo QTof MS offers confident assignment of the glycans. MS/MS fragmentation along with SimGlycan Software gives further confirmation on glycan structure. Figure 6 shows that glycan 4A/1F/4S was observed from both rFIX and pd-FIX; the LC retention time is the same for the isobaric tetra-antennary glycans. However, the CID fragmentation showed distinct difference, which resulted from the fucose location on the glycans (Figure 5B). Figure 7 shows another example on how CID was used to differentiate isobaric doubly fucosylated glycans from both rFIX and pd-FIX glycans, again distinct fragment ions were used to differentiate the location of fucose. Biological influence caused by the location of fucose was documented in the literature.

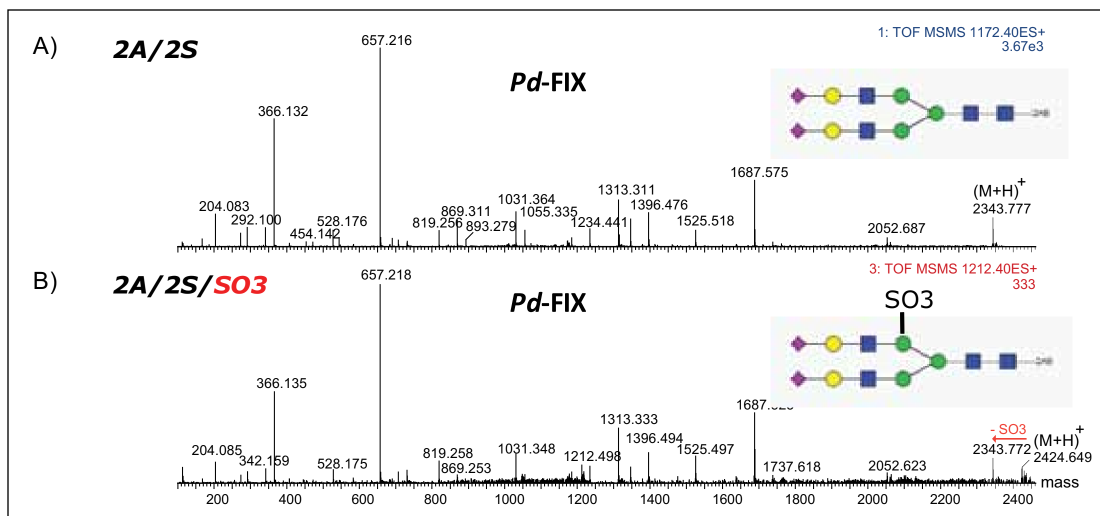


Figure 5. MS/MS fragmentation of sulfated glycans such as 2A/2S/SO3 were compared with its non-sulfated counterpart, 2A/2S. Facile loss of SO3 (-80 Da) was observed. The MS/MS data, after MaxEnt3 mass deconvolution, was submitted to SimGlycan software for structure assignment; and SimGlycan validated the proposed structures.

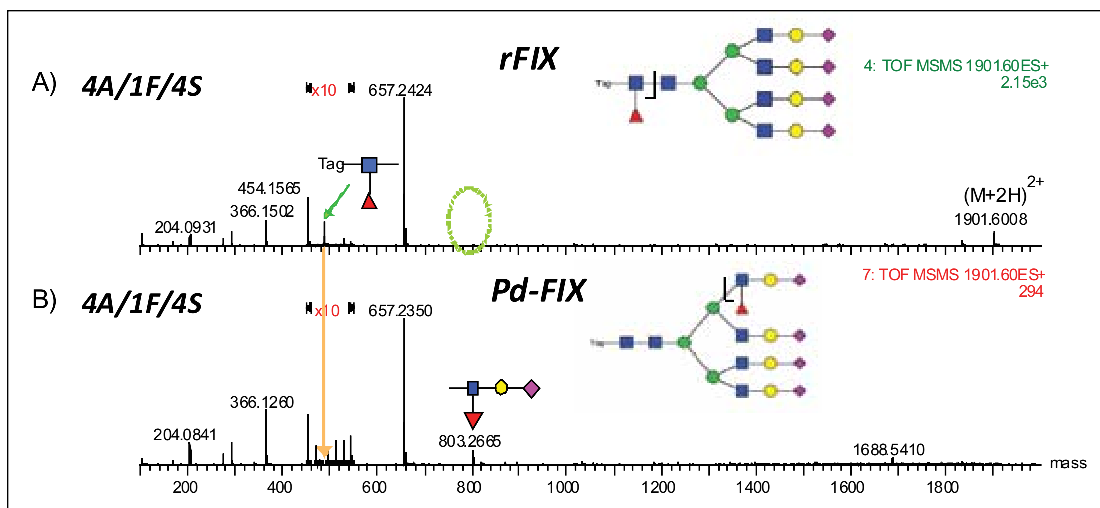


Figure 6. Differences in fucosylation were observed for rFIX and pd-FIX. Fucose from rFIX was located at the first GlcNAc residue in the core structure; while the majority of the fucosylation site for singly fucosylated pd-FIX glycans was located at the antenna. Fragment ions at m/z 488 and 803 were the diagnostic ions for probing the fucosylation sites.

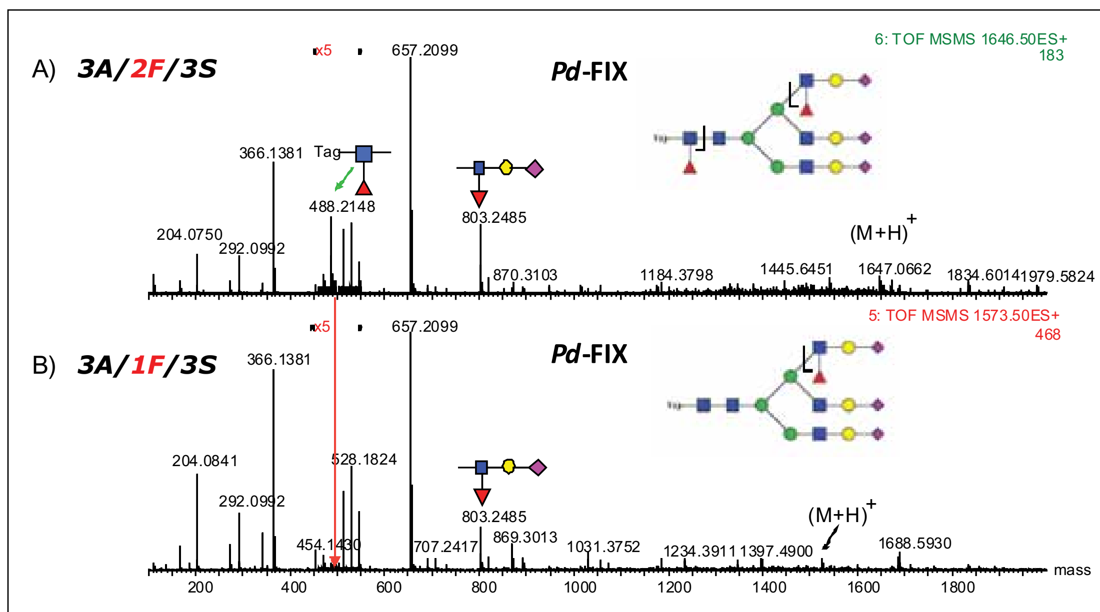


Figure 7. Some doubly fucosylated glycans were observed for pd-FIX sample. The two diagnostic ions at m/z 488 and 803 were observed, which indicated that one fucose was at the first GlcNAc residue (core structure) and the other one was located at the antenna.

CONCLUSION

Blood protein glycan characterization is known to be very challenging for scientists working in the biopharmaceutical field, since the glycans that attach to the protein backbone are highly heterogeneous and complex. Our solution for complex glycan separation and characterization is the UPLC/FLR/QTof MS analytical platform.

In addition to shortened run time enabled by ACQUITY UPLC technology, HILIC UPLC also offers significant improvement in peak resolution compares to conventional HPLC method; for example, positional sialic acid isomer separation is achieved, also the separation of sulfated and sialyated glycans were observed.

MS/MS fragmentation and database search using SimGlycan Software helped the glycan structure elucidation, adding more confidence to glycan structure assignment.

The ACQUITY UPLC/FLR/Xevo QTof MS analytical platform along with SimGlycan Software is a powerful and versatile tool for complex glycan characterization. Glycan profiling, mass confirmation, and structure elucidation can all be done in a single LC/MS system. High quality data are generated with shorter analysis and data interpretation time. This is a valuable tool for researchers working with glycoprotein drugs.

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2. Ying Qing Yu, Martin Gilar, Jennifer Kaska and John C. Gebler. *Deglycosylation and Sample Cleanup Method for Mass Spectrometry Analysis of N-linked Glycan.* Waters Application Note. 2007. 720001146EN.

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T: 1 508 478 2000
F: 1 508 872 1990
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UPLC Separation of 2-AB Labeled Fetuin Glycans Removed by Exoglycosidase

GOAL

To demonstrate benefits of UPLC® separations in HILIC mode for the glycans' linkage analysis of the released 2-AB labeled N-linked glycan pool of fetuin, sequentially digested by exoglycosidase array.

BACKGROUND

Many biotherapeutics are glycosylated. Glycoprotein characterization includes glycan profiling, which is an important step in the process of development and production of biopharmaceutical proteins.

The released glycan pool is of a great complexity due to branching and linkage isomers. Such structural heterogeneity of glycans is attributed to their biological role and requires thorough characterization for glycobiology applications and regulatory purposes.

At the same time, glycan complexity often hinders complete structural elucidation by conventional HPLC methods since many species remain unresolved. Entire oligosaccharide characterization is incomplete if structural or linkage isomers cannot be closely monitored in order to maintain consistency and high quality during therapeutic manufacturing.

In glycan analysis, UPLC delivers a clear advantage for resolving coeluting positional isomers, which are often difficult to separate with conventional HPLC.

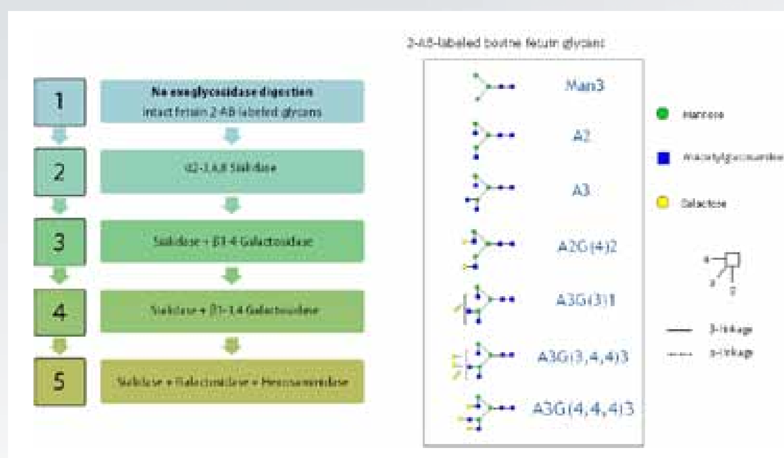

Figure 1. Workflow of the enzymatic array digestion and the structures of the released bovine fetuin N-glycans.

UltraPerformance Liquid Chromatography® (UPLC) in hydrophilic interaction chromatography (HILIC) separation mode has become a routine and widely-recognized technique for rapid, efficient, sensitive, and reproducible analysis of 2-aminobenzamide (2-AB) labeled glycans. UPLC Glycan columns packed with sub-2-μm particle sorbent offer significant improvements in separation power and allow enhanced resolution of complex glycans.

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THE SOLUTION

To compare resolving capabilities of HPLC and UPLC systems, a polysaccharide ladder of the released bovine fetuin N-glycan was created by a sequential digestion using enzyme array (Figure 1). Each monosaccharide was released based on the enzyme specificity (Figure 2).

LC experiments were run on a Waters ACQUITY UPLC® System with fluorescence detection (FLR) and an ACQUITY UPLC BEH Glycan Column (2.1 x 150 mm, 1.7 µm). The Waters Alliance® 2695 HPLC System with a TOSOH Bioscience TSKgel Amide-80 Column (2.0 x 150 mm, 3.0 µm) was used as the default HPLC method. The structures of the digested glycans were confirmed by glucose units (GU) values and specificity of the applied enzyme.

The top chromatograms represent the undigested total N-glycan pool, and the remaining chromatograms correspond to the sequential digestion of the N-glycan pool with the exoglycosidase enzymes indicated.

The first enzyme applied was Arthrobacter Ureafaciens sialidase, which releases sialic acids. This step yields two linkage isomers A3G(3,4,4)3 and A3G(4,4,4)3, which are hard to resolve under HPLC conditions.

As highlighted on the chromatogram, these 2-AB labeled isomers were separated using the 1.7-µm ACQUITY UPLC BEH Glycan Column on the UPLC system, but coeluted on the 3.0-µm HPLC column. Such an improvement in resolution is due to the higher peak capacity of sub-2-µm particle packing.

Method transfer between HPLC and UPLC is straightforward, since selectivity of neutral and siaylated glycans in both columns are comparable under the conditions used for this study.

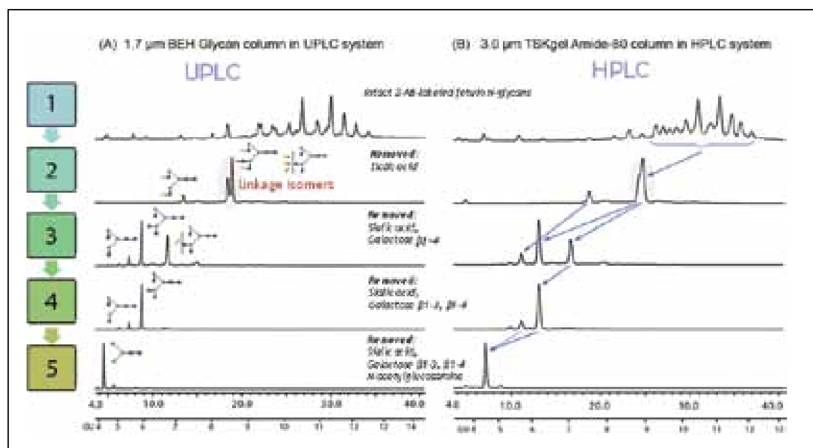

Figure 2. Sequential glycan removal from bovine fetuin. Linkage isomers not separated by HPLC are well-resolved using UPLC. Both UPLC and HPLC systems used solvent A: 100 mM Ammonium Formate, pH 4.5, solvent B: Acetonitrile; 72 to 57% B gradient in 45 min gradient time. Flow rate was 0.5 mL/min on the ACQUITY UPLC System and 0.45 mL/min on the Alliance 2695 HPLC System Figure 2. Sequential glycan removal from bovine fetuin. Linkage isomers not separated by HPLC are well-resolved using UPLC. Both UPLC and HPLC systems used solvent A: 100 mM Ammonium Formate, pH 4.5, solvent B: Acetonitrile; 72 to 57% B gradient in 45 min gradient time. Flow rate was 0.5 mL/min on the ACQUITY UPLC System and 0.45 mL/min on the Alliance 2695 HPLC System.

SUMMARY

Side-by-side comparisons of UPLC and HPLC methods of the fetuin N-glycan digest, obtained by exoglycosidase array, illustrates the suitability of UPLC separations for glycan identification. The results demonstrate UPLC's clear advantage for resolving coeluting positional isomers, which are often difficult to separate with a conventional HPLC column and system.

This highly efficient and fast UPLC separation of glycans meets regulatory obligations as a part of complete glycoprotein characterization, and conveniently offers users a seamless HPLC-to-UPLC method transfer.

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

[TECHNOLOGY BRIEF]

A Systematic Approach to Glycan Analysis using HILIC-UPLC and an Online Database of Standardized Values

GOAL

To enable the systematic analysis of fluorescently labeled N-glycan pools released from recombinant biotherapeutics and separated by HILIC-UPLC® chromatography. Data are interpreted with the aid of a robust online glycan database generated from standardized retention values.

BACKGROUND

Glycosylation is the most complex post-translational protein modification and it is estimated that more than half of all eukaryotic proteins are glycosylated. In order to elucidate the functional outcomes of glycosylation and to characterize the glycoprotein more fully, it is essential to define the monosaccharide sequence, linkage, and anomericity of the covalently attached oligosaccharides.

Glycosylation of biotherapeutics is a critical product attribute and adverse changes in glycosylation can significantly alter product efficacy and safety. Furthermore glycosylation of biotherapeutics can be influenced by dissolved oxygen, pH, carbon source, temperature during manufacture, as well as by the choice of expression system. These variations can put product integrity at risk and therefore the ability to monitor glycosylation accurately and rapidly is essential at all stages of the process.

The U.S. FDA, the EMA, and other regulatory bodies have started to increase pressure on manufacturers to analyze the glycosylation of therapeutics more fully, and also to demonstrate how their process can affect glycan composition. However, there are no robust workflows for systematic detailed, quantitative, sensitive, glycan analysis.

NIBRT's GlycoBase 3+ is a web-enabled proprietary resource that contains normalized retention data, expressed as Glucose Units, or GU values, for more than 600 2AB-labeled N-linked glycan structures.

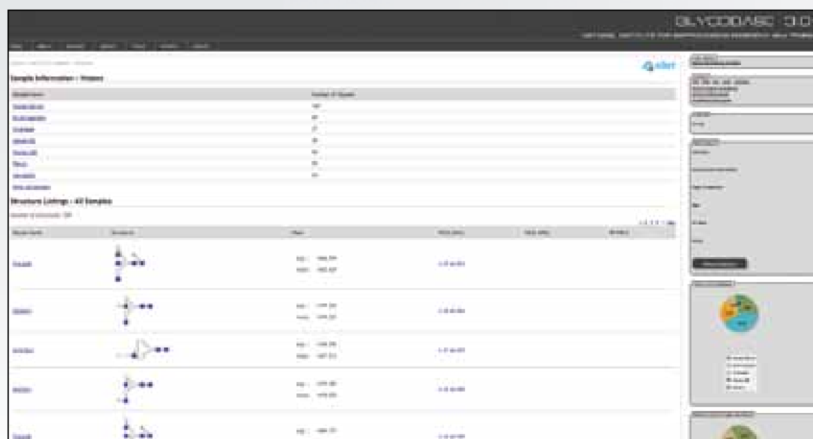

Figure 1. Overview of GlycoBase 3+, NIBRT's UPLC N-glycan database.

THE SOLUTION

The National Institute for Bioprocessing Research and Training (NIBRT) in Ireland is a world-class institute that provides training and research in the biopharmaceutical industry. NIBRT's mission is to provide a unique experience for trainees in an environment that replicates a modern bioprocessing facility. In parallel, NIBRT also undertakes leading bioprocessing research in collaboration with industry partners and provides contract analytical services.

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Waters Corporation and NIBRT, led by Prof. Pauline Rudd, have developed a comprehensive solution to glycan analysis that combines NIBRT's novel bioinformatics database solution, GlycoBase 3.0+, with the unique capabilities of the Waters ACQUITY UPLC® System and Glycan Separation Technology chemistries for HILIC-UPLC separations.

GlycoBase 3.0+ is a web-enabled proprietary resource that contains normalized retention data (expressed as Glucose Units, or GU values) for more than 600 2-AB labeled N-linked glycan structures. These values were obtained by systematic analysis of released glycans from a diverse set of glycoproteins using Waters HPLC and UPLC technologies and the NIBRT glycan analytical platform.

Two orthogonal technologies, exoglycosidase sequencing and mass spectrometry, were used to confirm every structure, generating a high-quality glycan library that can be used as a general tool or interrogated in various ways, e.g., for each of the biotherapeutic glycoproteins analyzed. Data stored in a web-accessible database is accessed through a customized software application with a simple intuitive interface.

Each entry/glycan is comprehensively annotated and includes:

- A pictorial representation of the structure depicting monosaccharide sequence, stereochemistry, and glycosidic linkages in a variety of fcolor or black-and-white formats (Oxford notation that includes embedded linkage information, CFG notation)
- GU retention time values and descriptive quality statistics
- Molecular mass (average and monoisotopic) for each glycan
- Monosaccharide composition
- Graphical representations of relative glycan composition and antennary structure
- Related reference information (biological context, taxonomy, diseases, enzyme specificities)
- Links from raw data to experimental acquisition conditions

Glycan Details - Waters - F(6)A2B

Physical-Chemical Properties

Monosaccharide Composition

Data

Summary | Raw Data | NIBRT Records | Public Records

Run	RP-HPLC**	NP-HPLC*	UPLC*	Sample	Profile	Taxonomy
1			6.24	Human Serum	see conditions	Homo sapiens
2			6.24	Human Serum	see conditions	Homo sapiens
3			6.24	Human Serum	see conditions	Homo sapiens
4			6.25	Human Serum	see conditions	Homo sapiens
5			6.25	Human Serum	see conditions	Homo sapiens
6			6.24	Human Serum	see conditions	Homo sapiens
7			6.25	Human Serum	see conditions	Homo sapiens
8			6.24	Human Serum	see conditions	Homo sapiens
9			6.24	Human Serum	see conditions	Homo sapiens

Figure 2. Detailed experimental and supporting data for the selected glycan, F(6)A2B.

Figure 3. A bank of Waters ACQUITY UPLC Systems in NIBRT's analytical laboratory.

SUMMARY

A glycan database, GlycoBase 3.0+, of protein N-linked oligosaccharides released from a range of biotherapeutic proteins has been developed for the biopharmaceutical industry using a combination of Waters and NIBRT technologies. Reproducible, sensitive, standardized and high-throughput glycan HILIC separations, in combination with an online glycan bioinformatics resource, GlycoBase 3.0+, allows the identification of glycans based on three orthogonal data attributes.

An additional feature of 2-AB fluorescently labeled N-linked glycans is that the data generated by chromatography can be used for relative quantitation, which when combined with Glycobase 3.0+ is a powerful tool for the biopharmaceutical industry. The dynamic range allows glycans present at <1% of the glycan pool to be quantified.

These novel capabilities will facilitate the more rigorous characterization of biopharmaceutical protein products required by today's regulatory environment

Waters would like to acknowledge Mark Hilliard, Weston Struwe, Giorgio Carta, John O'Rourke, Niaobh McLoughlin, and Pauline Rudd from NIBRT for this work.

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http://glycobase.nibrt.ie

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

[APPLICATION NOTE]

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Development of a Glycan Database for Waters ACQUITY UPLC Systems

Mark Hilliard,[1] Weston Struwe,[1] Barbara Adamczyk,[1] Radka Saldova,[1] Ying Qing Yu,[2] John O'Rourke,[1] Giorgio Carta,[1] and Pauline Rudd[1]
[1]National Institute for Bioprocessing Research & Training (NIBRT), Dublin, Ireland
[2]Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

ACQUITY UPLC/FLR analysis under the control of an Empower workstation represents a powerful and fully integrated analytical platform for analyzing the oligosaccharides attached to glycoproteins.

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC FLR Detector

Empower™ Software

GlycoBase 3.0 Database

KEY WORDS

HILIC-UPLC with florescence detection, 2AB-labeled glycans, glycan database

INTRODUCTION

Glycosylation is the most complex post-translational protein modification. More than half of all proteins are glycosylated, and, to elucidate their function, it is important to define the structures of the glycans that are covalently attached to their surfaces. The manufacturing of glycoprotein life sciences products can be challenging and a number of factors can have a major impact upon their glycosylation, including the cell type in which the protein is expressed, media composition, and processing parameters such as dissolved oxygen, pH, the carbon source, and temperature.

The glycosylation of biotherapeutics is often a critical product attribute for therapeutic efficacy and safety. Fluctuations in the process can put product integrity at risk and therefore it is important to monitor glycosylation accurately throughout the process.

www.nibrt.ie

In collaboration with Waters, the National Institute for Bioprocessing Research and Training (NIBRT), based in Ireland, has developed a glycan database for use in conjunction with UPLC glycan separations.

The analytical platform utilized by NIBRT is described in Figure 1. Briefly: N-glycans are released by PNGase F and fluorescently labeled with 2-aminobenzamide before separating the labeled glycan pool by hydrophilic interaction chromatography (HILIC).

The Waters ACQUITY UPLC® System applies an optimized hydrophilic interaction chromatographic separation (HILIC) using columns containing sub-2-μm particles. The combination of the ideal selectivity of diol-bonded Bridged Ethyl Hybrid (BEH) particles and exceptional peak capacity at higher flow rates results in overall increases in speed, sensitivity, and resolution compared to standard HPLC systems.

EXPERIMENTAL

Sample

The monoclonal antibody Herceptin was immobilized in acrylamide gel blocks and N-linked glycans released using peptide-*N*-glycosidase F. The glycan pool was labelled with 2-aminobenzamide (2AB). A linear gradient of 50 mM ammonium formate buffer, pH 4.4, and acetonitrile was used for glycan separation.

LC conditions

UPLC system: ACQUITY UPLC

Column: ACQUITY UPLC BEH Glycan Column (2.1 x 150 mm)

Column temp.: 40 °C

Sample temp.: 5 ± 5 °C

Flow rate: 0.561 mL/min

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

Mobile phase B: 100% acetonitrile

Gradient table

Time (min)	Flow rate	%A	%B	Curve
1. Initial	0.561	30.0	70.0	6
2. 1.47	0.561	30.0	70.0	6
3. 24.81	0.561	47.0	53.0	6
4. 25.50	0.400	70.0	30.0	6
5. 26.25	0.400	70.0	30.0	6
6. 26.55	0.400	30.0	70.0	6
7. 28.55	0.561	30.0	70.0	6
8. 30.00	0.400	30.0	70.0	6

Detection

UPLC detector: ACQUITY UPLC FLR Detector

Wavelengths: λ_{ex} =330 nm, λ_{em} = 420 nm

Data rate: 10 pts/sec

PMT gain: 20

Time constant: Normal

Auto Zero On Injection Start (check)

Data management

Empower Software

GlycoBase 3.0 database

RESULTS AND DISCUSSION

The preliminary assignment of glycan structures is obtained by interrogating the NIBRT database (http://glycobase.nibrt.ie) in which peak retention times are expressed in glucose units (GU values) by alignment to a dextran hydrolysate ladder. Chromatographic resolution and reproducibility play a vital role in obtaining accurate and reproducible GU measurements and quantitative data. In the ACQUITY UPLC System, the GU value standard deviation is below ± 0.01 GU from 8 to 10 independent analyses, illustrating the superb peak resolution and run-to-run reproducibility.

Improved peak capacity and resolution of glycan pools

An ACQUITY UPLC System combined with a BEH glycan column provides significant enhancement in terms of peak capacity and resolution in comparison to classical HPLC technologies.

In Figure 2, a 30-minute separation of N-glycans from human serum on a (1.7-µm) BEH glycan column is compared with chromatograms that were generated using a classical HPLC system with amide columns (5-µm and 3-µm particle size).

Figure 3 shows that the ACQUITY UPLC System rapidly resolves glycans that were released from Herceptin in 12 minutes. 24 glycans were identified and quantified using the NIBRT database and exoglycosidase array digestions; the major glycan species are annotated in the figure.

GlycoBase 3.0; an aid to data interpretation

Included in GlycoBase 3.0 (http://glycobase.nibrt.ie) is the Waters collection of UPLC GU values of the glycans of a diverse group of nine samples (Herceptin, Human and Mouse serum IgG, recombinant Erythropoietin, Bovine Fetuin, Bovine Ribonuclease B, Yeast Invertase and Human serum). These values were obtained by the systematic analysis of released glycans using Waters HPLC and UPLC technologies and the NIBRT glycan analytical platform. The profiles of the glycan pools and the enzymatic digestions can be obtained from a link (http://glycobase.nibrt.ie/glycobase/documents/enzyme.pdf). In brief, the N-linked glycan pool is separated equally into 5 (0.5 mL) eppendorfs and appropriate exoglycosidase enzymes are sequentially added with 10x 50 mM sodium acetate pH 5.5 buffer to build up the array. These are labeled (i) undigested control; (ii) ABS; (iii) ABS+BFK; (iv) ABS+BFK, BTG; and finally (v) ABS+BFK, BTG, GUH. Enzyme nomenclature and amounts are described below.

Figure 1. High-throughput UPLC-based method for N-linked glycan analysis.

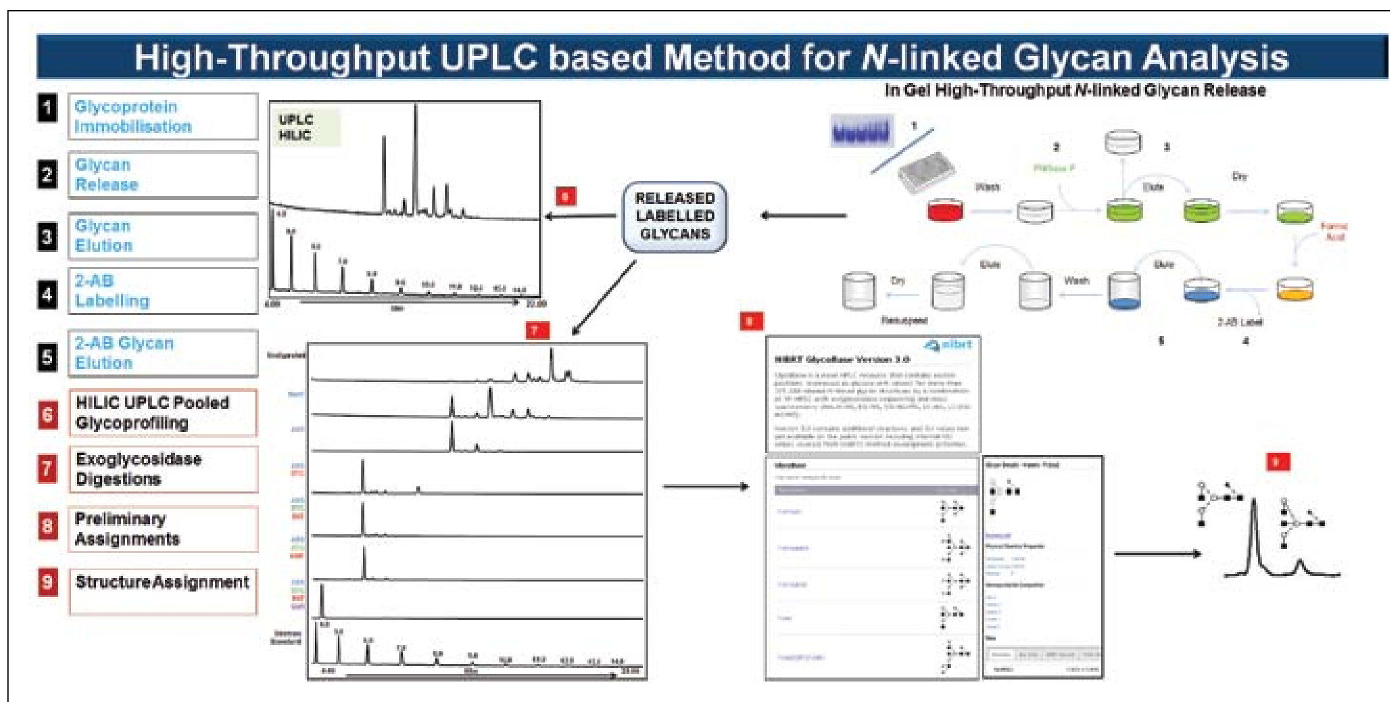


Figure 2A. Comparison of the 3-hour HPLC method and 1-hour HPLC separation of complex human serum glycans on (i) 5-μm and (ii) 3-μm TSK-GEL Amide-80 columns

2A

i

HPLC 3-hour separation
TSKgel Amide-80, 5 µm
4.6 mm x 25 cm

ii

HPLC 1-hour separation
TSKgel Amide-80, 3 µm
4.6 mm x 15 cm

iii

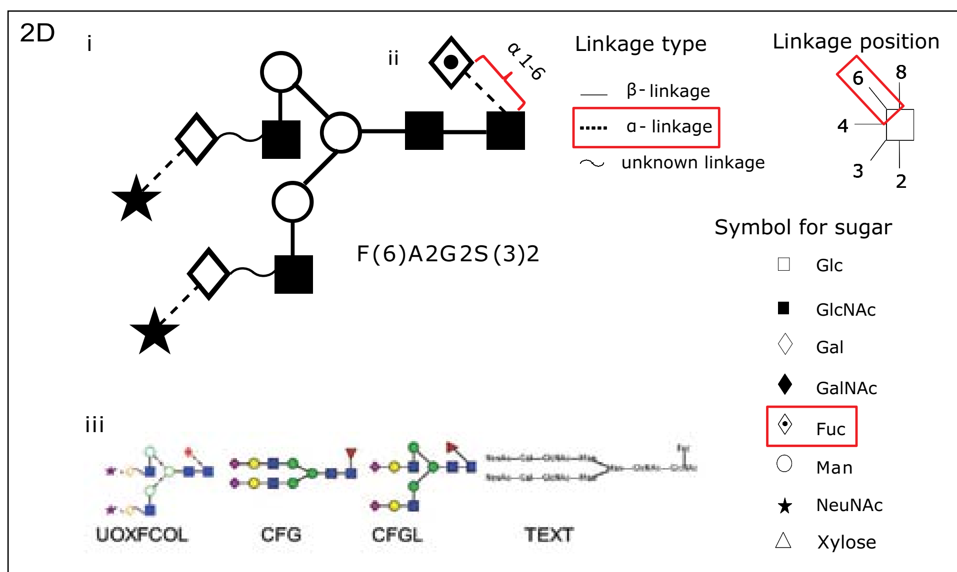
UPLC 30-minute separation
ACQUITY UPLC BEH
Glycan Column, 1.7 µm
2.1 mm x 15 cm

2B

2C

Minutes 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00

GU 5 6 7 8 9 10 11 12 13 14



using a 30 minute Waters UPLC method (iii) BEH glycan column 1.7-µm, 2.1 mm x 15 cm.

Figure 2B. Separation of human serum protein glycans with classical HPLC methods, TSK-GEL 3-µm Amide-80 column, 1 hour run time.

Figure 2C. The same human serum glycome analyzed on Waters ACQUITY UPLC. The most abundant of the 136 glycans that were identified are annotated (Figure 2D) There is significant decrease in run times and improved separation of the glycans compared with the classical HPLC methods.

Figure 2D. (i) A cartoon depicting the linkage type, linkage position and symbol for common sugars used with the Oxford notation. (ii) An example using F(6)A2G2S(3)2, where core fucose F(6) α 1-6 linkage is depicted with Oxford notation highlighted in red for linkage type, linkage position and symbol for associated sugar. (iii) Glycobase has a conversion function (developed by Eurocarb DB) that can represent glycans in other common formats as can be see with F(6)A2G2S(3)2.[1,2]

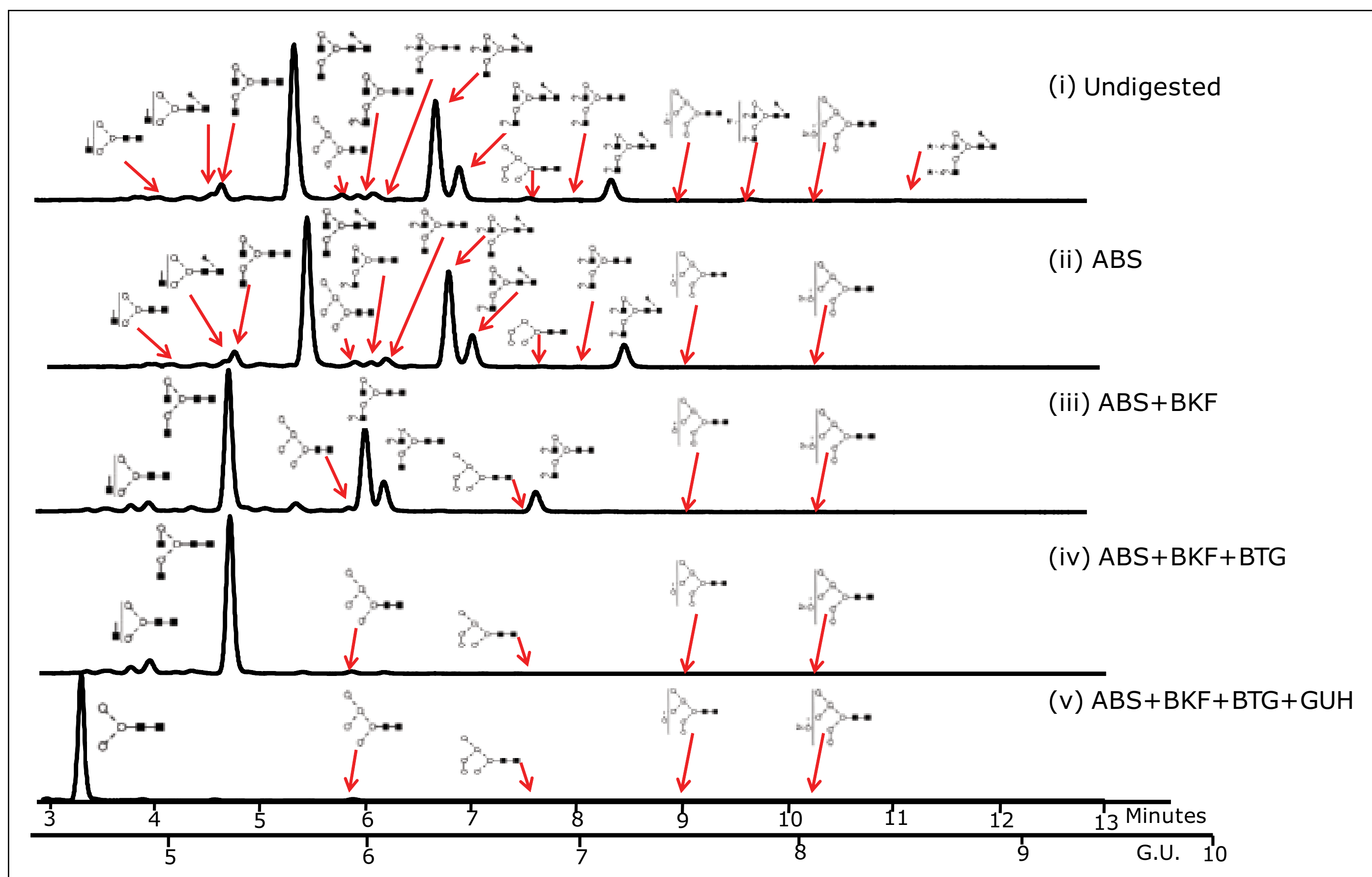


Figure 3. UHPLC analysis of the pool of glycans released from Herceptin (Trastuzumab) monoclonal antibody and the exoglycosidase array digestions that provide monosaccharide sequence and linkage information. 10 ug of Herceptin was used for the initial release by PNGaseF and intact pool represents approximately 2% of the total released N-glycan pool.

(i) Intact N-glycan pool released by PNGase F; (ii) ABS, Arthrobacter ureafaciens α-sialidase *(1 U/mL) digestion. ABS releases α2-3 /6/8 sialic acids; (iii) ABS + BKF, bovine kidney alpha-fucosidase (BFK) (1 U/mL) array digestion. BKF releases α1-6/2 fucose (iv) ABS +BKF+ BTG, bovine testes beta-galactosidase (BTG) (1 U/mL) array digestion. BTG releases galactose β1-3>1-4 linkages and (v) ABS+BKF+BTG+N-acetylglucosaminidase (GUH) (4 U/mL) array digestion.*

CONCLUSIONS

- HPLC technologies are relatively straightforward, robust, and inexpensive. They can separate arm specific isomers and when coupled with exoglycosidase digestions they provide monosaccharide sequence and linkage information.
- Non-selective fluorescent labeling (2AB) provides quantitative data and high sensitivity (glycans from <1 ng protein on a 2D gel can be analyzed)[3, 4]
- Enhancements in peak resolution, peak capacity, and reproducibility of fluorescently labeled (2AB) glycan separations can be achieved using an ACQUITY UPLC System and a BEH Glycan Column operated in HILIC mode.
- The enhanced resolution of UPLC enables the more accurate assignment of GU values compared to conventional HPLC because overlapping peaks are better separated.

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

Analysis of Isobaric Carbohydrate Structural Isomers with the MALDI SYNAPT G2 HDMS

GOAL

To demonstrate that isobaric oligosaccharide mixtures can be resolved and identified using ion mobility TOF mass spectrometry with the MALDI SYNAPT® G2 HDMS™ System.

BACKGROUND

Glycosylation plays a vital role in stability, *in vivo* activity, solubility, serum half-life, and immunogenicity of many recombinant therapeutic proteins. Because therapeutic glycoproteins are typically manufactured using non-human expression cell systems, the glycoforms from recombinant proteins and from human sources can be very different.

For example, glycosylation can change significantly under poorly-controlled culture conditions, making it a key indicator of process robustness. It is therefore essential to demonstrate that glycosylation is consistent, showing control over the production process, and to establish acceptable variation limits for biotherapeutic production.

Oligosaccharides (sugars) from glycoproteins frequently exist as sets of isomers, where differences in glycosidic linkage or in position isomeriation are present. These apparently minor differences may significantly impact the safety and efficacy profile of a biotherapeutic. Organizations that fail to comprehensively profile the glycans may risk expensive and damaging product recalls or difficulties with regulatory approval. Therefore a comprehensive characterization of the glycan profile is required to clarify the variation in these structural and spatial isomers.

Ion mobility mass spectrometry is a rapid and efficient technique for differentiating structural isomers of oligosaccharides.

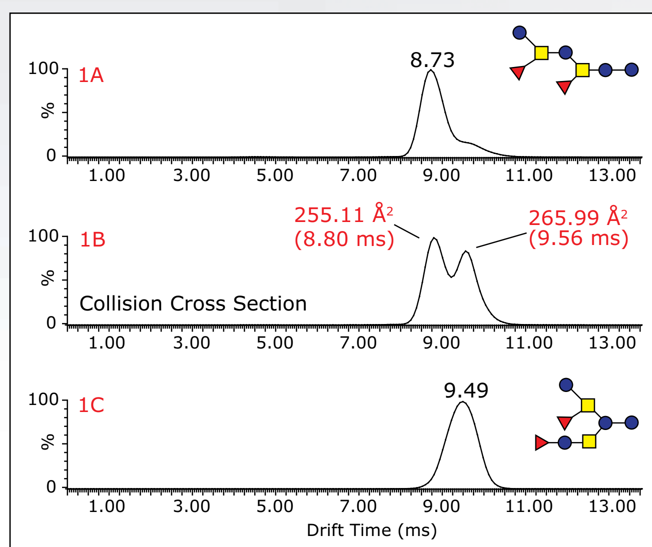

Figure 1. Ion mobility drift time vs. intensity plots for the ions (MNa+, m/z 1365.4278,) detected during the analysis of carbohydrate structural isomers by MALDI SYNAPT G2 HDMS. The upper and bottom traces (Figure 1A and 1C) are from individual carbohydrate. The middle trace is from the analysis of a mixture of the carbohydrate at 1:1 ratio. The collisional cross section (CCS) value for each glycan is also labeled at the apex of the peak.

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THE SOLUTION

Oligosaccharide standards difucosyl-p-lacto-N-hexaose II and difucosyllacto-N-hexaose-a (V-labs Inc.) were used. Each oligosaccharide standard was dissolved in water (0.2 mg/mL) and was mixed at 1:1 ratio to generate a sample containing both isobaric oligosaccharides. The individual and mixture samples were mixed 1:1 (v/v) with a 2,5-dihydroxybenzoinc acid (DHB) MALDI matrix solution (20 mg/mL in ethanol), respectively, before spotting onto a stainless steel MALDI target plate. All samples were analyzed in positive ion MS and MS/MS mode, and MS signals reported are from the sodiated adducts (MNa^+: 1365.4278). Data processing was accomplished using either MassLynx™ 4.1 or DriftScope™ 2.1 software, which supports data viewing, processing, and collisional cross section measurements for ion mobility mass spectrometric analysis.

Collisional cross-sections of each oligosaccharide were automatically calculated after the system was calibrated using a polyalanine calibration standard.

The unique ability of the SYNAPT G2 HDMS mass spectrometer to differentiate isobaric structural isomers of oligosaccharides is illustrated in Figure 1, where the drift time distributions for each of the oligosaccharides, as well as the oligosaccharide mixture are shown. The drift time distribution for the oligosaccharide mixture (Figure 1B) shows two distinct peaks with drift times of 8.80 and 9.56 ms, whereas the pure difucosyllacto-N-hexaose-a sample generates one mobility peak at 9.49 ms (Figure 1A) and difucosyl-p-lacto-N-hexaose II sample at 8.66 ms (Figure 1C). Thus, the ion mobility separation enables clear resolution and assignment of the two isomeric peaks.

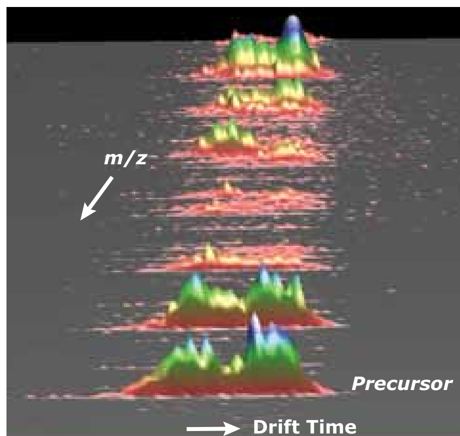

Figure 2. Three-dimensional representation of data for isobaric carbohydrate precursor ions and their fragment ions (drift time and m/z domain). The fragmentation process takes place after ion mobility separation, thus all the attendant ions are aligned up against the precursor in the drift time dimension.

Overall, this figure demonstrates that travelling wave ion mobility can be used to rapidly separate isobaric oligosaccharides in mixtures submitted to MALDI IMS-TOF analysis.

Fragmentation following ion mobility separation is further capability of the SYNAPT G2 HDMS platform that offers additional information for structural assignment confirmation. Because fragmentation can be performed following the ion mobility separation, the drift time of all of the attendant fragment ions will be conserved with the intact glycan precursor ions (Figure 2). Thus, multiple species can be simultaneously fragmented for further analysis. Enabling fragmentation to be performed both prior to and following the ion mobility separation (data not shown) further enhances the information content in the analysis of complex oligosaccharides.

SUMMARY

Ion mobility mass spectrometry is a rapid and efficient technique for differentiating structural isomers of oligosaccharides. The Triwave® Technology of the SYNAPT G2 HDMS offers enhanced functionality by performing fragmentation either before and/or after ion mobility separation to elucidate the complex structures of oligosaccharides. These oligonucleotides can be described not only by their mass or mass-to-charge but by their collisional cross-sectional areas. Organizations wishing to increase their productivity, protect their intellectual property, or improve the characterization of their complex biotherapeutic products will see immediate benefits by providing their scientists access to ion mobility-enabled mass spectrometry platforms.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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[APPLICATION NOTE]

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Electron Transfer Dissociation of N-Linked Glycopeptides from a Recombinant mAb using SYNAPT G2-S HDMS

Jonathan P. Williams, Jeffery M. Brown, Stephane Houel, Ying Qing Yu, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

ETD fragmentation enhanced by a "super charging" reagent improves the overall glycopeptide fragmentation efficiency. SYNAPT® G2-S HDMS™ System equipped with an ETD source proves to be an indispensible tool for protein glycosylation analysis.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

SYNAPT G2-S Mass Spectrometer

ACQUITY UPLC BEH300 C_{18}
2.1 x 150 mm, 1.7 µm Column

KEY WORDS

ETD, *m*-NBA super charging, glycopeptides, trastuzumab

INTRODUCTION

Protein glycosylation is a major requirement in the production of recombinant therapeutic antibodies and plays a vital role in its efficacy and safety. Typical glycosylation analysis consists of a released glycan assay, glycosylation site determination, and site heterogeneity analysis.

Throughout the years, biopharmaceutical laboratories have developed and optimized the glycan profiling analysis using a variety of liquid chromatographic separation modes with optical detectors. Tandem mass spectrometry based approaches represent a rapid and sensitive approach to obtain site-specific characterization of glycosylation. Electron transfer dissociation (ETD) is a powerful fragmentation technique known to be particularly useful for determining modification sites of labile post-translational modifications (PTMs), which are often more difficult to characterize using collision induced dissociation (CID). This application note offers details of glycopeptide characterization using ETD fragmentation.

Selected precursor ion charge-state is important in ETD, since this affects the dissociation efficiency.[1] Non-dissociative electron transfer is often observed as a function of decreasing precursor ion charge; therefore, *m*-nitrobenzyl alcohol (*m*-NBA) was added post-column to increase the charge-state of the ESI-generated ions to enhance ETD efficacy.[2]

We demonstrate ETD-based MS technologies used for structural *N*- and *O*-glycan analysis, describing the modification site determination, and sequence information that can be readily obtained. A recombinant mAb, trastuzumab, is used as a test case for this study.

EXPERIMENTAL

Mass Spectrometry Conditions

Mass Spectrometer:	SYNAPT G2-S HDMS
Capillary:	2.0 kV
Reagent glow discharge current:	70 µA
Source temp.:	100 °C
Sampling cone:	25 V
Extraction cone:	3 V
Reagent make-up gas flow:	25 mL/min
Desolvation temp.:	200 °C
Cone gas flow:	30.0 L/h
ETD reagent:	1,3-dicyanobenzene (*m/z* 128)
Trap wave height:	0.25 V
Trap RF:	500 V

Sample Description

3-Nitrobenzyl alcohol (*m*-NBA), was purchased from Fluka (Part number 73148), and the ETD reagent, 1,3-Dicyanobenzene, was purchased from Sigma-Aldrich (Part number 145858). Trastuzumab was reduced and alkylated using diothiothreitol and iodoacetamide, followed by overnight trypsin digestion.

UPLC Conditions

An ACQUITY UPLC H-Class Bio System was directly coupled to the standard ESI interface of a SYNAPT G2-S HDMS Mass Spectrometer equipped with ETD. Reversed phase chromatographic separation of 1 to 10 pmol of tryptic peptides was performed on an ACQUITY UPLC BEH300 C_{18}, 2.1 x 150 mm, 1.7-µm Column. Mobile phases A and B were water and acetonitrile, both containing 0.1% formic acid. The tryptic peptides were eluted from the column using a gradient from 5% to 35% B over 35 min at a flow rate of 100 µL/min. The gradient was further ramped to 50% B for 10 min, then to 80% B for 1 min, and held for 5 min at a flow rate of 200 µL/min. Post-column addition of ~0.4% *m*-NBA, a 'super-charging' reagent, was achieved using a Valco T-piece.

RESULTS AND DISCUSSION

ETD has been used as a complementary tool to CID for protein post-translational modification analysis. CID fragmentation of glycopeptides is known to favor neutral losses (cleavage of regions within the attached sugar); consequently, intense oxonium ions are detected at the low *m/z*. Since CID is usually dominated by glycan-related product ions, this hampers peptide sequence information for precise site-specific identification of this important class of PTM.

Fortunately, ETD fragmentation follows a different fragmentation mechanism where the glycan moieties are preserved on the c and z fragment ions. Since fragmentation is conducted post separation, these fragment ions are generated at the same retention time as those in the glycan moiety CID analysis.

Tryptic recombinant monoclonal IgG, trastuzumab, was used for the ETD analysis. Trastuzumab is known to have one N-linked glycosylation site on the heavy chain in the Fc region. Glycopeptide chromatographic retention times were determined from a UPLC®/MS^E experiment. Since the common oxonium ions, e.g., *m/z* 204.09, 366.14, from the high energy fragmentation data channel are the dominant fragments, as shown in Figure 1, the *m/z* extracted ion chromatograms allow identification of the retention times of the glycopeptides and obtain their *m/z* values. The retention times and precursor *m/z* values were used to set up the subsequent ETD experiment.

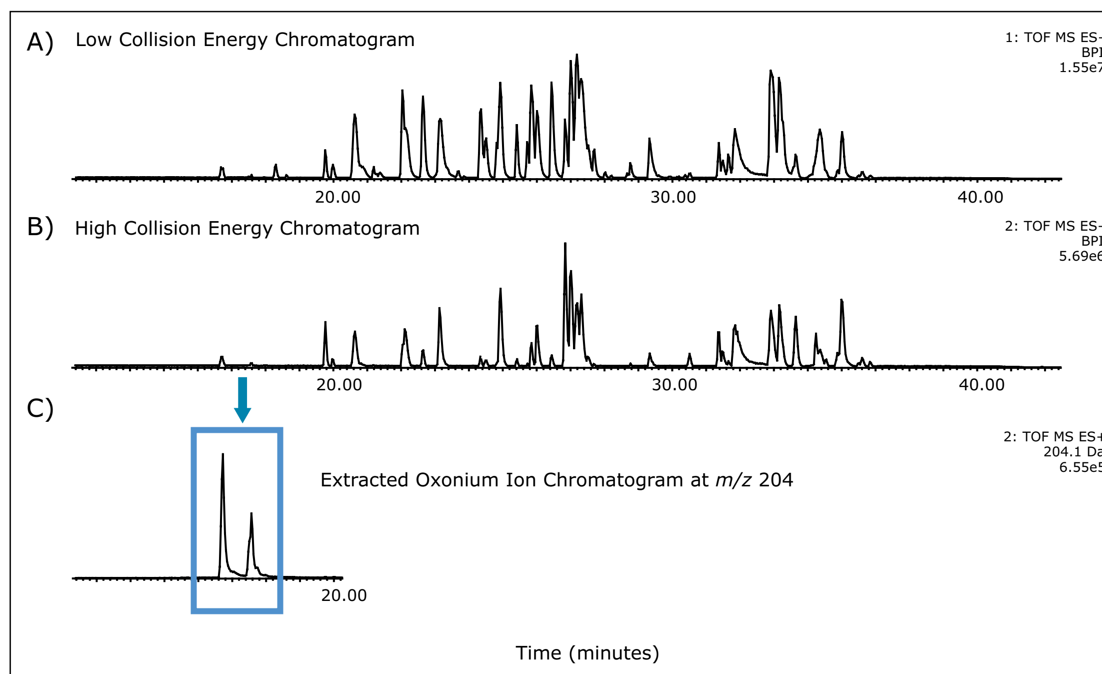

Figure 1. UPLC/MS^E base peak intensity chromatograms. A) The low collision energy data channel; B) The high collision energy data channel; C) The extracted ion chromatogram at m/z 204 (oxonium ion) shows that the glycopeptides are the early eluting peaks.

By post column mixing the super-charge reagent, *m*-NBA, the overall charge envelopes of the targeted glycopeptides are shifted to favor higher charges, as shown in Figure 2. Non-dissociative electron transfer is often observed as a function of decreasing precursor ion charge. Figure 3A shows the fragmentation of the triply charged precursor ion (peptide contains G0F) which generates charge-reduced to 2^+ and 1^+ ions, with little peptide backbone fragmentation. Figure 3B demonstrates that intense c and z ions were produced when *m*-NBA was used to promote the quadruply charged precursor ion for ETD. The net mass difference between the c_4 to c_5 (and z_4 to z_5) ions matches the mass of Asn plus G0F (total mass difference of 1558.6 Da).

In addition to G0F, the other two dominant glycoforms from trastuzumab are G1F and G2F. Figures 4A-B illustrate the ETD fragmentation of the quadruply charged precursor ions from these glycopeptides. The c and z ions in these spectra are used to confirm the peptide backbone with high confidence. In addition, we observed little to no neutral loss from the glycan moiety; hence, the intact glycan mass can be assigned to the glycosylation site with single amino acid resolution.

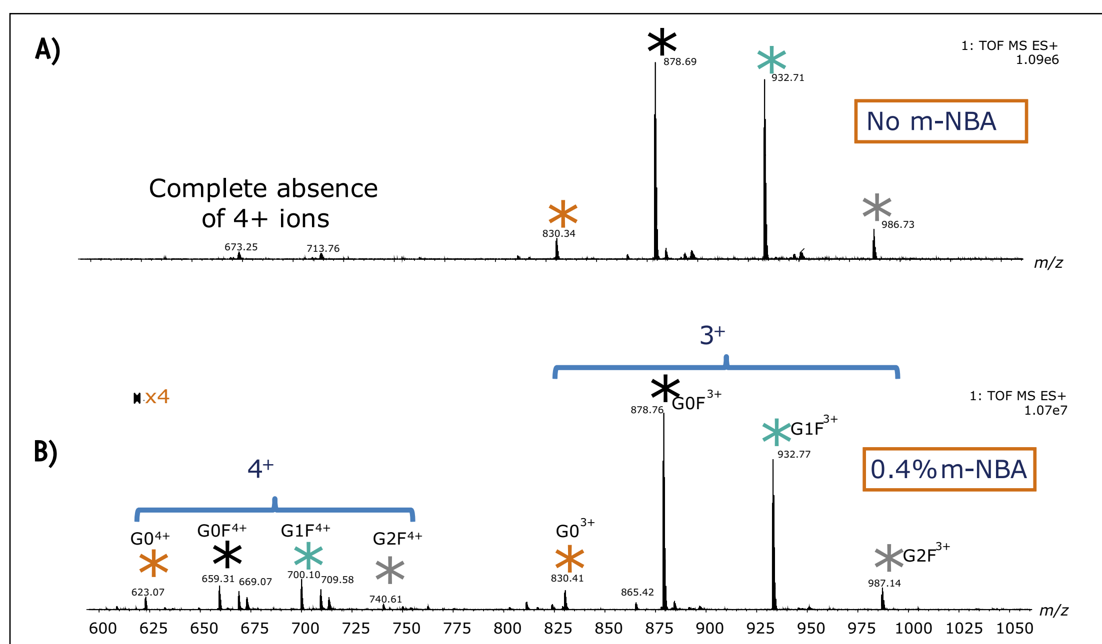

Figure 2. Summed MS spectra for glycopeptides eluting between 15 to 18 minutes. A) No 4^+ charge peaks are observed when the super base is not added. B) Supercharge reagent m-NBA shifts the charge states of the glyopeptides to 4^+ (see the zoomed in region).

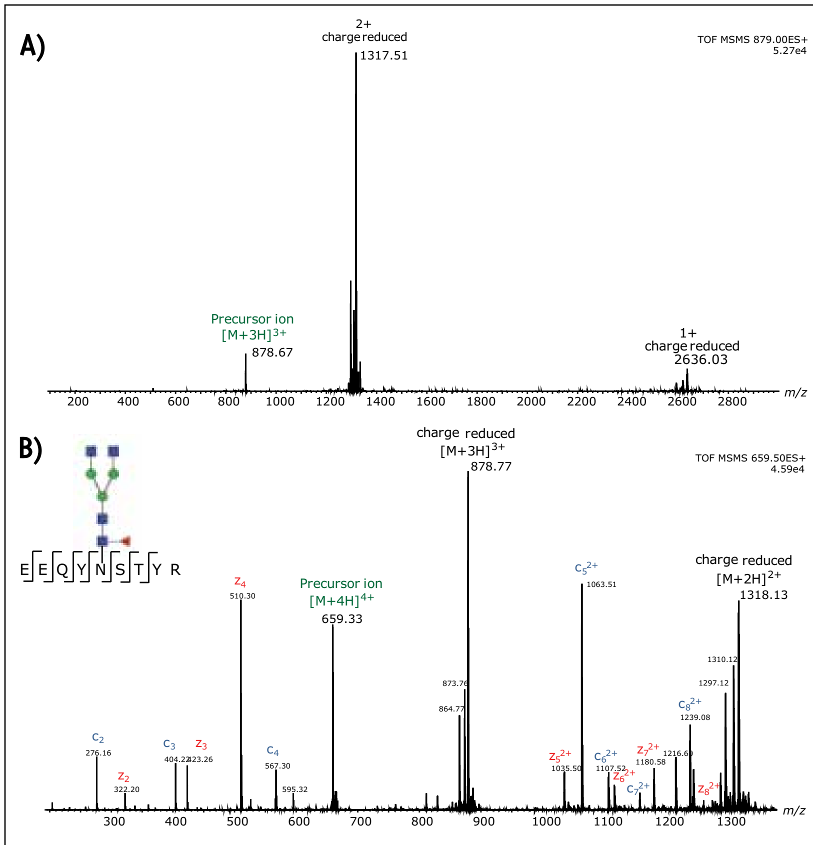

Figure 3. A) No m-NBA added, ETD of 3+ ion of G0F containing peptide at m/z 878.7 shows No sequence-specific ions. Supercharging is a good possibility since ions with higher charges may be better suited to ETD. B) With m-NBA, ETD of 4+ ion is possible, and intense c/z ions are observed.

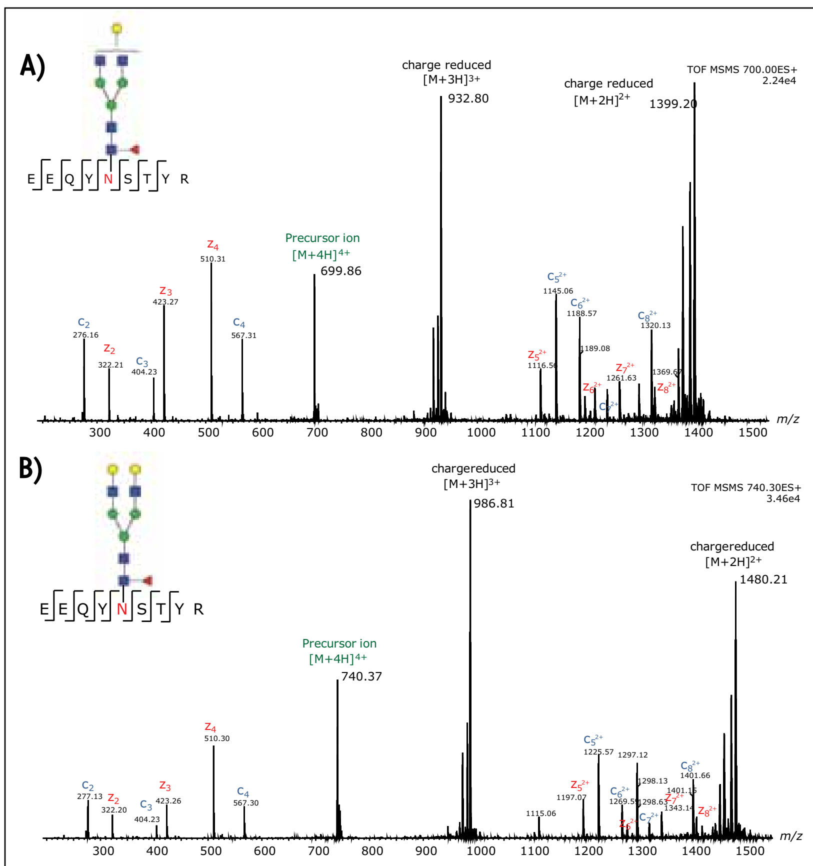

Figure 4. Two additional ETD spectra from 4+ precursors of G1F (A) and G2F (B) containing glycopeptides. Super base m-NBA is critical for generating peptide backbone fragmentations.

CONCLUSIONS

ETD proved to be an indispensable tool for characterizing post translational modifications of biotherapeutic proteins. The data from this application demonstrate that glycopeptide site heterogeneity from a monoclonal antibody, trastuzamab, can be achieved using ETD on a SYNAPT G2-S HDMS System with post-column mixing of *m*-NBA.

- Both *de novo* sequence information, glycan linkage site, and the glycan mass can be confidently assigned from the fragmentation data.
- The combined UPLC/MS^E method, using both CID and ETD, is a powerful tool for protein biotherapeutic characterization.
- In addition, this ETD method can be performed using analytical scale chromatographic setup and analytical scale MS ion source, hence, minimizing the amount of time spent on method optimization.

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

HIGHER ORDER STRUCTURE

Higher Order Structure (HOS) studies are an important part of characterizing biotherapeutic products. These studies go beyond primary structure verification to provide information on protein folding, protein interactions, and aggregation. HOS tools have become indispensable for biotech companies, particularly in the light of recent U.S. FDA biosimilar guidelines that outline the need for HOS characterization as part of complete characterization or comparability submission.

COMPREHENSIVELY DESIGNED FOR PERFORMANCE

The study of HOS has become increasingly accessible through advancements in analytical technologies in high-resolution size exclusion chromatography (SEC), ion mobility separation (IMS), disulfide bond mapping, and hydrogen deuterium exchange with mass spectrometry (HDX MS, H/D exchange, HDX, or HXMS). Waters' application-focused and customer-driven innovations in UPLC® Technology, mass spectrometry, and informatics are enabling more researchers to take advantage of powerful HOS studies.

AGGREGATION AND PROTEIN-CLIP ANALYSIS

Higher resolution UPLC size exclusion chromatography (SEC) techniques based on sub-2-micron-particle column technology now enable laboratories to provide size-based separations with robustness and reproducibility previously not achievable on HPLC systems. The identification and quantification of low levels of aggregated and clipped protein variants are now routine on columns that have lifetimes and batch-to-batch reproducibility far surpassing HPLC technology.

ION MOBILITY MASS SPECTROMETRY (IMS)

IMS has opened up new doors to biotherapeutic analysis by providing orthogonal gas-phase molecule separations based on charge and cross-sectional area within the mass spectrometer. The ability to separate by size and shape has allowed greater discrimination of complex mixtures and resolution of components of common mass-to-charge ratios. IMS has shown application to glycan structural determination, improved peptide assignment within rapid peptide maps, and for the assessment of protein folding and aggregation. The value of IMS analysis has been established by hundreds of peer-reviewed articles that are testament to the utility of the technique for biotherapeutic protein and vaccine studies.

PEPTIDE MAPPING AND DISULFIDE BOND MAPPING

Regulators have defined a standard that the expected pattern of disulfide linkages in a biotherapeutic protein should be confirmed, and that scrambled (or mispaired) disulfide structural variants be absent or well-controlled by the production process. The mapping of disulfide bonds as part of a peptide map has recently become automated by innovative informatics tools where the combinatorial search for scrambled disulfide variants can be managed by using accurate mass precursor and fragmentation data from a non-reduced peptide map.

HYDROGEN DEUTERIUM EXCHANGE MASS SPECTROMETRY (HDX/MS)

Recent innovations in LC/MS, automation, and informatics have evolved HDX/MS from a complex academic exercise to a robust tool for biotherapeutic development. Waters launched the first commercialized HDX/MS system that was designed through an academic/industry partnership. This HDX/MS system transformed a highly manual process requiring highly skilled specialists, into an integrated and automated system solution accessible to those with routine knowledge of LC/MS operations. This system leverages UPLC separations, application specific cooled fluidics, and high-resolution MS^E mass spectrometry to answer the most common questions about biotherapeutic protein dynamics, conformation, and interactions. The value of the system for epitope mapping, protein-drug binding, protein-protein interactions, aggregation studies, the effects of mutation on protein conformation and dynamics, and the localization of conformational changes as a result of formulation changes has been demonstrated by our customers in peer-reviewed literature and regulatory filings.

HDX/MS DATA ANALYSIS SOFTWARE

A major bottleneck for scientists tasked with HDX/MS studies has been removed with the introduction of application-specific informatics, DynamX,™ which processes, displays, and reports peptide deuterium uptake from even the most complex HDX studies. Innovative visualization tools enable rapid communication of structural findings to non-specialists. Typical data processing times are reduced from weeks or even months of manual processing to just hours of automated data processing and user verification of results. Ion mobility separations (IMS) used within an HDX/MS workflow enable resolution of interfering ion distributions and improved quantization and coverage for the increasingly complex molecules and protein interactions now accessible to study by hydrogen deuterium exchange.

Waters nanoACQUITY HDX.

Hydrogen Deuterium Exchange MS (HDX MS) for Calmodulin Protein Conformation

GOAL

To determine conformational changes between apo- and holo-calmodulin using hydrogen deuterium exchange combined with high resolution MS.

BACKGROUND

The correct protein conformation is essential for biological function. A large number of highly diverse diseases are caused by inherited or acquired modifications in protein structure. Protein conformation is a major concern in the biopharmaceutical industry where it is important to assure lot-to-lot reproducibility of a protein product or its integrity following storage. Changes in protein conformation are also of interest to pharmaceutical researchers who study the effect of an active pharmaceutical ingredient (API) on its target protein.

The hydrogen deuterium exchange (HDX) MS analysis is used to measure changes in protein conformation. When HDX is combined with UPLC® separations and high-resolution MS, it's possible to discover subtle changes in conformation. Well established techniques, such as x-ray crystallography and NMR, determine protein structure. However, these technologies require substantial amounts and are challenged by higher molecular weight and dynamic proteins. The UPLC/MS approach described here measures global or local changes in protein conformation using very small amounts of wide-ranging proteins.

THE SOLUTION

A typical workflow of an HDX reaction involves incubating a protein in solution with deuterium oxide (D_2O) where hydrogen atoms on the protein backbone exchange with the deuterium in solution. The more accessible portions of the protein exchange rapidly compared to the less exposed parts which may be buried in the center of a protein or involved in hydrogen bonding. When the exchange reaction is complete, the pH is decreased to 2.5 to quench the reaction.

After pH reduction, the protein may be analyzed intact for global analysis, or may be exposed to an acidic protease, such as pepsin, to digest the protein for local analysis. Another important requirement is to perform highly resolving separations rapidly at 0 °C. Operating at 0 °C minimizes deuterium losses due to back-exchange with the chromatographic eluents.

To meet these analytical challenges, the nanoACQUITY UPLC® System with HDX Technology reproducibly performs peptic digestion and UPLC separation before MS analysis. The nanoACQUITY UPLC System with HDX Technology consists of an ACQUITY UPLC Binary Solvent Manager, Auxiliary Solvent Manager, and HDX Manager. The HDX Manager is capable of online pepsin digestion, online desalting, and fast, highly resolving chromatographic separations at 0 °C. A Xevo® QTof MS with ProteinLynx Global SERVER™ 2.4 (PLGS) Software provides secure identification of peptic peptides.

To test this technology, conformation and dynamics of calmodulin (CaM) upon the calcium binding was studied. CaM is a Ca^{+2} receptor that regulates biological activities of many target proteins. Once Ca^{+2} binds to CaM, several intracellular processes are activated. CaM was incubated with Ca^{+2} and exposed to D_2O to monitor changes in its conformation.

The nanoACQUITY UPLC System with HDX Technology was interfaced with a Xevo QTof MS via a standard electrospray source for these experiments. CaM was solubilzed in physiological buffer and incubated in the absence (control) and presence

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of Ca^{+2} in deuterium labeling solution. Aliquots were taken at timed intervals from the control and Ca^{+2} labeled CaM solutions. In each case, the sample was reduced from pH 7.0 to pH 2.5 and immediately placed into 0 °C in the HDX Manager for peptic digestion and resolution by UPLC.

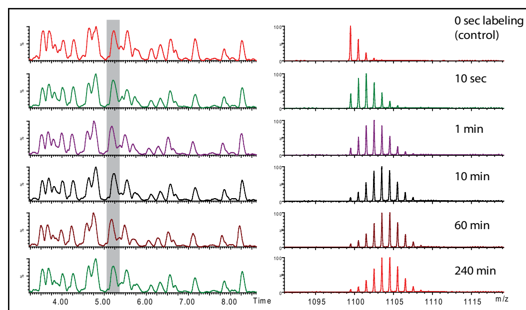

Figure 1. Reproducible chromatographic separations of calmodulin calmodulin peptides following online pepsin digestion. The peptides derived from online digestion were resolved on an ACQUITY BEH (1 mm x 10 cm) column at 0 °C in the HDX Manager. The typical separation profile is a linear gradient from 97% A to 60 % A at 40 µL/min, where A= water/ 0.1% formic acid and B= acetonitrile/ 0.1% formic acid. The left-side panel shows the elution profile of the calmodulin peptic digest at times: 0, 10 s, 1 min, 10 min, 60 min, and 240 min following exposure to D_2O. The panel on the right shows the resultant Xevo QTof spectra from the peptides at the same time points. Notice the increase in m/z of the isotopic distribution as a result of longer exposure to D_2O.

The nanoACQUITY UPLC System with HDX Technology.

These data indicate that there was an increase in deuterium uptake into the calmodulin molecule as a function of exposure time to calcium and the conversion of apo-calmodulin to holo-calmodulin. This is represented in the binding diagrams below.

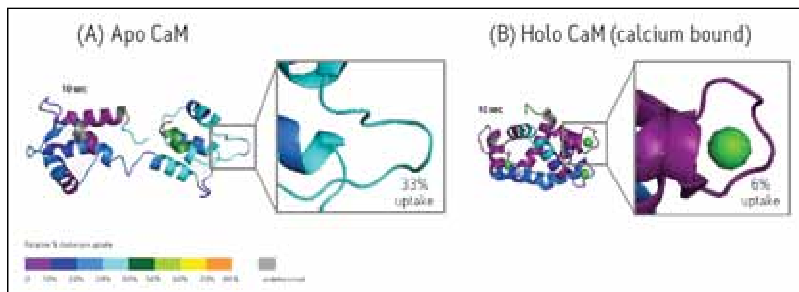

Figure 2. Crystal structure of CaM color coded to represent deuterium uptake. (A) Apo CaM and (B) holo CaM at 10 s deuterium labeling were compared. A significant difference in deuterium uptake was observed in the highlighted region where the conformation has been changed due to the calcium binding.

CONCLUSION

The nanoACQUITY UPLC System with HDX Technology when combined with high resolution MS can effectively be used to determine small changes in protein conformation. This innovative approach to structural conformation analyses can augment data obtained from x-ray crystallography and NMR by providing in-depth knowledge about minor, local changes in protein structure solution that may be used to study function.

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[APPLICATION NOTE]

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Conformational Characterization of Calmodulin by Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS)

Joomi Ahn, Martha Stapels, Michael Eggertson, Keith Fadgen, and Ying Qing Yu
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

To determine changes in conformation between apo- and holo-calmodulin using hydrogen deuterium exchange combined with high resolution MS.

WATERS SOLUTIONS

nanoACQUITY UPLC® with HDX Technology

Xevo® QTof MS

ProteinLynx Global SERVER™ Software

KEY WORDS

Hydrogen Deuterium Exchange Mass Spectrometry, HDX Technology, protein conformation

INTRODUCTION

Correct protein conformation is essential for proper biological function of protein therapeutics. Changes in protein conformation are a major concern in the biopharmaceutical industry and conformational characterization is a difficult task. Many analytical tools such as circular dichroism (CD), differential scanning calorimetry (DSC), and analytical ultracentrifugation (AUC) are used to study the higher-order structure of proteins. These methods sample global conformation and cannot determine where conformational changes occur. Nuclear magnetic resonance (NMR) and X-ray crystallography determine protein structure with high spatial resolution, but both technologies require substantial amounts of sample and are not routinely applied to biopharmaceutical products due to the significant time and effort required.

Hydrogen deuterium exchange (HDX or HX MS) mass spectrometry has proven to be a useful analytical method for the study of protein dynamics and changes to protein conformation.[1] Successful HDX studies require an LC/MS system that can perform rapid chromatographic separations at 0 °C and make accurate mass measurements of small quantities of deuterium labeled proteins and peptides.[2]

When an HDX experiment is performed with UPLC® separation technology and high-resolution mass spectrometry, subtle conformational changes can be revealed and the locations of these changes are determined at the peptide level. Such innovative technology with a commercially available system solution makes conformational analyses a more practical method. Waters HDX Technology has been adopted as an

Figure 1. nanoACQUITY UPLC System with HDX Technology. HDX Manager, Binary Solvent Manager (BSM), and Auxiliary Solvent Manager (ASM) are shown from top to bottom, respectively. The HDX Manager is a key component, which can perform the online pepsin digestion and maintain the low temperature at 0 °C required for HDX MS.

EXPERIMENTAL

Protein preparation:

Bovine calmodulin purchased from Sigma

Protein buffer solution:	20 mM HEPES in 100% H_2O pH 7.40 with and without 50 µM calcium[3]
D_2O labeling solution:	20 mM HEPES in 99.99% D_2O at pD 7.40 with and without calcium[3]
Quenching solution:	33 mM HCl 100 % H_2O pH 2.50

Method

LC conditions:

LC system:	Waters nanoACQUITY UPLC with HDX Technology including HDX Manager
Columns:	ACQUITY UPLC BEH C_{18} 1.7-µm 1.0 x 100 mm for peptides ACQUITY UPLC BEH C_4 1.7-µm 2.1 x 50 mm for intact proteins
Trapping column:	ACQUITY VanGuard® Pre-Column, BEH C_{18}, 2.1 x 5 mm
Desalting column:	MassPREP™ Desalting Column, 2.1 x 5 mm
Column temp.:	0 °C
Online digestion column:	Immobilized pepsin column 2.1 x 30 mm from Applied Biosystems
Analytical flow rate:	40 µL/min
Mobile phase A:	0.1 % formic acid in water, pH 2.1
Mobile phase B:	0.1 % formic acid in acetonitrile, pH 2.1
Gradient:	3 to 40 % B in 7 min
Auxiliary mobile phase A:	0.05 % formic acid in water pH 2.50 for digestion flow
Trapping condition:	3 min at 100 µL/min

MS conditions:

MS system:	Waters Xevo QTof MS
Ionization mode:	ESI +
Capillary voltage:	3.0 kV
Collision energy:	6 V
Sampling Cone voltage:	35 V
Source temp.:	80 °C
Desolvation temp.:	175 °C
Lock Mass:	Glufibrinopeptide, 100 fmol/µL in 50% acetonitrile, 0.1% formic acid at 5 µL/min

Data management:

MassLynx 4.1 Software, ProteinLynx Global SERVER (PLGS) Software

effective analytical tool for biotech researchers who are studying higher-order structure of protein drugs. Characterizing higher-order structure is one of the key factors in drug discovery and early development phases and adopting this technology will offer a distinct advantage in understanding the safety and efficacy of biopharmaceuticals.

In this application note, we introduce the nanoACQUITY UPLC System with HDX Technology (Figure 1). This system consists of a nanoACQUITY UPLC Binary Solvent Manager (BSM), Auxiliary Solvent Manger (ASM), and HDX Manager. The HDX Manager is capable of rapid online protein digestion, desalting, and highly resolving chromatographic separations at 0 °C. Operating at 0 °C is required to reduce the deuterium loss during analyses. With this system, subtle differences in deuterium uptake can be adequately determined with very reproducible HDX conditions. A Xevo QTof MS, utilizing MS^E with ProteinLynx Global SERVER (PLGS) Software, provides an ideal tool for accurate mass analysis and reliable peptic peptide assignment.

To demonstrate this technology, the conformational changes in bovine calmodulin (CaM) were studied. Calmodulin is an important intracellular calcium receptor and it is known that it undergoes a conformational change upon calcium binding.[3] This change regulates the biological activities of target proteins. Here we describe the HDX workflows using the nanoACQUITY UPLC System with HDX Technology at both whole protein and peptide levels to show how the conformational study is performed.

RESULTS AND DISCUSSION

Sample

Holo and apo bovine calmodulin were prepared with and without calcium, respectively, in protein buffer solution. Both proteins were labeled with 20-fold dilution with D_2O labeling solution and incubated for 10 seconds, 1, 10, 60, and 240 minutes at room temperature. The labeling reaction was quenched by adding quenching solution to reduce the pH to 2.5. The quenched samples were immediately placed at 0 °C.

Global HDX analysis for intact protein

Global HDX analysis provides information about overall conformational changes by comparing the deuterium uptake of control vs. analyte proteins. The intact HDX workflow is illustrated in Figure 2. First, the protein is labeled with deuterium and incubated for selected amounts of time between 10 seconds and 4 hours.

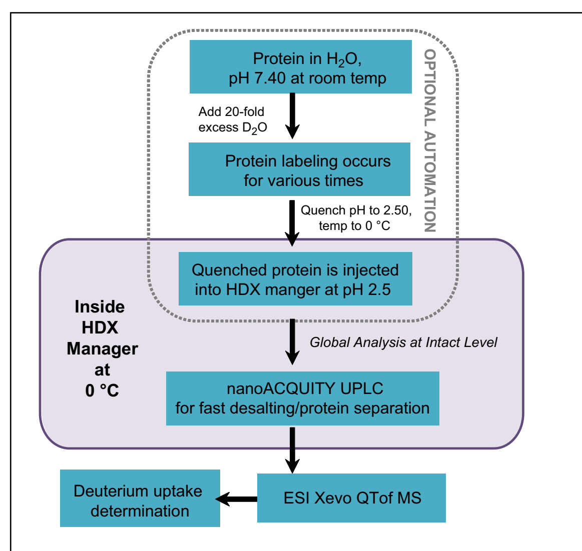

Figure 2. Global HDX workflow for intact protein analyses. The labeled protein is quenched to pH 2.50, and directly injected into the HDX manager, where online desalting and separation is performed at 0 °C.

During the exchange reaction in solution, amide hydrogens on the protein backbone exchange at a rate that depends on both solvent accessibility and hydrogen bonding. The deuterium uptake as a function of time will be different between control and analyte if there is a difference in higher-order structure. The exchange reaction is quenched with cold acidic (pH 2.50) buffer and then quickly injected into the HDX Manager. The temperature inside the HDX Manager is set at 0 °C to minimize back-exchange. Back-exchange must be controlled because deuterium can exchange back to hydrogen in the 100 % H_2O LC solvents. Therefore, low temperature, acidic pH, and a rapid run time are needed to minimize back-exchange. The HDX Manager in conjunction with the nanoACQUITY UPLC System is designed to satisfy specific HDX requirements such as fast 0 °C operation.

The Waters HDX System can be fully automated for sample preparation (Figure 2, highlighted in dashed line). The laborious preparation steps such as labeling, time measurement, quenching, and injection are handled by robotics. This system configuration saves a significant amount of time for laboratories performing high-throughput analyses.

Apo-calmodulin is known to be an open structure without bound calcium (Figure 3, left top panel). When calcium ions are bound to calmodulin (holo-), the protein adopts a more compact conformation (Figure 3, left bottom). The deuterium uptake curves display the relative deuterium level and compare the difference of apo and holo at each labeling time point (Figure 3, right panel). After structural conversion from apo to holo, a lower amount of deuteration was observed in holo calmodulin, indicating that fewer amide hydrogens were protected from exchange compared to the apo state.

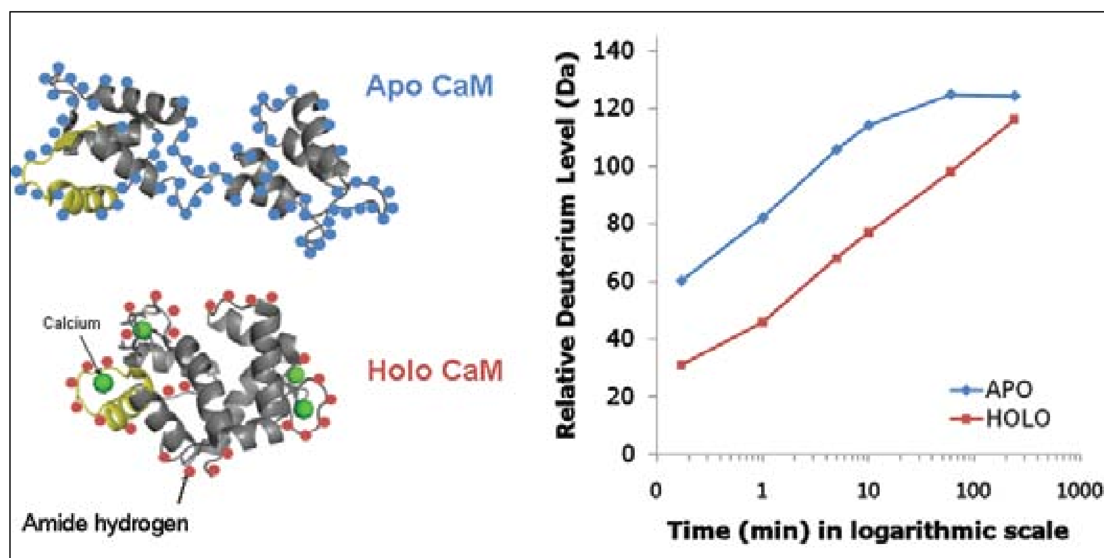

Figure 3. Deuterium uptake curves for intact apo and holo calmodulin with corresponding tertiary structures known from crystallography or NMR. The blue and red dots represent amide hydrogens that are available to exchange with deuterium in apo and holo, respectively. Lower deuterium uptake was observed in holo calmodulin due to its closed conformation.

Local HDX analysis for peptides

To locate where deuterium has been incorporated in a protein, the labeled and quenched protein is digested into peptides with an acidic protease (pepsin). Pepsin digestion of an undeuterated sample is performed to create a peptide map. In this peptide workflow (Figure 4), the HDX System performs an efficient pepsin online digestion and separates the peptides via UPLC with MS spectra collected in MS^E mode from the Xevo QTof MS. Finally, ProteinLynx Global SERVER (PLGS) Software processes the MS^E data to identify the peptides generated by pepsin. This UPLC/MS^E analysis offers several unique advantages compared to typical HPLC/MS.

- Robust online pepsin digestion
- Rapid peptide separation at 0 °C in less than 5 to 6 minutes
- UPLC resolution to be able to separate complex peptide digest of large proteins
- Independent temperature control for digestion and separation
- MS^E and PLGS for reliable peptic peptide ID

Linear sequence coverage of 94% was observed in this study. Pepsin is active at low pH and maintains activity at low temperatures, which are ideal for HDX quench conditions. Pepsin also generates many overlapping peptides and short peptides helpful for localizing deuterium incorporation. The reproducible peptides of calmodulin by online pepsin digestion were used to construct a peptide map. These peptides shown in Figure 5 were found in all digestions in apo and holo samples. Identifying reproducible peptides is necessary because more robust analysis is possible when the deuterium uptake of the same peptide is compared in deuterated apo and holo samples.

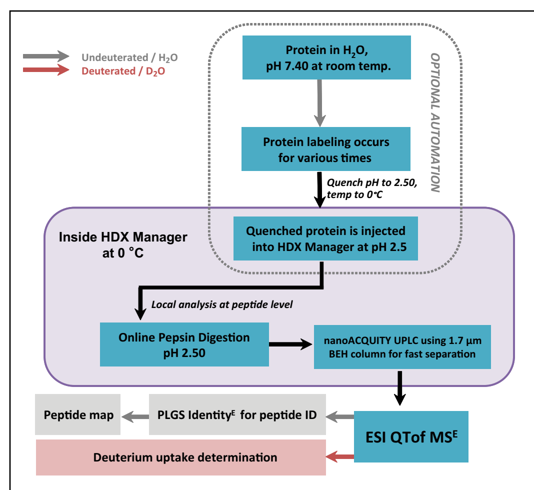

Figure 4. Peptide ID workflow. The peptide ID step is in gray and the HDX experiment is in red. The peptide ID step is needed to identify what peptides are produced during online digestion and to construct the coverage map.

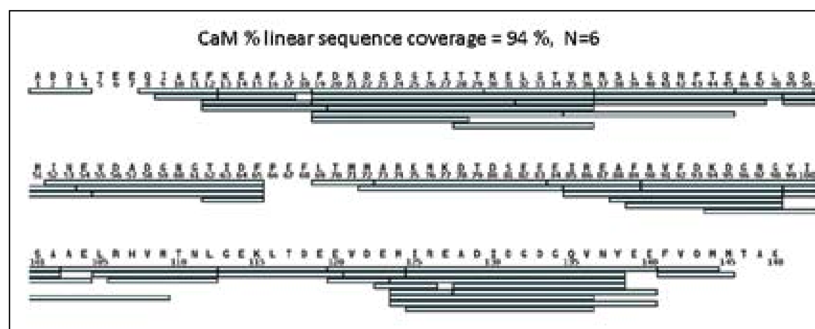

Figure 5. Calmodulin online pepsin digestion coverage map. Each bar under the sequence represents an identified peptide. These peptides were reproducibly observed (N=6).

The deuterated protein was digested and separated at 0 °C in same way as undeuterated protein (Figure 4, red arrows). Reproducible chromatographic separations were obtained throughout the labeling time-course (Figure 6, left panel). MS spectra showed the expected mass increase in m/z as a result of longer exposure to D2O (Figure 6, right panel). The intensity weighted average mass of each isotopic envelope was found and compared to that of the undeuterated protein.

Peptide HDX data interpretation

Deuterium uptake into each peptic peptide was compared for apo and holo calmodulin. These data indicate that there was a change in deuterium uptake for certain peptides from apo to holo form. An example is shown in Figure 7. The highlighted yellow arrows point out the different deuterium uptake in one peptide after 10 minutes of labeling.

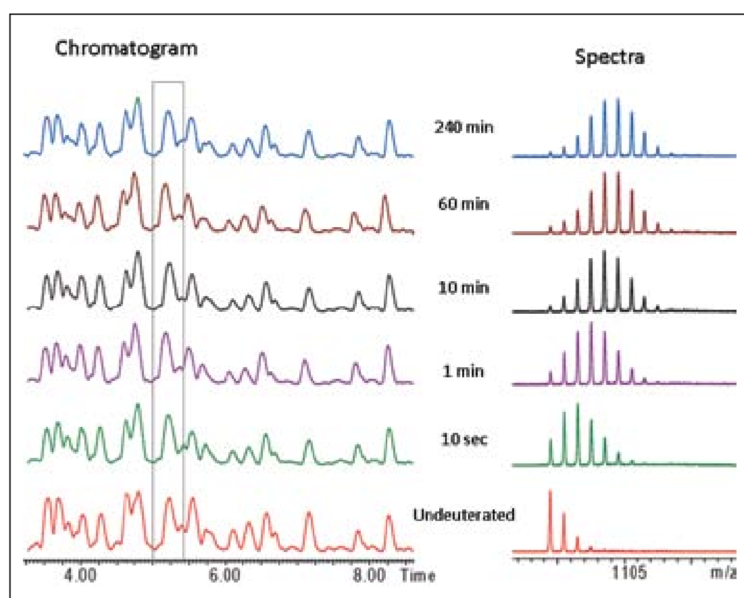

Figure 6. Reproducible chromatographic separations of calmodulin peptides following online pepsin digestion (left panel). The right panel shows the spectra of peptide (ADIDGDGQVNY) labeled from 10 seconds to 240 minutes. The m/z increases as a result of longer exposure to D_2O.

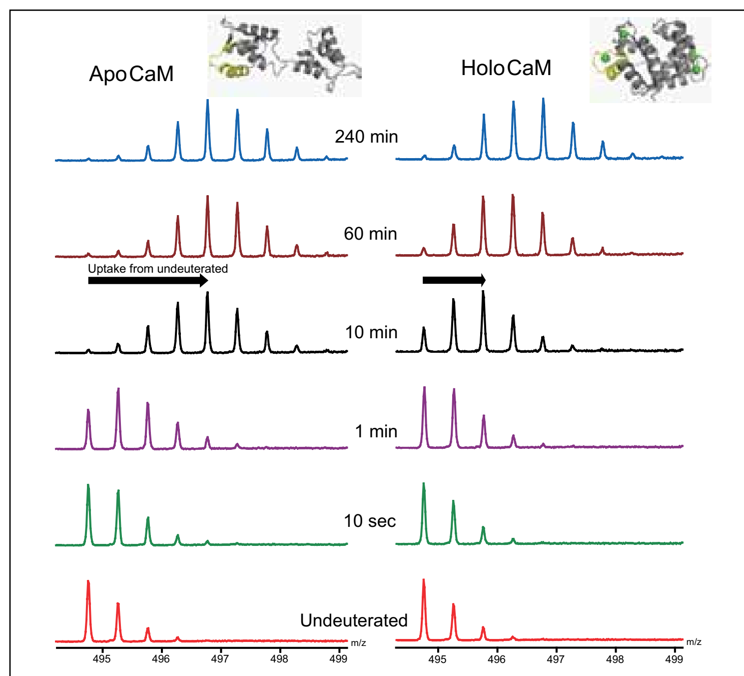

Figure 7. Different deuterium uptake of the same peptide found in Apo and Holo CaM. At 10-minute labeling, this peptide (FKEAFSLF, 12-19) showed a higher deuterium uptake in Apo (left panel) than in Holo (right panel).

Peptide HDX analysis typically generates hundreds of peptic peptides depending on the size of protein, therefore manually determining the deuterium uptake of each peptide is a time-consuming process. With Waters software, data interpretation is automated, and the data processing time is significantly reduced.

The measured deuterium incorporation can be plotted as deuterium uptake curves for each peptide (Figure 8). These charts show where the difference /no differences in deuterium uptake occur for all peptides. Figure 9 is one of the representations of HDX results. The relative percent deuterium uptake was color coded on 3D structure, thus overall dynamics of apo and holo calmodulin can be easily compared over time. Three dimensional (3D) structures confirmed that those peptides with significant differences in deuterium uptake were located primarily the calcium binding region.

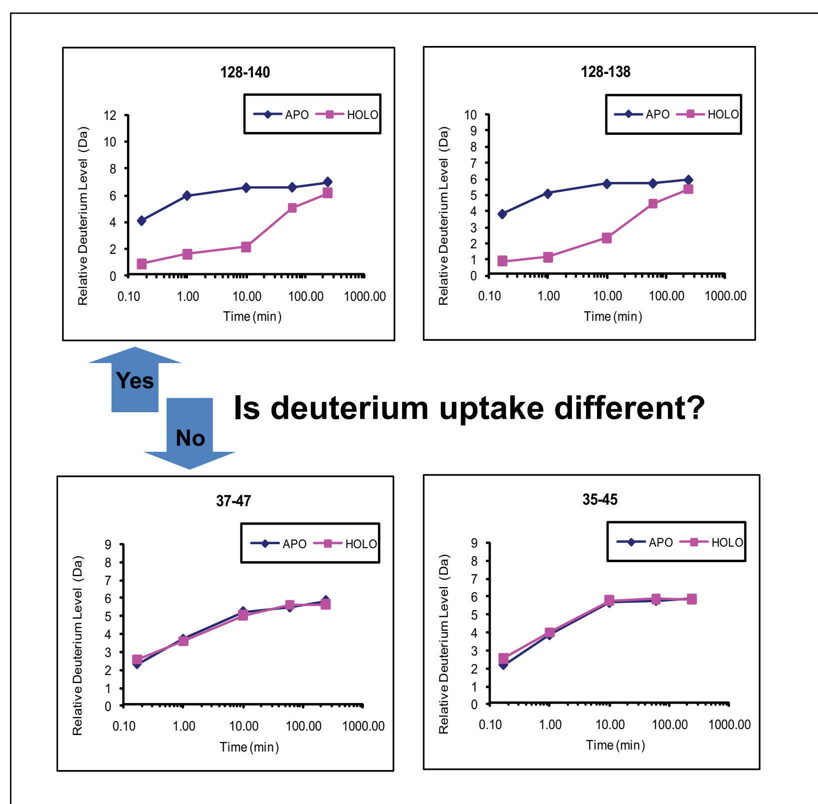

Figure 8. The Example of deuterium uptake curves for apo and holo calmodulin. Upper curves (overlapping peptides covering 128-140 and 128-138) illustrate that there was distinct change in deuterium uptake between apo and holo calmodulin. The lower curves (peptides 37-47 and 35-45) showed no change in deuterium uptake between apo and holo calmodulin. Every peptide represented in Figure 5 has its own uptake curve.

For example, the highlighted box on the left panel in Figure 9 indicated 32% deuterium uptake for apo calmodulin whereas the same peptide region in holo form only showed 8% deuterium uptake at 10 seconds of labeling time. Figure 10 is another example of data representation with corresponding 3D structures. This HDX heat map overlays the deuteration on the sequence. 3D structures obtained from X-ray crystallography or NMR are complementary to the HDX results if available.

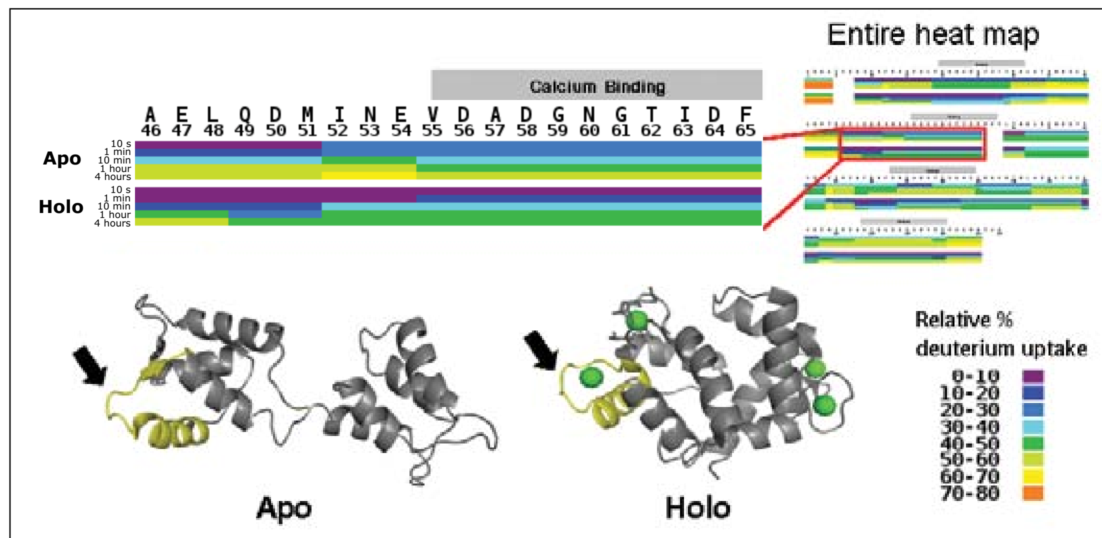

Figure 9. HDX heat map display for assessing conformational difference in localized region (46 - 65). Two sets of colored bands of the heat map represent the apo (top band) and holo (bottom band) calmodulin of % deuterium uptake. Cooler color represents less exchange, thus greater protection from exchange. The 3D structures with black arrows are the corresponding regions of the displayed heat map (46-65). Represented holo region shows much cooler color in mostly purple and dark blue in early time points (for example at 10 seconds) than in apo calmodulin.

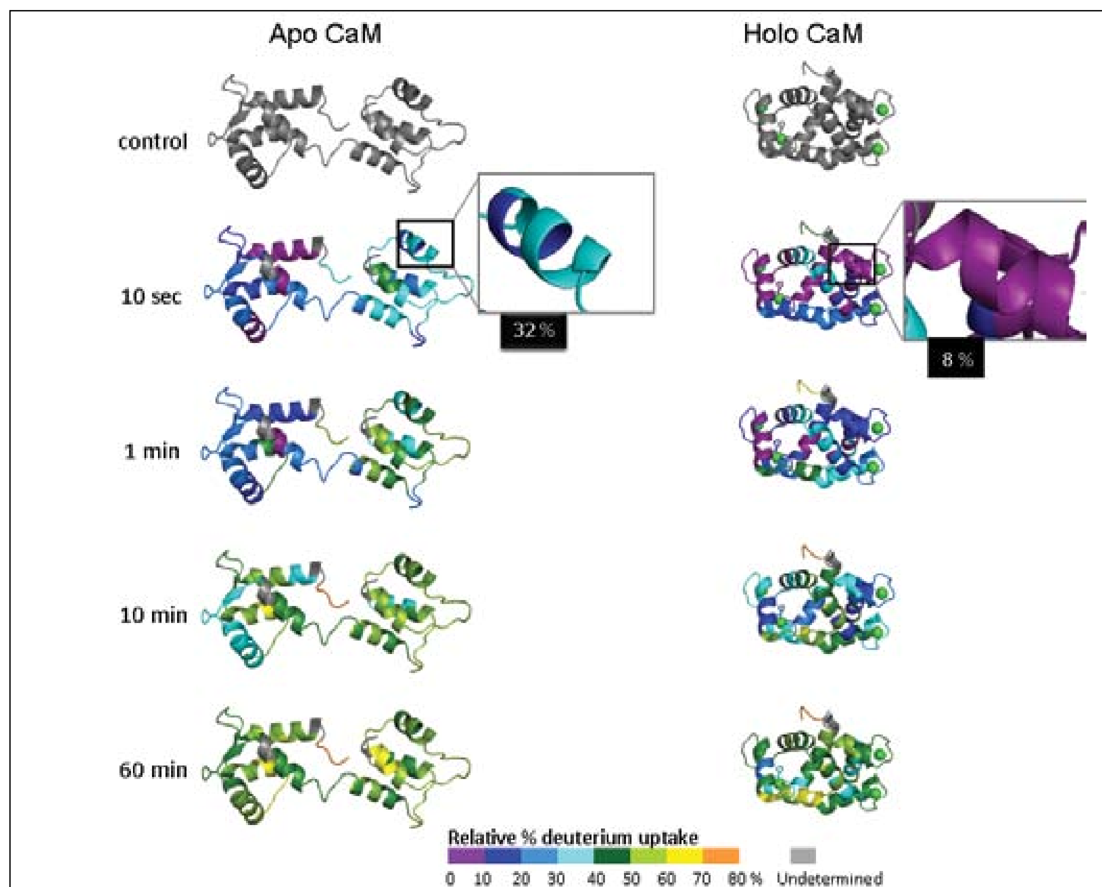

Figure 10. Deuterium uptake information represented on calmodulin apo and holo 3D structures (apo = 1CFD.pdb, holo = 1PRW.pdb). At 10 seconds labeling, two regions of calmodulin were compared in highlighted boxes. Overall, the apo structure shows more rapid exchange due to greater protein flexibility without calcium binding.

CONCLUSIONS

The Waters nanoACQUITY UPLC System with HDX Technology, when combined with high-resolution MS, was effectively used to determine changes in calmodulin conformation upon calcium binding. This system provides unique features that improve the quality of HDX analysis.

- HDX workflow for global and local HDX analyses.
- Robust online pepsin digestion
- Rapid reproducible UPLC separation at 0 °C
- Accurate identification of peptic peptides by MS^E and PLGS
- Automated HDX data processing

Waters HDX Technology facilitates the study of protein conformation and protein dynamics at global and local levels. The information obtained helps researchers understand protein function and related biological activity. The HDX System is the informative analytical tool for biopharmaceutical drug discovery and development.

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Solutions in practice

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

[TECHNOLOGY BRIEF]

Localized Conformation Analysis of Mutated Human IgG1 by HDX MS and nanoDSC

GOAL

Probing mAb conformation change caused by amino acid mutation using both Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS) and nano Differential Scanning Calorimetry (DSC) technologies.

BACKGROUND

Conformational changes in biotherapeutics may impact their safety and efficacy. Therefore it is important to regulators and manufacturers to understand what affects conformational changes. Guidelines also stipulate that manufacturers must produce a well-characterized biotherapeutic product (WCBP), including knowledge of higher order structure (HOS). The analytical tools described here help organizations more effectively discover and develop biotherapeutics in order to maintain their competitiveness.

A human immunoglobulin G (IgG) was developed with a site-specific mutation. When the higher order structures of non-mutated and mutated IgGs were compared using alternative methods, the localized conformational changes at the substitution sites were not easily detected. Two techniques were used in this study: nano Differential Scanning Calorimetry (DSC), and hydrogen deuterium exchange with mass spectrometry. Both techniques contribute to the understanding of the higher order structure of biomolecules and biotherapeutics.

HDX MS provides insights in days that may be challenging to achieve in months with other techniques.

The Nano DSC instrument from TA Instruments can detect changes in heat content down to the nanowatt range from 2 micrograms of protein. The heat signal on a DSC is used as a measure of the folding or unfolding process and can therefore provide insights into the overall structure. DSC is useful in providing rapid and robust measurements with little sample preparation.

HDX MS can be used to locate the region where the conformational change has taken place. HDX MS experiments provide extremely high detail and leverage the power of UPLC®/QTof-MS^E to maximize coverage of a protein. Here, a nanoACQUITY UPLC® System with HDX Technology was used along with a Xevo® G2 QTof MS. The local HDX workflow demonstrates the ability to detect differences in deuterium uptake at the peptide level. The differences in deuterium uptake between different conformations allowed us to locate precisely where the mutated and non-mutated forms differed.

HDX data processing used to be tedious and time-consuming when performed manually. DynamX™ software was developed by Waters to automate data processing and provide efficiency that had not previously existed. DynamX automatically calculates deuterium uptake. Results are displayed in convenient comparative views: uptake curves, butterfly charts, and difference plots.[1] The processing time is thereby reduced from months of specialist interpretation to a day of reporting and curation. We report efficient HDX studies of a control and of two batches of mutated IgG1.

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THE SOLUTION

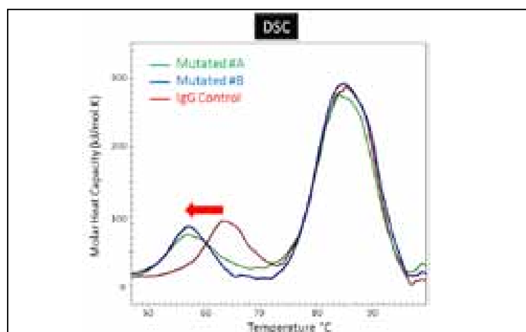

Figure 1. Background corrected DSC scan data of two mutated batches (green and blue traces) and control (red trace). Both mutated batches, #A and #B, had same amino acid mutation. The major difference is the low temperature transition indicating that the difference between the mutated samples and the control sample only affects a specific domain of the IgG. The shift to lower temperature (red arrow) of a portion of the trace indicates that the mutation has decreased the stability of this responding region of the IgG structure, i.e., that less energy is required to make it fold or unfold because the unfolding temperature is directly related to the free energy of the protein.

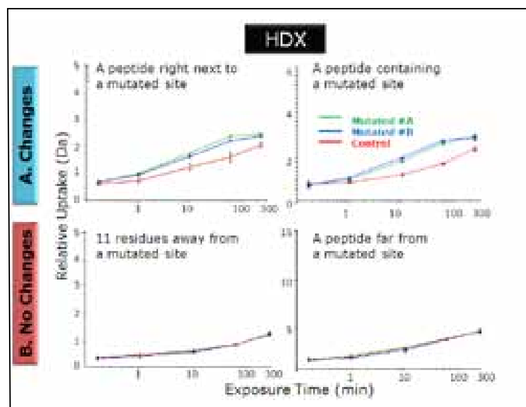

Figure 2. The deuterium uptake curves from the HDX MS study with (top two panels) and without changes (bottom two panels) comparing the control to the mutated samples for four selected peptides. The key finding in this figure is that HDX MS has located at the peptide level where the conformational changes have taken places in the mutant species. Each panel shows the corresponding peptides from the Control, Mutant A, and Mutant B batches. The deuterium uptakes of the peptides were measured at exposure times 0.17, 1, 10, 60, 240 min. (A) The top two panels show that the peptides from the mutated samples had faster exchange rates compared to the ones from the control. These peptides were found nearby or at the mutated site. (B) Conversely, two other peptides located far from the mutated site showed the same deuteration uptake as the control. This indicated that there were no conformational changes at those locations. In both the heavy and light chains only the mutation sites showed changes.

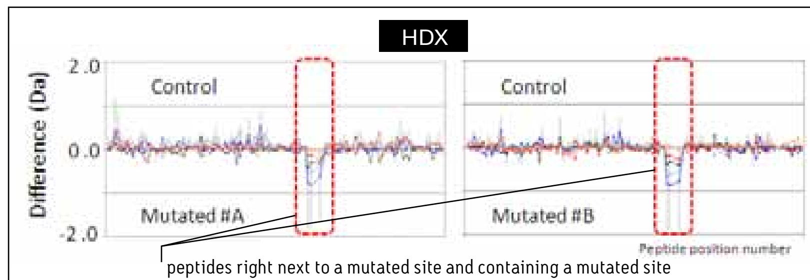

Figure 3. Comparison of deuterium difference between peptides from the IgG heavy chains. DynamX "Difference" charts comparing the mutated IgG #A and mutated IgG #B to the control in the left and right panels, respectively. This visualization helps to locate which peptides contain different uptakes in a comparative view. The red boxes highlight the peptides near and at a mutated site, showing where the largest changes were detected. Each data-point indicates the difference in uptake between the control and a mutated batch. The vertical bar represents the sum of the uptake differences across the time-points; the longer the vertical bar, the larger difference found between the control and mutated IgGs. The values of vertical bars in red boxes were significantly different compared to the other regions, indicating this region is responsible for the local conformational effect.

SUMMARY

Waters' tools for high order structure of protein allow the determination of conformational changes for all sectors of a biopharmaceutical company. Benchtop scanning calorimetry provides a robust and rapid measure of the change in folding across different temperatures. Rapid comparisons can be made for different states, to be used in discovery or development. HDX MS provides highly detailed information to locate where conformational changes occur.

HDX MS provides insights in days that may be challenging to achieve in months with other techniques. Both tools contribute to efficiency in an industry that requires robust, rigorous, and rapid information. Using these tools will help a pharmaceutical organization manage their pipeline effectively, satisfy regulators, and protect better against competition.

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F: 1 508 872 1990
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[APPLICATION NOTE]

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Method Development for Size Exclusion Chromatography of Monoclonal Antibodies and Higher Order Aggregates

Paula Hong and Kenneth J. Fountain
Waters Corporation, 34 Maple St., Milford, MA, USA

APPLICATION BENEFITS

- Robust analysis of mAb monomer and aggregates
- High throughput SEC separation
- Consistent purity profile
- Reproducible quantitation of higher order aggregates
- Easy SEC method development

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class Bio System
- ACQUITY UPLC BEH200 SEC 1.7 µm Column
- Auto•Blend Plus™ Technology
- Empower™ 2 Software

KEY WORDS

Size exclusion chromatography, UPLC, monoclonal antibody, method development, aggregates

INTRODUCTION

Since the early introduction of biologic based therapeutics, the presence of protein aggregates can compromise safety and efficacy.[1] Given these factors, protein aggregates are typically monitored throughout the production of a biotherapeutic. While a variety of analytical techniques have been used to analyze soluble aggregates, the dominant technique continues to be size exclusion chromatography (SEC).[2]

While SEC has been performed with silica-diol coated columns and HPLC instrumentation, the introduction of UPLC® or low dispersion systems in combination with sub-2 µm particles has allowed for improvements in these isocratic separations, including improved resolution, higher throughput, and sensitivity.[3] However, as in any SEC method, a variety of parameters can be adjusted to improve resolution and method robustness. In the following application, we will investigate the impact of some of these parameters, including mobile-phase composition, flow rate, and column length on a SEC separation. Evaluation of the separation will be based on a variety of criteria such as column calibration, resolution, and aggregate quantitation.

EXPERIMENTAL

Sample Description

The protein standard (BioRad) containing bovine thyroglobulin (5 mg/mL), bovine γ –globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 mg/mL) and Vitamin B12 (0.5 mg/mL) in de-ionized water was analyzed. A murine monoclonal antibody, purified by Protein A affinity chromatography, was analyzed. The sample concentration was 10 mg/mL in 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3. Samples were not controlled for inter-experiment conditions.

Method Conditions

LC Conditions:

System:	ACQUITY UPLC H-Class Bio System with TUV and Titanium flow cell
Wavelength:	214 and 280 nm
Column:	ACQUITY UPLC BEH200 SEC 1.7 µm, 4.6 x 150 mm, part number 186005225
Column Temp.:	30 °C
Sample Temp.:	4 °C
Injection Volume:	2 µL (unless otherwise specified)
Flow Rate:	0.4 mL/min (unless otherwise specified)
Mobile Phase:	Prepared using Auto•Blend Plus technology
Final Composition:	25 mM sodium phosphate, pH 6.8, 200 mM sodium chloride, (unless otherwise specified)

Data Management

Software:	Empower 2

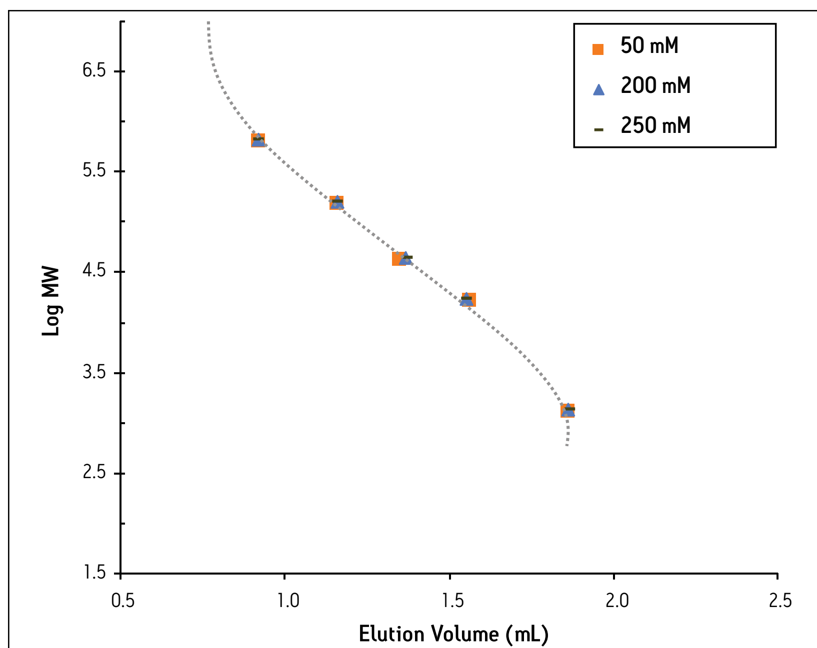
RESULTS AND DISCUSSION

A number of factors need to be evaluated in SEC method development. Ideally SEC separations are based on the size of the proteins in a solution. For this reason, size exclusion chromatography of biomolecules is performed under aqueous, native conditions. However, the presence of mixed mode interactions can obscure size measurements.[4] More specifically, the charged sites on the packing material can interact with the proteins, resulting in an 'ion- exchange' effect. To determine the influence of these effects the mobile-phase conditions of the separation need to be evaluated. However, the conditions of the chromatographic separation can alter the protein structure and state. The concentration and identity of the salt and pH can affect the 3-D structure and the protein-protein interactions. For these reasons, evaluation of a SEC method must incorporate information of the biological activity of the biomolecule.

In the following discussion, we will outline considerations and parameters for developing a SEC method. While the SEC method development steps are illustrated on UP-SEC, the same principles apply to any HP-SEC separation. Methods will be evaluated based on peak shape, resolution, calibration accuracy, and quantitation. Optimization of the mobile-phase ionic strength and pH can easily be accomplished with a quaternary eluent management system in combination with software that can take advantage of this four eluent blending system.[5] This approach was used throughout the studies described.

Mobile-phase Ionic Strength

The ionic strength of the mobile-phase should be adjusted to minimize any secondary interactions between the packing material and proteins. To determine the effect of mobile-phase concentration on the calibration curve, a set of protein standards was analyzed at 50-250 mM sodium chloride. Sodium chloride was selected since it is the most common salt used in SEC separations. The buffer concentration (sodium phosphate) and pH were kept constant at 25 mM and pH 6.8, respectively. Over the concentration tested, the retention times for each protein were within 0.07 minutes with the greatest retention time variability observed for ovalbumin (Figure 1). These results indicate the calibration curves are not sensitive to salt concentration.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 1. Effect of sodium chloride on a SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

In addition to protein standards, the SEC separation of a murine monoclonal antibody (mAb) was evaluated at 50-250 mM sodium chloride (Figure 2). As is commonly observed with gel filtration packing materials,[2] higher ionic-strength mobile phases lead to decreased peak tailing and narrower peaks for the mAb monomer. With increasing sodium chloride concentrations from 50-200 mM, the mAb peak height increases from 0.189 – 0.289. The USP tailing factor also decreases from 1.64 to 1.22. Changes are less pronounced as the ionic strength of the mobile phase is increased from 200 to 250 mM sodium chloride (USP Tailing = 1.20).

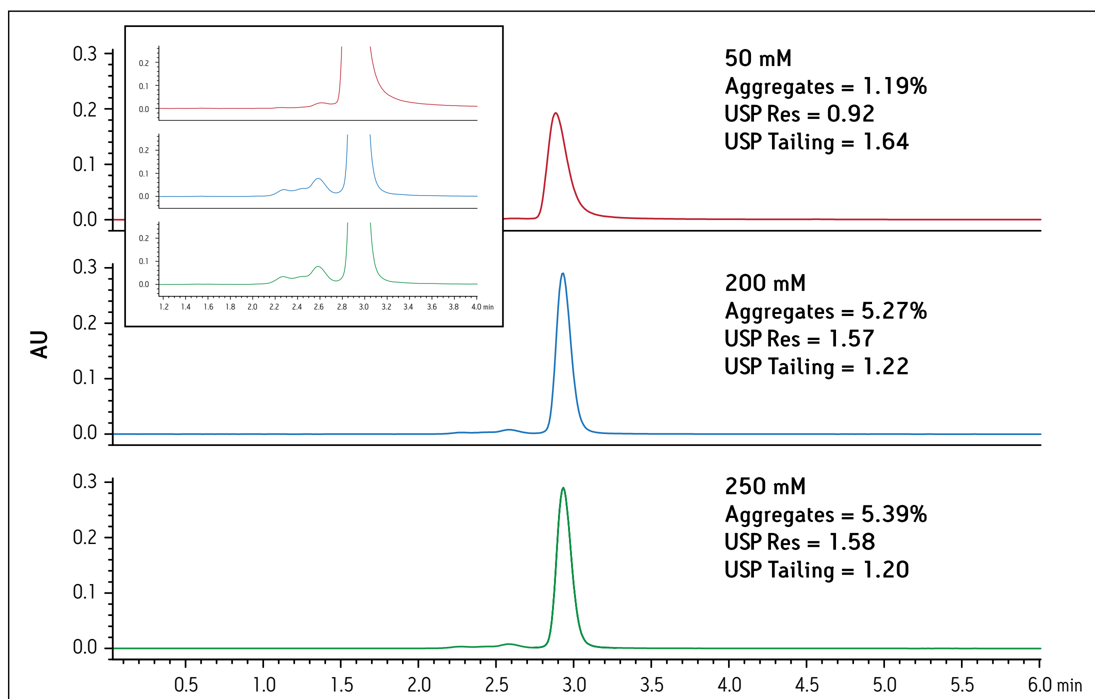


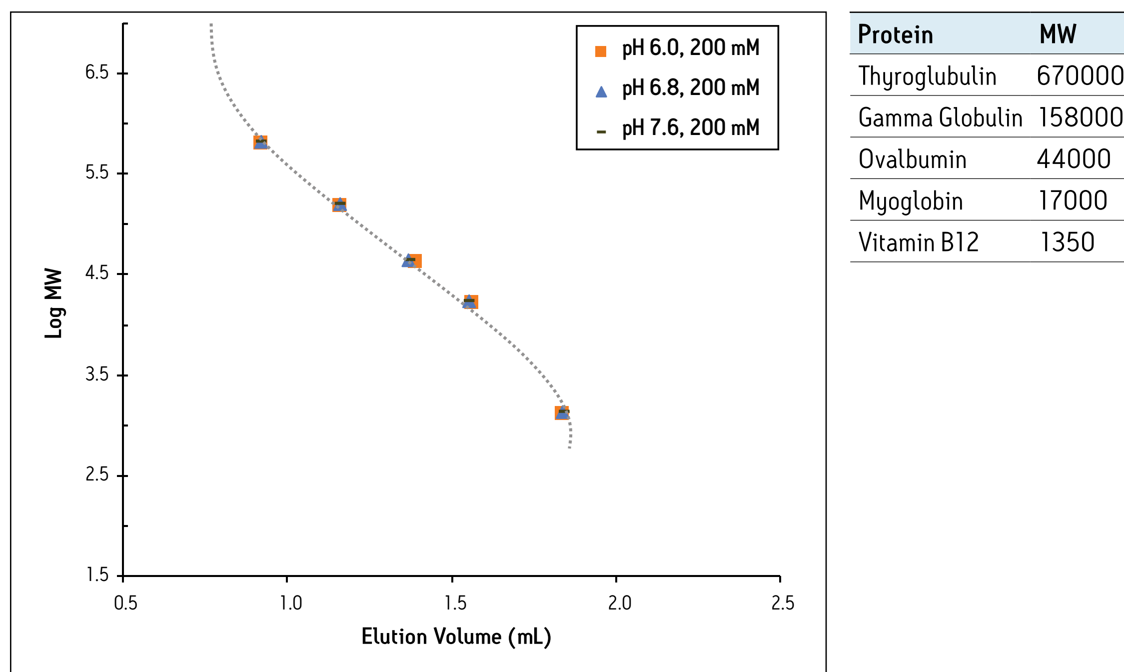
Figure 2. Effect of sodium chloride on the SEC separation of a murine mAb. Inset shows how aggregate quantitation can change with salt concentration due to secondary interactions.

The effect of buffer ionic strength on the observed amount of aggregate was also analyzed. In the experiments previously described, increasing sodium chloride concentrations from 50 -200 mM results in greater observed recovery of aggregates (see inset). The aggregate % area increased from 1.18% to 5.27%. However, at concentrations above 200 mM sodium chloride, aggregate quantitation did not change significantly. This suggests minimal secondary interactions above this concentration.

The variability in retention time and changes in peak shape indicate secondary interactions between the protein and the column packing material, as has been observed for the materials used to prepare SEC packings. These interactions, which can lead to increased retention and irregular peak shape, are easily minimized by increasing the ionic strength of the buffer.

Mobile-phase pH

Given the influence of pH on both secondary interactions and the structure of the protein, SEC method development should also evaluate pH and its influence, if any, on the separation and quantitation of the biomolecule. The BEH200 column was evaluated with the protein standard mix from pH 6.0 – 7.6. This analysis was performed to evaluate the effect of pH on the column calibration. The pH range was based on the buffering capacity range of the sodium phosphate buffer. The sodium chloride concentration was kept constant at 200 mM. The results show no significant shift in retention times were observed for the proteins. All of the retention times were within 0.02 minutes (Figure 3), suggesting pH has no significant affect on calibration under the conditions tested.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 3. Effect of mobile-phase pH on a SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

To test the effect of pH on a typical biotherapeutic, the mAb was analyzed under the same conditions (pH 6.0 to 7.6, 200 mM sodium chloride) (Figure 4). As the pH increases from 6.0 to 7.6, the mAb monomer peak height decreases and shifts to earlier retention time (Figure 4). However, the aggregate quantitation over the pH range from pH 6.0 – 7.6 was within 0.4% (5.7- 5.3%), indicating mobile phase pH has no effect on the measured proportion.

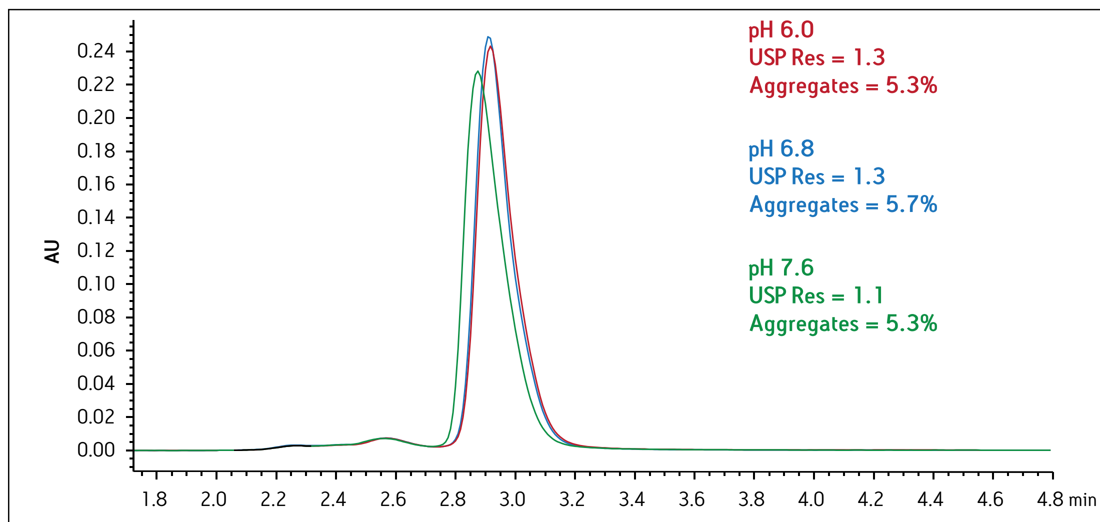


Figure 4. Effect of mobile-phase pH on a SEC separation of murine mAb. Mobile phase: 25 mM sodium phosphate, 200 mM sodium chloride pH 6.0-7.6.

The buffer pH can influence secondary interactions. In this case we observe changes for monomer elution profile but not for the dimer. This suggests a change in the hydrodynamic radius rather than a change in the secondary interactions.

Flow Rate

Resolution in size based separations can be influenced by linear velocity. Although using lower flow rates results in longer run times, the increased resolution gives greater confidence in aggregate quantitation. In addition, the use of sub-2 µm particles for this application allows the use of shorter columns. Thus, the throughput achieved with UPLC-SEC is still greater than that of traditional HP-SEC.[3]

In order to test the reliability and robustness of the method, the effect of flow rate on the SEC separation of a mAb was analyzed. Triplicate injections of the mAb were analyzed at flow rates of 0.2 and 0.4 mL/min (Figure 5). Analysis of the separations shows no significant change in aggregate quantitation with flow rate. However, decreasing the flow rate did increase the monomer/dimer resolution by 15%. While the lower flow rates allow for increased resolution, higher flow rates allow for greater throughput and faster analyses times.

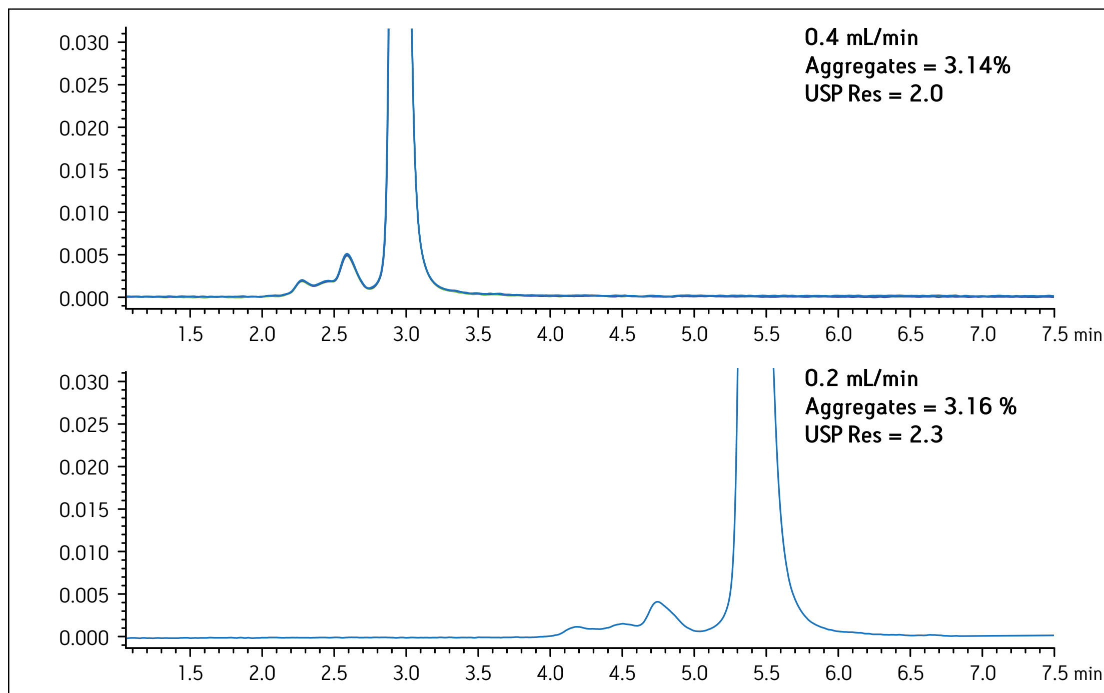
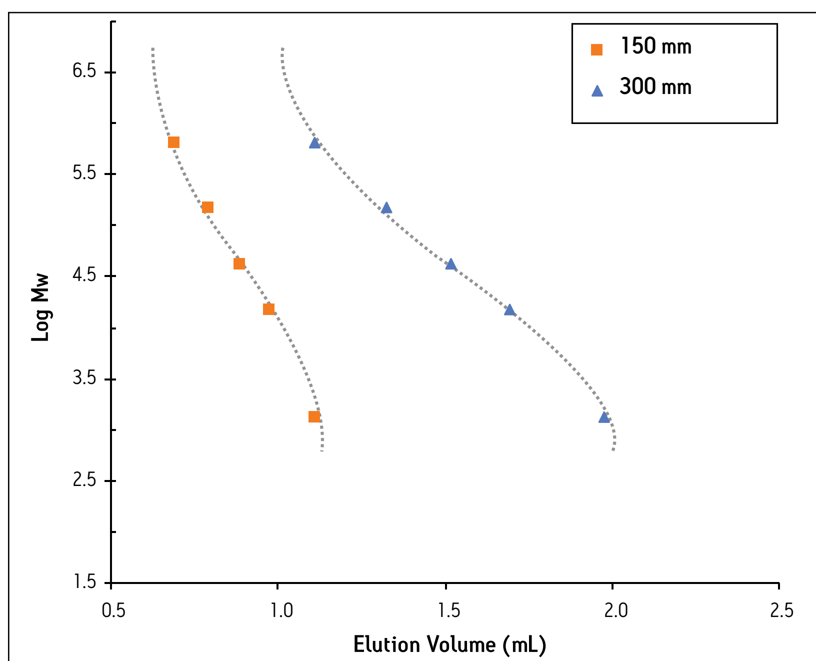


Figure 5. Effect of flow rate on a SEC separation of a murine mAb.

Column Length

Improvements in SEC resolution can also be gained by increasing column length. SEC separations are based on diffusion into and out of the pores of the column's packing material. The larger proteins cannot access the pores and thus elute earlier. The smaller the protein, the longer the residence time within the pores, which results in longer retention times. These principles allow for greater resolution with longer column lengths.

To demonstrate these effects, a set of protein standards were run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Comparison of the calibration curves reveals a shallower slope for the 300 mm column as compared to the 150 mm, demonstrating the higher resolving power achievable on a longer column (Figure 6).



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 6. Effect of column length on SEC calibration curve. Note: Calibration points deviate from a straight line because of protein shape in solution.

The effect of column length was also tested for the SEC separation of a murine mAb run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Under the same conditions, the longer column provided improved resolution for the monomer/dimer (2.07 to 2.80) (Figure 7) with comparable aggregate quantitation. The improved resolution is also apparent in the monomer peak tail, in which a small, lower molecular weight peak is partially resolved on the 300 mm but not on the 150 mm column. However, the improved resolution is accompanied by an increase in retention time (from 3.0 to 6.0 minutes).

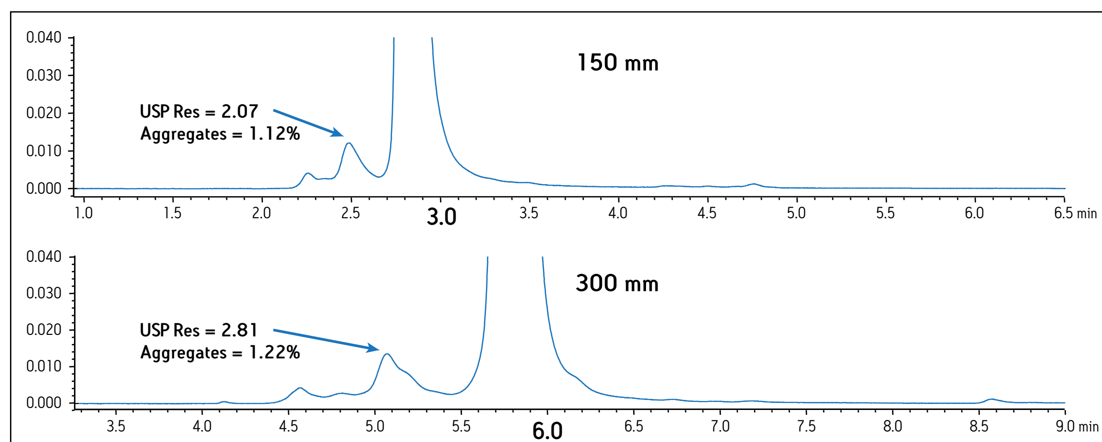


Figure 7. Effect of column length on a SEC separation of murine mAb.

These results indicate that column length can be a useful tool in method development. Depending on the method requirements, column length can be chosen to either provide improved resolution or higher throughput. For example, in a manufacturing environment a longer column allows for improved resolution. While in discovery or development, a shorter column allows for faster analysis time and high throughput.

CONCLUSIONS

Size exclusion chromatography continues to be a standard technique for the analysis of monoclonal antibodies and their aggregates. However, as in any SEC method, a thorough evaluation needs to be performed to develop an optimum separation. While HP- SEC can be time consuming, the use of UP-SEC allows method optimization to be predicted in less time with a high level of efficiency and higher degree of confidence. In addition, the use of Auto•Blend Plus technology makes it easier and less labor intensive to systematically examine the effects of mobile phase on protein structure and on secondary interactions.

As described, optimization should evaluate a number of conditions, including mobile phase (pH and ionic strength), flow rate, and column length. In addition – although not described in detail – injection volume, mass load and temperature can also affect SEC separations. Therefore, a suggested set of experiments should evaluate:

1. ionic strength
2. pH
3. column length
4. flow rate
5. other variables (mass load, injection volume, temperature, etc.)

These experiments should incorporate information on the biological activity of the protein. If factors affecting the proteins biological activity are limited, PBS is the recommended starting mobile phase.

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Probing Conformational Changes of Cytochrome C Induced by Acidification using SYNAPT High Definition Mass Spectrometry

Weibin Chen and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The function of protein-based drugs strongly depends on three-dimensional structures. Conformational changes such as protein denaturation generally result in the loss of drug potency and the alternation of the pharmacological properties of the product. Thus, physicochemical characterizations of the higher-order structures of protein drugs are very important to drug development. Methods that allow fast determination of protein conformations or conformation changes in drug formulations for protein therapeutics are of high value.

Conventional NMR and X-ray methods can elucidate protein geometries in detail but are slow and generally require large quantities of pure protein. These methods are unsuitable to directly analyze real biological matrixes (e.g., drug formulation) that often contain many additives in addition to the drug protein itself.

The conformation of proteins can also be analyzed by optical spectroscopy, such as UV spectroscopy, or circular dichroism. However, these methods only provide information about a particular structural or functional feature of a protein and are generally not sensitive enough to detect the subtle conformational changes caused by small alterations in the protein structure. The need to rapidly identify and quantify a mis-folded protein from a real biological matrix is a particular challenge.

Electrospray ionization mass spectrometry (ESI-MS) has been widely adopted for the study of proteins. Coupled with a time-of-flight (TOF) analyzer, ESI-MS not only enables accurate mass measurement of intact proteins, but also provides useful information on the number of charges and the charge-state distributions of protein ions. However, MS methods are generally insensitive to the three-dimensional structure of proteins and protein complexes.

Ion mobility spectrometry (IMS) separates gas-phase ions with different collision cross-areas and/or charge states. When subjected to IMS separation, a tightly-folded protein conformer with a smaller cross section would travel faster (higher mobility) in an IMS cell, and hence is separated from a more extended, less-folded conformer of the same protein. Similarly, protein ions arising from the same protein conformer but with different charge states may also be separated, with the more highly charged species having a shorter mobility (drift) time. Thus, a combination of IMS and ESI-MS offers great potential to resolve and identify protein conformations in the gas phase that cannot be assessed by MS alone.

Waters SYNAPT™ High Definition Mass Spectrometry™ (HDMS™) System.

Here we present a study that uses a novel ion mobility mass spectrometer, the Waters® SYNAPT™ High Definition Mass Spectrometry™ (HDMS™) System, to probe the conformational structures of a model protein, cytochrome c. Results demonstrate that the instrument is a powerful tool for resolving the population of coexisting conformational states of cytochrome c and for revealing the conformational changes induced by the addition of acid or organic solvent, thus providing direct evidence that the protein is denatured.

EXPERIMENTAL

Cytochrome *c* (bovine heart) was purchased from Sigma. The protein was prepared at a concentration of 2.0 pmol/μL, either in 2.5 mM ammonium acetate (pH 6.6 or 3.0) or in 50:50 MeOH/5.0 mM ammonium acetate (pH 6.6). Samples were introduced to MS directly by infusion using a syringe pump (Harvard Apparatus) at a flow rate of 10 μL/min. MS conditions used for the analysis are listed below.

MS conditions

MS system:	Waters SYNAPT HDMS System
Ionization mode:	ESI Positive
Capillary voltage:	2.8 kV
Cone voltage:	40 V
Desolvation temp.:	50 °C
Desolvation gas:	250 L/hr
Source temp.:	50 °C
Acquisition range:	500 to 3000 m/z
Trap collision energies:	6 V
IMS gas:	N2 gas
IMS gas pressure:	0.7 mbar
Pulse height:	Variable, 9 to 12 v

RESULTS

An ESI-TOF mass spectrum of cytochrome *c* (Figure 1A) was obtained by infusion of an acidic solution (pH 3.0). The mass spectrum exhibits six major peaks and several minor low-intensity peaks, each one corresponding to a different charge (protonation) state of cytochrome *c*. The highest charge state is 7+. The occurrence of more than one charge-state distribution implies the presence of a mixture of folded and less-folded protein conformers or conformational groups as initially suggested in the literature.[1] This implication is confirmed when these ion species are subjected to analysis by SYNAPT HDMS.

Figure 1B shows a two-dimensional plot of m/z vs. drift time (DriftScope™ Software), which displays ion species separations for each charge state of cytochrome *c*. Because ion species with the same charge state migrate at different times through the IMS cell, the data provide direct evidence that these ions have different collision cross sections (conformations), thus demonstrating that multiple, co-populated conformers of cytochrome *c* exist among all the ions generated. The number of distinct species associated with each charge state can be readily visualized with DriftScope.

The ability of the SYNAPT HDMS to differentiate protein conformers is further illustrated in Figure 1C, which shows IMS separations of compact and less-folded conformers at three different charge states (8+, 9+, and 10+).

The 8+ ions show three distinct signals with drift times of 8.3, 11.6, and 12.5 ms; the 9+ ions show three signals with drift times of 7.4, 9.2, and 9.9 ms; and the 10+ ions show a predominant signal with a drift time of 8.4 ms together with a signal of low intensity at 6.6 ms.

In each case, the signal with the shortest drift time (i.e., higher mobility) can be attributed to a more compact conformation, compared with the signals detected at longer drift time (i.e., lower mobility), which can be assigned to a less-folded, more extended conformation. Thus the 8+ ion species exhibit a higher percentage of the folded conformation compared with the less-folded species. In comparison, the 9+ and 10+ ion species predominately have the less-folded species.

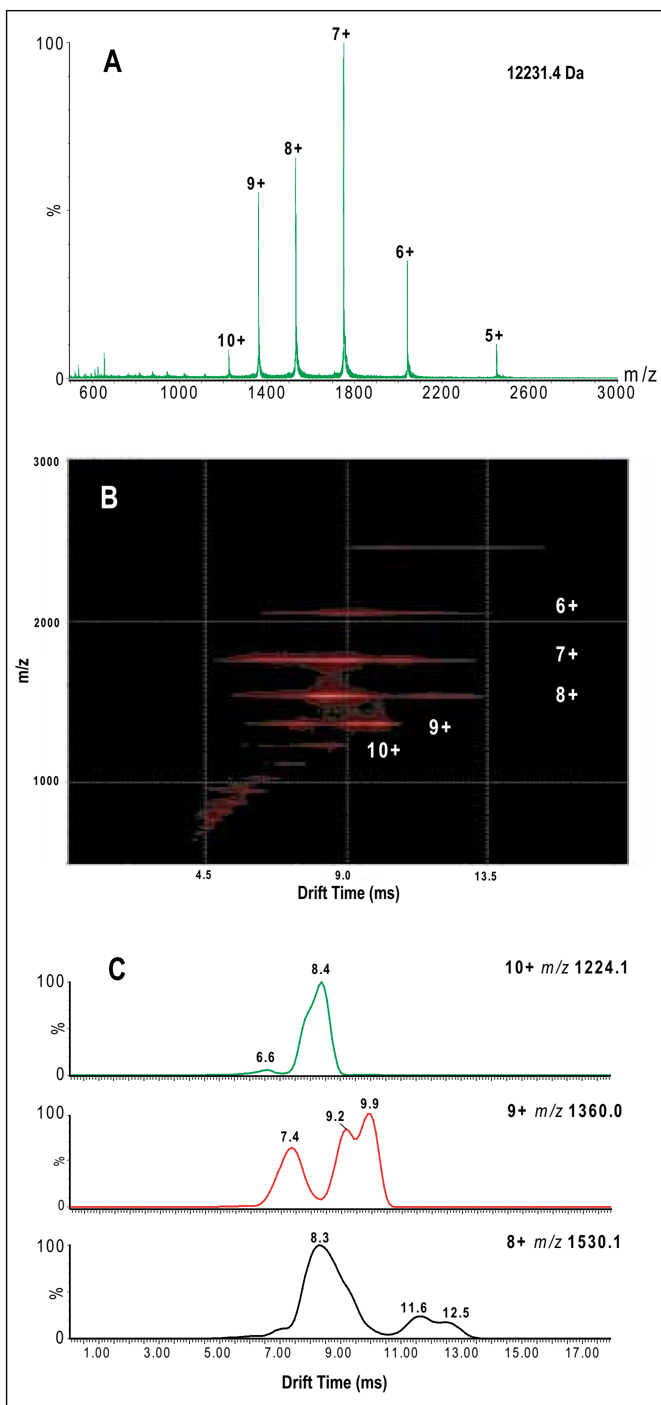

Figure 1.

A. Summed ESI-TOF-MS mass spectrum of cytochrome c in 2.5 mM aqueous ammonium acetate solution (pH 3.0) acquired using the SYNAPT HDMS System incorporating the enabling Triwave™ Technology.

B. DriftScope shows drift time (x axis) vs. m/z (y axis) for the analysis of cytochrome c in 2.5 mM ammonium acetate at pH 3.0.

C. Ion mobility drift time vs. intensity plots for the ions with m/z of 1224.1 (10+ ions; upper), 1360.0 (9+; middle) and 1530.1 (8+; lower) detected during the analysis of cytochrome c in 2.5 mM aqueous ammonium acetate solution at pH 3.0.

Charge-state distributions of proteins from ESI processes depend on the pH of the solution. For a cytochrome *c* solution with a nearly neutral pH (6.6), the distribution peaks around the 7+ charge state (Figure 2A). When an acid is added, the distribution shifts to higher charge states (Figure 2B). The change in the charge-state distribution is interpreted as reflecting a change in the high-order structure in the solution phase.[1] Cytochrome *c* is denatured in acidic solutions, and the appearance of the higher charge states is believed to be caused by more basic sites available for protonation from the unfolding structure.

To test whether the conformational information obtained from SYNAPT HDMS can be used to correlate with the high-order structure changes of protein in solution, two cytochrome c solutions from a neutral (pH 6.6) or an acidic solution were analyzed under identical experimental conditions.

Figure 2C shows drift-time distributions recorded for the 8+ charge state of bovine cytochrome c from the two solutions. Although the 8+ charge state from both solutions displays a similar pattern in the drift-time distribution, the percentage of ion species with more extended conformational forms is clearly higher from the acidic solution than that from the neutral solution, implying that the acidic cytochrome *c* solution contains more unfolded species. This observation closely correlates with the fact that cytochrome c undergoes conformational changes (denaturation) in an acidic solution.[2]

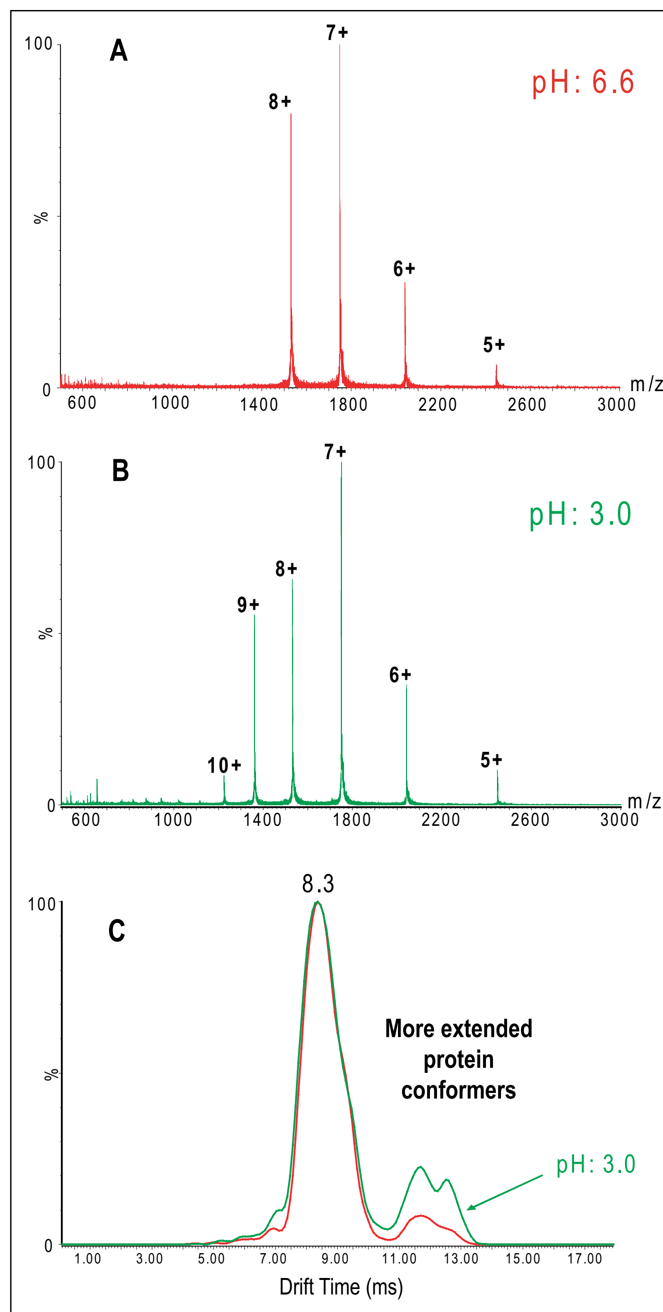

Figure 2. Mass spectra and drift-time distributions for the 8+ charge states of cytochrome c, measured at two different pH levels (6.6 and 3.0).

A. Summed ESI-IMS-MS spectrum of 2.0 pmol/µL of bovine cytochrome c in 2.5 mM ammonium acetate aqueous solution (pH 6.6).

B. Summed ESI-IMS-TOF-MS spectrum of 2.0 pmol/µL of bovine cytochrome c in 2.5 mM ammonium acetate aqueous solution (pH 3.0).

C. Comparison of the drift-time distribution of the 8+ charge states of bovine cytochrome c from the two aqueous solutions with different pHs (6.6 vs. 3.0), showing the correlation between the gas-phase ion conformations and solution denaturing changes.

The role of the SYNAPT HDMS System in probing high-order structure changes of proteins is additionally demonstrated in the analysis of cytochrome *c* solution containing 50% methanol, which is a relatively mild denaturant.

The protein is completely denatured at methanol concentrations greater than 40% (in the absence of an acid). However, unlike acid denaturation at low ionic strength, which yields extended unfolded states, alcohol denaturation leads to a denatured form with increased helical content.[3]

Mass spectra recorded for cytochrome c in a 1:1 solution of 5.0 mM ammonium acetate (pH 6.6) and methanol show essentially the same charge state distribution as recorded for a pure aqueous solution (2.5 mM ammonium acetate, pH 6.6), which suggests cytochrome c takes folded conformations (Figure 3A and 3B).

However, the drift-time distribution recorded for the 8+ charge state from the 1:1 methanol/5.0 mM ammonium acetate solution (Figure 3C) clearly shows an increase in the more-extended, less-folded conformational forms compared to the drift-time distribution of the same charge state with the aqueous solution. This peak increase presumably results from a solution conformation with increased helical content.

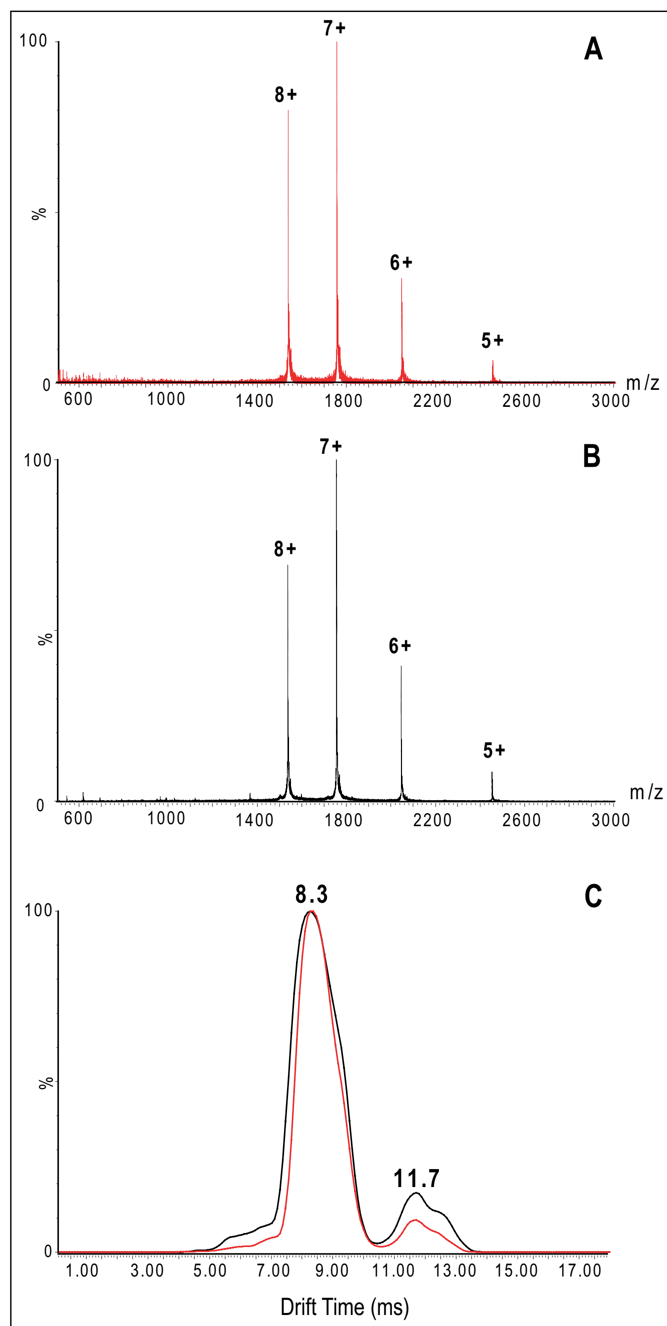

Figure 3. Mass spectra and drift time distributions for the 8+ charge states of bovine cytochrome c, measured at two different solutions (aqueous and 50% MeOH).

A. Summed ESI-IMS-TOF-MS spectrum of 2.0 pmol/µL of bovine cytochrome c in 2.5 mM ammonium acetate aqueous solution (pH 6.6).

B. Summed ESI-IMS-MS spectrum of 2.0 pmol/µL of bovine cytochrome c in 50% MeOH/50% 5.0 mM ammonium acetate aqueous solution (pH 6.6).

C. Comparison of the 8+ charge states of bovine cytochrome c from the two solutions, showing the correlation between the gas-phase ion conformations and solution denaturing changes.

CONCLUSION

When applied to an analysis of a well-studied model system, cytochrome *c*, the SYNAPT HDMS System has demonstrated that it can be used to directly monitor and separate gas-phase ion conformers based on their cross sectional areas. These results indicate SYNAPT HDMS is a potentially powerful tool to correlate conformation changes of proteins under different solution conditions, and can be used to quickly probe the conformations or conformational changes of protein therapeutics in drug formulations.

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

[APPLICATION NOTE]

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Improved Ion Mobility Separation of Protein Conformations in the Gas Phase with SYNAPT G2 HDMS

St John Skilton, Iain Campuzano, Kevin Giles, Hilary Major, and Weibin Chen
Waters Corporation, Milford, MA, U.S. and Manchester, UK

APPLICATION BENEFITS

This application note illustrates that the SYNAPT G2 HDMS System, which provides an orthogonal separation technique with ion mobility mass spectrometry, can clearly separate different conformations of equine cytochrome c within minutes, matching that of published work.

WATERS SOLUTIONS

SYNAPT G2 HDMS

KEY WORDS

IMS, T-Wave, collision cross-sectional area Ω

INTRODUCTION

Biomolecules introduced to a mass spectrometer by electrospray ionization (ESI) exhibit a number of different conformations depending on charge states, eluent pH, and size. Understanding the higher order structure of biomolecules is important for the biopharmaceutical industry because different conformations may affect biological activity.

Methods for determining changes in higher order structure are valuable to stability studies and purification development, especially those that can be implemented in a high throughput manner. It is often time-consuming to separate or identify different conformations. Recent work on hydrogen-deuterium (HD) exchange shows how ion mobility separation (IMS) can be used to differentiate conformations of heterogeneous biomolecules such as IgG.[1]

It is known that specific conformations are preferentially susceptible to biological activity.[1,2] The ability to reliably distinguish different forms is vital for organizations that need to discover which conformations of a biomolecule are important. Reducing the need for time-consuming workup for crystallography or NMR work can save an organization valuable time and resources.

Different conformations of isobaric biomolecules cannot be separated by mass spectrometric resolution alone. With the inclusion of IMS in Waters SYNAPT® HDMS™ instruments, an orthogonal separation technique is added to the capability of the mass spectrometer.

IMS is well-established for analysis of small and large molecules.[3-6] In its most recent commercial implementation, the SYNAPT G2 HDMS improves IMS resolution by a factor of three to four. Conformations that were not distinguishable in the first-generation instrumentation become apparent using SYNAPT G2 HDMS. This work describes how different conformations of equine cytochrome c can be clearly separated within minutes.

Cytochrome c was chosen as a model compound because multiple conformations have been observed in the published work using a standard drift-tube ion-mobility instrument.[6,7] Here, we demonstrate that SYNAPT G2 HDMS achieves equivalent separation on a more compact platform than a previous research instrument.

EXPERIMENTAL

MS conditions

MS system:	Waters SYNAPT G2 HDMS
Ionization mode:	ESI positive
Nanoflow capillary voltage:	1.0 kV
Cone voltage:	40 V
Extraction cone:	2 V
Trap collision energy (CE):	6 V
Transfer CE:	4 V
Trap/Transfer gas:	2.0 x 10-2 mbar (Argon)
IMS gas:	N2
IMS gas pressure:	2.7 mbar

METHOD

Cytochrome c (Bovine heart) was purchased from Sigma. The protein was prepared at a concentration of 2 pmol/µL either in 2.5 mM ammonium acetate (pH 6.6 or 2.6) or in 50:50 MeOH/ammonium acetate (5.0 mM pH 6.6) for mass spectrometric analysis. Samples were introduced to MS directly by infusion using a syringe pump (Harvard Apparatus, Holliston, MA, U.S.) at a flow rate of 10 µL/min. Myoglobin solution (sperm whale, 2 pmol/µL in 50% MeOH/H2O, 0.1% FA) was used for TriWave IMS calibration for cross-section measurement.

RESULTS AND DISCUSSION

Ion Mobility Separation (IMS) is a technique that allows the separation of ions in a neutral buffer gas based on their collision cross-section Ω. IMS in the SYNAPT G2 HDMS is implemented by use of a travelling wave ion guide.[3, 8] The technique is therefore orthogonal to liquid chromatography and to mass analysis, providing additional information.

The instrument is a hybrid tandem quadrupole IMS-TOF; ions with different charge states can be readily isolated at the first stage by the quadrupole. By selecting a single charge state of biomolecule ions, ions with different conformations can be separated.

The samples were analyzed on both SYNAPT HDMS and SYNAPT G2 HDMS to ensure comparability between instruments. Figure 1 illustrates that exactly the same charge-state envelope is apparent on each of the instruments, showing the same characteristics and in the same ratios. In the SYNAPT G2 HDMS data, the time-of-flight (TOF) resolution has increased to 40,000 resolution (FWHM), providing monoisotopic resolution of the 9+ charge state.

The 8+ charge state ions were selected and transferred to the ion mobility cell, where ions with different conformation were separated. In Figure 2, a comparison is shown between the equivalent data on a SYNAPT HDMS and a SYNAPT G2 HDMS. The same separation time window was compared overall and the extracted mobilograms are comparable. Although the data from the first-generation instrument implies that existence of multiple protein conformers for this charge state, certain conformations (e.g., peaks at 11.0 ms) were not completely separated. In the SYNAPT G2 HDMS, at least three different conformers are clearly resolved with a fourth conformation appearing at the base of the major peak at 7.86 milliseconds of drift time.

To probe the different conformations of cytochrome c in aqueous solution, the pH level was adjusted to two different values: pH 2.6 and pH 6.6, reflecting denaturing and native solution conditions. At pH 2.6, the protein is denatured and is therefore expected to produce more open conformations because of the repulsive charge effects. The reverse is expected at pH 6.6 and therefore the conformations are expected to be more compact.

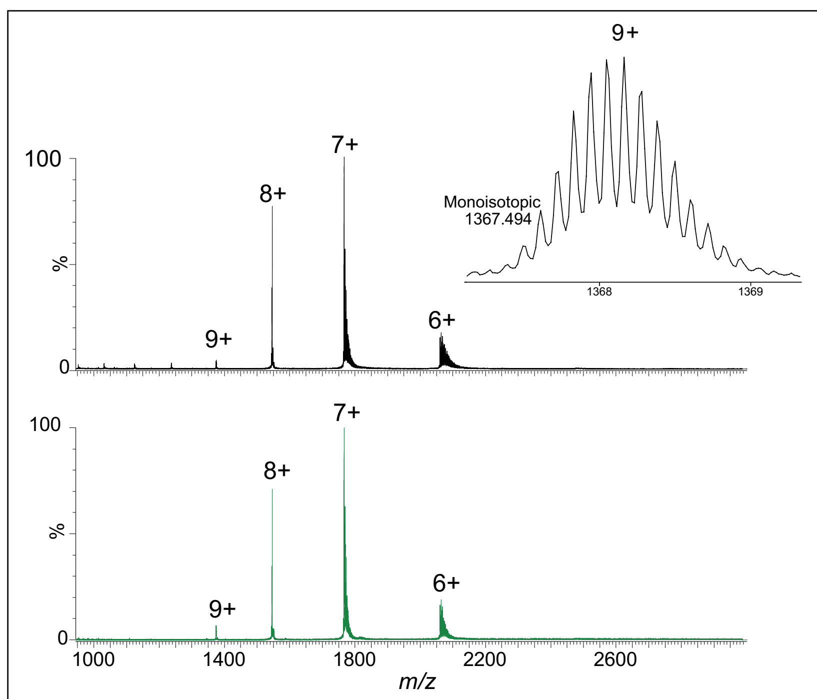

Figure 1. Comparisons of the most abundant charge states of cytochrome c in ESI mode on SYNAPT HDMS (bottom) and SYANPT G2 HDMS (top). Inset is a zoom of the increased resolution of the 9+ charge state at 40,000 resolution FWHM.

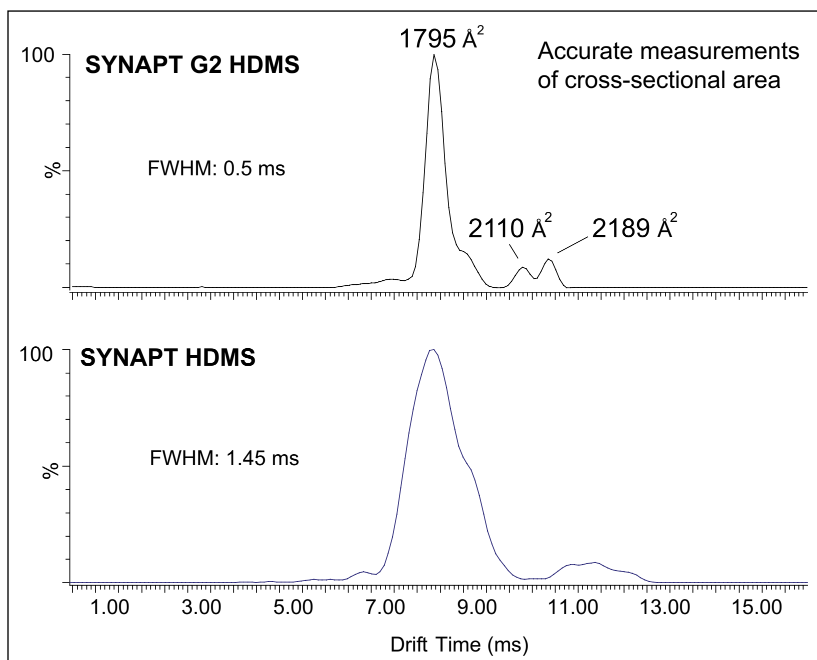

Figure 2. Comparative extracted ion mobilograms of the 8+ charge state of cytochrome c in ESI mode on SYNAPT HDMS (bottom) and SYNAPT G2 HDMS (top). The drift time at the half height of the major peak indicates that the mobility resolution has increased by a factor of three on SYNAPT G2 HDMS. The collision cross sections for each resolved peaks are shown on the apex of the G2 peaks.

Figure 3 shows the extracted ion mobilograms of charge state 8+ from both pH solution conditions are superimposed on each other. The traces are normalized to the most intense peak at 7.86 ms. In the more acidic state at pH 2.6 the peak heights at a drift time of 9.79 ms and 10.35 ms are proportionally higher relative to the main peak when compared to the trace at pH 6.6. Therefore, as expected, changes in pH change the proportions of conformations.

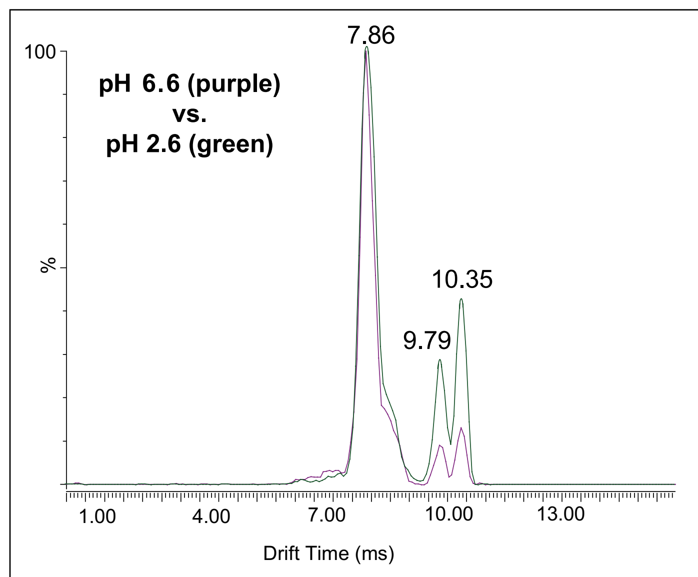

Figure 3. Comparative extracted ion mobilograms of cytochrome c at different pH values on SYNAPT G2 HDMS.

The result is consistent with ion mobility separation: the more mobile peak at 7.86 ms is the most compact form and therefore migrates earlier because it effectively has less resistance to being moved along the wave guide. In physical terms, the more compact conformation is being subjected to fewer rollover effects than more extended/unfolded conformations.[3] The other two peaks are more extended forms (9.79 ms, 10.35 ms) and migrate more slowly (less mobile) in the Travelling Wave Ion Guide (TWIG). There is a greater proportion of the more extended forms at pH 2.6, consistent with the expected effect of acidic solutions producing more denatured forms. Ion mobility is able to make this difference apparent where mass analysis alone would be blind to this effect.

The data indicates that there are at least three forms easily separable according to shape (only). The spectra of all of these forms are the same because they are all isobaric.

Measurement of cross-sectional areas

The increased mobility resolution allows easier measurement of collision cross-sectional area Ω. Collision cross-section measurements of proteins on the first generation instruments have been shown to match well with mobility separation.[9] Additionally, the latest implementation of software that provides visualization of ion mobility separations, DriftScope™ 2.1, allows for an automatic T-Wave™ ion mobility calibration to be performed. This enables the Ω determination of an unknown. In this case, T-Wave ion mobility calibration was performed using the known Ω values of sperm whale myoglobin. The calibration was validated with equine cytochrome c (Table 1).

z	***m/z***	**Clemmer Ω**	**SYNAPT G2 HDMS Ω**	**Diff (%)**
20	618	2889	2952	2.1
19	651	2800	2871	2.5
18	687	2766	2817	1.8
17	727	2723	2719	-0.1
16	773	2679	2643	-1.3
15	824	2579	2532	-1.7
14	883	2473	2457	-0.6
13	951	2391	2389	-0.1
12	1030	2335	2303	-1.3
11	1124	2303	2235	-2.9
10	1236	2226	2202	-1.0

Table 1. A comparison of SYNAPT G2 HDMS derived and drift cell Ω values.[7]

The T-Wave derived Ω values match well with published cytochrome c data8 and confirm that the commercially -available instrument provides exactly the same information in a short time frame: The sample introduction was by simple infusion in ESI positive mode with no special instrument conditions. Further increases in the TOF resolution of the instrument mean that even high charge states can be isotopically resolved.

CONCLUSION

- The data produced by the SYNAPT G2 HDMS significantly improves what was possible on previous instruments.
- Bovine cytochrome c conformations and Ω values were found to match that of previous, published work.
- The data was obtained easily and with no special conditions needed – these experiments can be performed by non-experts.
- The implementation of ion mobility on SYNAPT G2 HDMS integrates an orthogonal dimension with a commercially-available, high-resolution, tandem MS instrument.
- This improvement reduces the risk of missing important tertiary structure information for any organization that needs to characterize biomolecules.

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

AMINO ACID ANALYSIS

Amino acid analysis (AAA) is an established analytical technique that is used in the analysis of protein and peptide hydrolysates and cell culture for biopharmaceuticals. Amino acid analysis can be used to determine the amount of protein in a sample and the proportions of amino acids in a protein. This information confirms the identity of the protein and can detect modifications.

Another important application is the analysis of mammalian cell culture. Determination and control of critical nutrients in cell culture is important to maximize protein yield and provide a consistent manufacturing process.

Amino acid analysis can represent a difficult analytical problem. Amino acids have a wide range of chemical properties (e.g., acidic, basic, neutral), yet resolution of individual pairs having only minor structural differences is required. Analysis is further complicated by the absence of common chromophores, necessitating use of a derivatization chemistry to enable analyte detection.

Waters offers a turnkey system solution for accurate, high-throughput analysis of amino acids. This solution provides better resolution and sensitivity in a shorter analysis time than with previous methodologies. Its enhanced separation ensures that the analysis yields accurate and precise qualitative and quantitative results and that the methods are exceptionally rugged.

Waters UPLC Amino Acid Analysis Solution

- AccQ•Tag™ Ultra derivatization and separation chemistries, including quality-control tested columns, reagents, and eluents.
- ACQUITY UPLC,® ACQUITY UPLC H-Class, or ACQUITY UPLC H-Class Bio systems with a Tunable UV Detector, with enhanced chromatographic resolution and maximum sensitivity detection.
- Empower™ 3 Software with configured projects, methods, and report formats.
- Installation, Operation, and Performance Qualification (IQ/OQ/PQ) protocols that ensure every element of the system is performing, for reproducible results.
- Connections INSIGHT® Remote Services for proactive instrument diagnostics that ensure continuous, consistent, and reliable operation.

COMPREHENSIVELY DESIGNED FOR PERFORMANCE

The UPLC Amino Acid Analysis Solution provides control over the major factors that commonly cause variability in amino acid analysis. From optimized instrumentation and quality controlled chemistries to standardized software projects, methods, report templates, and qualification tools, rely on a method that is both rugged and reliable – a comprehensive and detailed approach that ensures successful analysis of every sample.

The UPLC AAA System brings together industry-leading technologies – instrumentation, chemistries, software, qualification tools, and services – that are specifically optimized for exceptional, application-focused performance. Designed by scientists for scientists, these solutions remove the guesswork in choosing the best solution for your application's needs.

COLUMNS AND CHEMISTRIES FOR AAA

AccQ•Tag Ultra Chemistries are a comprehensive and fully tested set of reagents, columns, and eluents optimized for use with the UPLC Amino Acid Analysis Solution. This chemistry is based on Waters' popular and rugged AccQ•Tag pre-column derivatization method. High-resolution separations are achieved using performance-qualified AccQ•Tag Ultra UPLC columns and mobile phases. Derivatized amino acids are quantified to sub-picomole levels with single wavelength UV detection.

[APPLICATION NOTE]

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UPLC Amino Acid Analysis Solution

Thomas E. Wheat, Eric S. Grumbach, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Analysis of amino acids is required in several different areas of research and is also a fundamental tool in various product analysis activities. These applications impose different requirements on the analytical method because the amino acids play different roles.

Amino acids are the basic constituents of proteins. For that reason, qualitative and quantitative analysis of the amino acid composition of hydrolyzed samples of pure proteins or peptides is used to identify the material and to directly measure its concentration.

Amino acids are also intermediates in a myriad of metabolic pathways, often not directly involving proteins. The amino acids are, therefore, measured as elements of physiological and nutritional studies. This has proven particularly important in monitoring the growth of cells in cultures, as used in the production of biopharmaceuticals. Similar considerations lead to the analysis of foods and feeds to ensure that nutritional requirements are met. These diverse sample applications will all benefit from improved amino acid methods.

A comprehensive system-based solution for the analysis of amino acids has been recently developed. This solution provides better resolution and sensitivity, all achieved in a shorter analysis time than with previous methodologies. Its enhanced separation ensures that the analysis yields accurate and precise qualitative and quantitative results and that the method is exceptionally rugged.

This application solution is based on the well-understood and widely-used Waters® AccQ•Tag™ pre-column derivatization chemistry. The derivatives are separated using the Waters ACQUITY UltraPerformance LC® (UPLC®) System for optimum resolution and sensitivity. System control, data acquisition, processing, and flexible reporting are provided within Empower™ Software. The integrated total application solution ensures successful analyses.

UPLC Amino Acid Analysis Solution.

METHODS AND DISCUSSION

Ultimately, a new amino acid method must provide the right answer. Increased ruggedness, preferably with reduced labor and run times, are also desired characteristics of a successful laboratory system. These needs are met by combining AccQ•Tag Ultra amino acid analysis chemistries with the proven separation technology of the ACQUITY UPLC System; together they comprise the turnkey application solution called the Waters UPLC Amino Acid Analysis Solution.

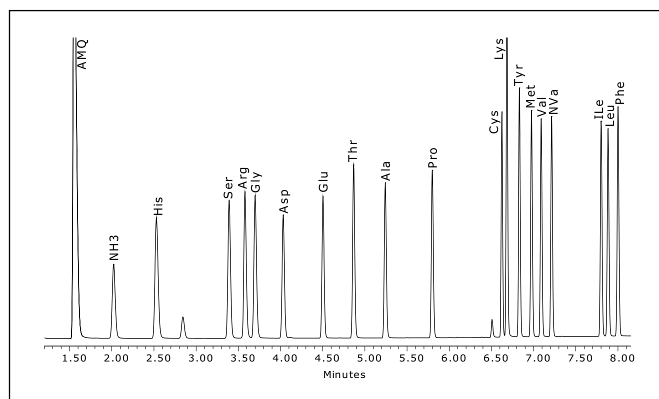

Figure 1. Separation of 50 pmoles of the amino acid hydrolysate standard with the UPLC Amino Acid Analysis Solution.

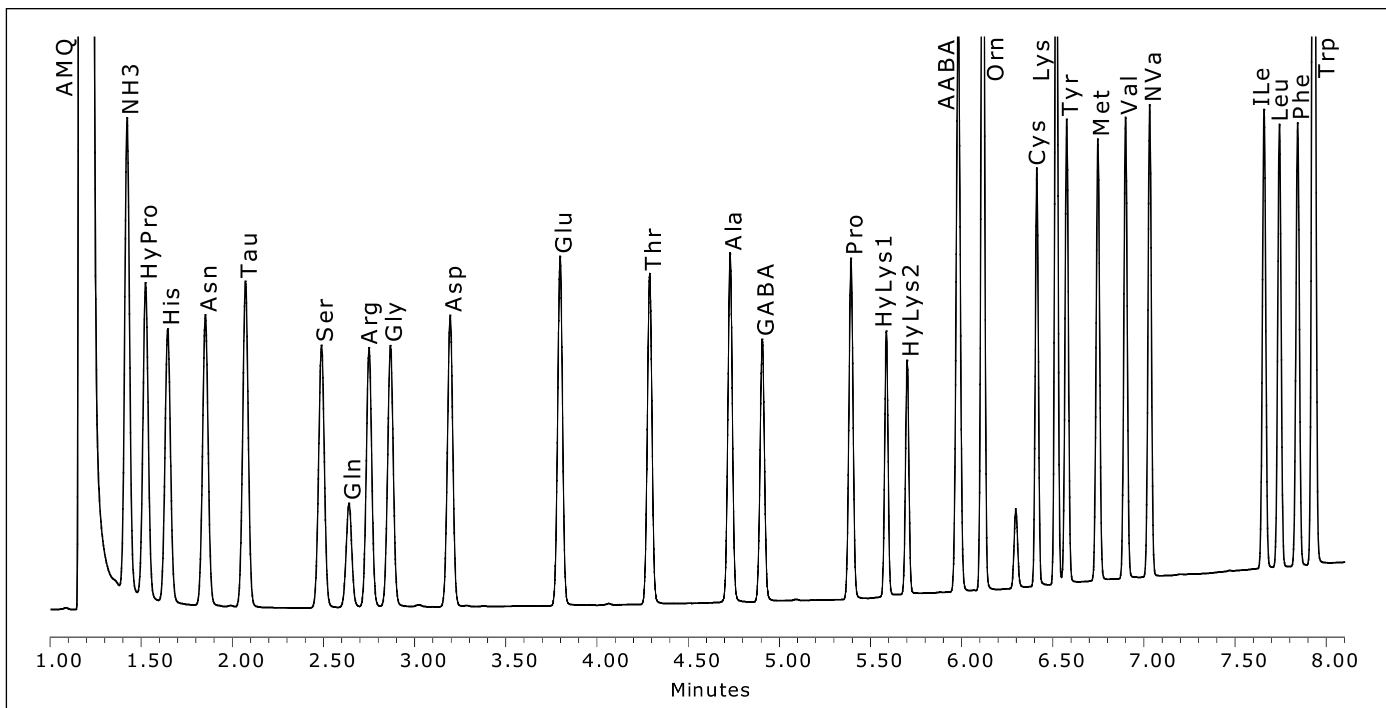

Figure 2. Separation of 10 pmoles of the amino acids commonly found in cell culture media. The UPLC Amino Acid Analysis Solution includes this modified separation method.

Analysis of a hydrolysate standard is shown in Figure 1. The amino acids are derivatized using AccQ•Fluor™ Ultra Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Both primary and secondary amino acids react in a simple batch-wise derivatization, and samples are stable for several days. No special sample preparation is required, and the reaction occurs in a largely aqueous solution so it is very tolerant of buffer salts and other sample components. The excess reagent naturally hydrolyzes, and the by-product is chromatographically resolved from the derivatives. No special handling or extraction is required.

The derivatives are separated on an AccQ•Tag Ultra Column, 2.1 x 100 mm, a bridged ethyl hybrid (BEH) C_{18} 1.7 µm particle specifically tested for separation of the amino acids. Packaged eluents are quality control (QC) tested with amino acid separations. They are provided as concentrates requiring only dilution with water before use. The instrument is the ACQUITY UPLC System with UV detection at 260 nm.

The resolution of the amino acids is 1.6 or greater to ensure accurate quantitation. Retention time reproducibility is on the order of hundredths of minutes, much less than a peak width, to ensure unambiguous identification of the amino acids. The detection is linear, over more than three orders of magnitude, to permit quantitative analysis of samples with disparate ratios of amino acids with an ample margin for samples of different concentration. The sensitivity of the method gives adequate signal-to-noise to quantitate at the level of 50 femtomoles on-column.

The method can be successfully used for a range of applications. The standard method shown in Figure 1 can also resolve the derivatives of cysteine commonly used in protein structure analysis. The products of performic acid oxidation that are part of assessing the nutritional quality of foods and feeds are also well-separated.

For monitoring the composition of media during the growth of cells in culture, additional amino acids must be resolved. This requires a different dilution of the AccQ•Tag Ultra Eluent A Concentrate and a higher separation temperature. The chromatogram used for monitoring cell culture media is shown in Figure 2.

CONCLUSION

These results describe the new Waters UPLC Amino Acid Analysis Solution, an Assured Performance Solution (APS), that come complete with application-focused chemistries, innovative UltraPerformance LC and MS technologies, methodology, documentation, and support to deliver the answers you need about amino acids, every time. Successful results are assured through the use of pre-tested derivatization and separation chemistry and the high resolution provided with the ACQUITY UPLC System.

This integrated analytical approach will give accurate and precise qualitative and quantitative results for a wide range of applications including protein and peptide hydrolysates, monitoring cell culture media, and measuring the nutritional value of food and feeds.

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

Analysis of Pure Protein Hydrolysate with the UPLC Amino Acid Analysis Solution

Hillary B. Hewitson, Thomas E. Wheat, and Diane M. Diehl
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Amino acid analysis is used in the protein structure laboratory to provide two kinds of information. First, the total quantity of amino acids is a direct measure of the amount of protein in a sample. Second, the measurement of the proportions of amino acids provides information to confirm the identity of the protein and to detect modifications. Both applications require robust, accurate, and sensitive measurements that both identify and quantitate the amino acids. There is increasing need for these labs to provide these correct results faster and more economically.

The Waters UPLC® Amino Acid Analysis Application is a turnkey solution to address these needs. This total system solution includes a well established and understood sample derivatization kit, eluents, chromatographic column, and a separation system based on the ACQUITY UPLC® using UV detection under Empower™ Software control.

In this experiment, this system solution is used to measure the composition and concentration of a known protein. The accuracy of the determination is compared to the known correct results.

Figure 1. Waters UPLC Amino Acid Analysis Solution.

EXPERIMENTAL

Sample

Acid-hydrolyzed bovine serum albumin (BSA) samples were prepared in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl sealed under argon in sealed ampoules. Samples were stored at -80°C until analysis.

Sample derivatization

The sample was diluted 1:10 with 0.1 M HCl prior to derivatization. The standard derivatization protocol was modified to include neutralization of excess acid with 0.1 M NaOH. Conditions for derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Application System Guide (P/N 71500129702).

- 10 µL of samples diluted 1:10 with 0.1 M HCl
- 10 µL 0.1 N NaOH
- 60 µL AccQ•Tag™ Ultra Borate Buffer
- 20 µL AccQ•Tag Ultra Reagent

LC conditions

LC system:	Waters ACQUITY UPLC System with TUV detection at 260
Column:	AccQ•Tag Ultra 2.1 x 100 mm, 1.7 µm
Column temp.:	55 °C
Flow rate:	700 µL/min
Mobile phase A:	1:20 Dilution of AccQ •Tag Ultra Eluent A concentrate
Mobile phase B:	AccQ•Tag Ultra Eluent B
Gradient:	AccQ•Tag Ultra Hydrolysate Method
Injection volume:	1 µL

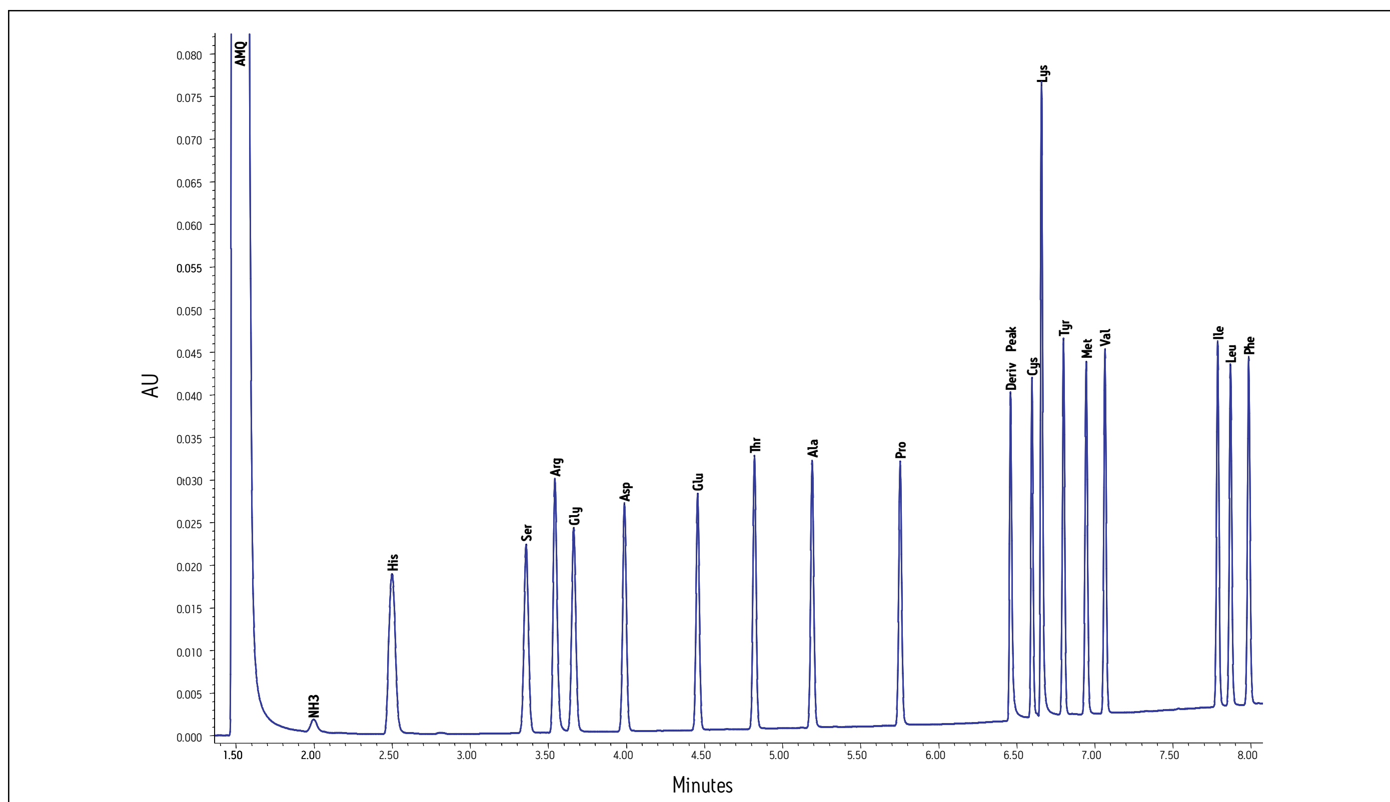

Figure 2. Analysis of amino acid hydrolysate standard, 10 pmoles each on-column.

RESULTS

Figure 2 shows the chromatogram of a standard that contains amino acids typically found in protein hydrolysate samples. Each amino acid is 10 pmoles on column. For quantitative analyses, a three point calibration at 0.5, 10, and 25 pmoles on column was applied.

The analysis of a typical BSA hydrolysate is shown in Figure 3. The estimated starting concentration is consistent with this chromatogram representing a total of 9 ng of protein on column. This analysis was repeated a total of 75 times, over five separate days, with two columns and a total of five mobile phase preparations. The 75 injections represent five independent sample dilutions, each dilution derivatized five separate times. Each derivatized sample was injected in triplicate.

The amino acid composition, expressed as residues/mole of protein, was compared to the value expected from the known sequence. Table 1 shows the mean and standard deviation for each amino acid over all 75 analytical injections. Tryptophan and cysteine/cystine are excluded from the calculation because they are destroyed by acid hydrolysis. The measured molar composition agrees well with the expected values from the sequence. The reliability of the UPLC Amino Acid Analysis Application is confirmed by the reproducibility of results over the large number of determinations that intentionally includes the variability that could arise from multiple columns, eluents, and derivatizations.

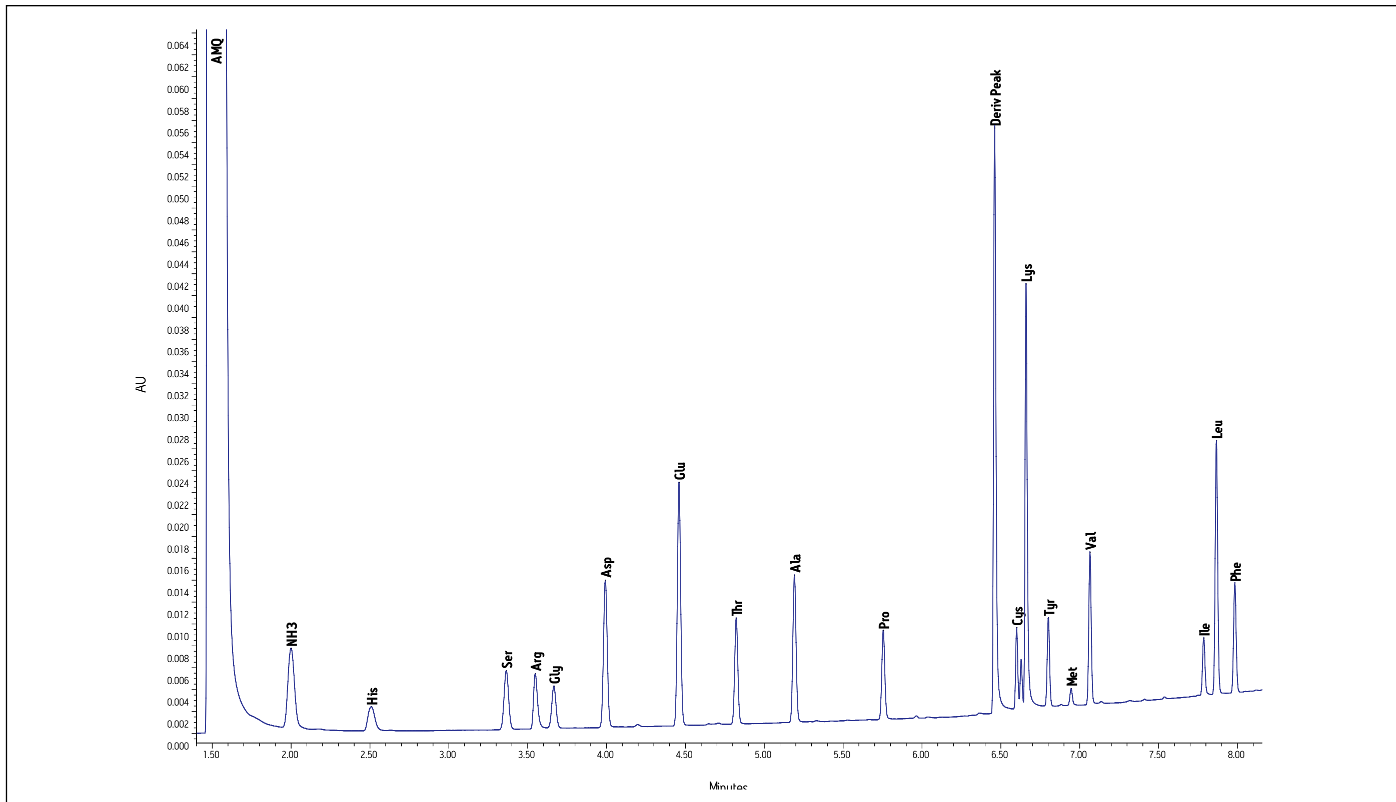

Figure 3. Analysis of BSA hydrolysate sample, approximately 9 ng on-column.

Amino Acid	Expected Residues	*Observed Residues
His	17	15.36 ± 0.19
Ser	28	26.00 ± 0.08
Arg	23	22.37 ± 0.08
Gly	16	17.68 ± 0.20
Asp	54	55.47 ± 0.21
Glu	79	80.68 ± 0.20
Thr	33	31.92 ± 0.06
Ala	47	47.51 ± 0.15
Pro	28	28.35 ± 0.14
Lys	59	57.78 ± 0.38
Tyr	20	20.19 ± 0.08
Met	4	4.16 ± 0.15
Val	36	35.67 ± 0.16
Ile	14	13.15 ±0.15
Leu	61	63.13 ± 0.19
Phe	27	26.57 ± 0.13

Table 1. Comparison of observed with expected composition derived from known sequence of bsa.

*Average of 75 data points (25 derivatizations, each injected in triplicate; mean value ± standard deviation)

Sample ID	Total µg AA/ mL Hydrolysate
BSA 1-1	699.64
BAS 1-2	694.09
BSA 1-3	697.88
BSA 1-4	698.21
BSA 1-5	695.43
BSA 2-1	716.66
BSA 2-2	717.87
BSA 2-3	715.65
BSA 2-4	709.75
BSA 2-5	707.93
BSA 3-1	714.93
BSA 3-2	711.50
BSA 3-3	708.46
BSA 3-4	708.14
BSA 3-5	708.10
BSA 4-1	674.43
BSA 4-2	678.43
BSA 4-3	683.07
BSA 4-4	678.62
BSA 4-5	678.94
BSA 5-1	591.40
BSA 5-2	604.30
BSA 5-3	571.78
BSA 5-4	571.57
BSA 5-5	599.18
Mean	677.43
Standard Deviation	47.98
% RSD	7.08

Table 2. Reproducibility of method for amount of bsa protein in sample.

The analytical data was used to calculate absolute amount of protein in the sample. The amount of each amino acid were expressed as the residue molecular weight. The sum of weights of the amino acids is equal to the weight of the protein. Table 2 summarizes the result of the 75 determinations with the mean for the triplicate injections shown for each derivatization. The measured amount corresponds to 0.7 mg/mL in the starting material. The estimated amount used to prepare the hydrolysate has not been independently verified. It should be noted in addition that this measurement of protein amount does not include the contribution of cysteine/cystine and tryptophan, as they are mostly destroyed by the hydrolysis of the protein.

The reproducibility of determination of the 75 analyses gives a RSD of 7%. Detailed examination shows that much of this variance is due to the difference of experiment 5 from the other four analyses. Since all of the replicates in experiment 5 are lower than the others, this variance is consistent with a difference in pipetting in the initial sample preparation. The addition of an internal standard to the sample to be hydrolyzed will improve the reliability of the final analytical result. Norvaline is the preferred internal standard for this purpose.

CONCLUSION

Protein structure and biopharmaceutical laboratories rely on accurate quantitation of amino acids to confirm the identity and amount of protein in their samples. The analyses shown here demonstrate that Waters UPLC Amino Acid Analysis Application can provide assured results for these laboratories.

The molar ratios of amino acids are reproducible over multiple derivatizations and replicate injections. The measured composition agrees with that expected from the sequence of BSA.

Determination of the absolute amount of protein in the samples is determined by summing the residue weights of amino acids. The reproducibility of this measurement is on the order of ±7%. The largest contribution to this variance is the initial sample dilution. Incorporation of an internal standard in hydrolysis will improve the precision of the determination. Norvaline is recommended as an internal standard for the method.

The Waters UPLC Amino Acid Analysis Application provides a complete turnkey analytical method for analyzing protein hydrolysate samples. The ACQUITY UPLC system gives very high resolution for certain peak identification and ease of integration. With standard UV detection, all the derivatized amino acids have similar extinction coefficients to facilitate quantitative analysis. Sensitivity levels corresponding to nanograms of protein can be achieved routinely. The ruggedness of this turnkey system solution ensures rapid and unequivocal identification of proteins, with no interference or ambiguity. The pre-tested column, eluents and reagents ensure that the user will not spend time adjusting the method. The small amount of sample required for good analyses contributes to long column life and minimizes the chance of failure during a series of runs. The high resolution ensures reliable peak identification and quantitation so that runs need not be repeated. The pre-defined methods and reports simplify reporting of results. These analytical benefits are obtained with a short analysis time for the high throughput required for the routine determination of protein composition and concentration.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Monitoring Cell Culture Media with the UPLC Amino Acid Analysis Solution

Paula Hong, Thomas E. Wheat, Jeffrey R. Mazzeo, and Diane M. Diehl
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Cell culture techniques are routinely used to produce proteins intended for use as biopharmaceuticals. The culture conditions must be optimized to ensure that the protein is produced without structural modification and in the highest possible yield. These preferred conditions will often be different for each clone investigated, so a large number of optimization experiments may be required. This assessment of growth conditions must also consider the changes in the media that occur as a consequence of cell growth, that is, the consumption of nutrients and the release of waste products. The monitoring and optimization are complex because of the large number of physical and chemical parameters that have an effect. The experiments described here are focused on one particular class of components, the free amino acids.

Amino acids are important as the constituents of proteins, but they also serve as intermediates in many metabolic pathways. They are provided as individual amino acids in the growth media to satisfy both types of nutritional requirements. The concentration of amino acids in the media changes both from consumption of some amino acids and release of others by the growing cells. Monitoring these dynamic conditions is part of the optimization process, and the observed changes in concentration can be used to schedule a "feeding" of the culture or replacement of the medium. The Waters UPLC® Amino Acid Analysis Solution (Figure 1) provides a suitable way to monitor these changing nutrient levels.

The Waters UPLC Amino Acid Analysis Solution is a turnkey offering that encompasses instrumentation, derivatization chemistry, separation chemistry, software, and support. The solution includes defined conditions suitable for the assay of the amino acids commonly found in mammalian cell culture media. We show here the use of this defined method in monitoring a growing culture.

EXPERIMENTAL CONDITIONS

Conditions for derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Solution System Guide.

Samples of serum-free cell culture medium were obtained at daily time intervals from a bioreactor that was actively producing a biopharmaceutical protein. The medium was diluted 1:4 with 0.1 M HCl. A 10 µL aliquot of the dilution, with no additional sample preparation, was derivatized using the standard AccQ•Tag™ Ultra protocol.

LC conditions

LC System:	Waters ACQUITY UPLC® System with TUV detection at 260 nm
Column:	AccQ•Tag Ultra 2.1 x 100 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	700 µL/min
Mobile phase A:	1:10 Dilution of AccQ•Tag Ultra A concentrate
Mobile phase B:	AccQ•Tag Ultra B
Gradient:	AccQ•Tag Ultra Cell Culture Method
Injection volume:	1 µL

Figure 1. Waters UPLC Amino Acid Analysis Solution.

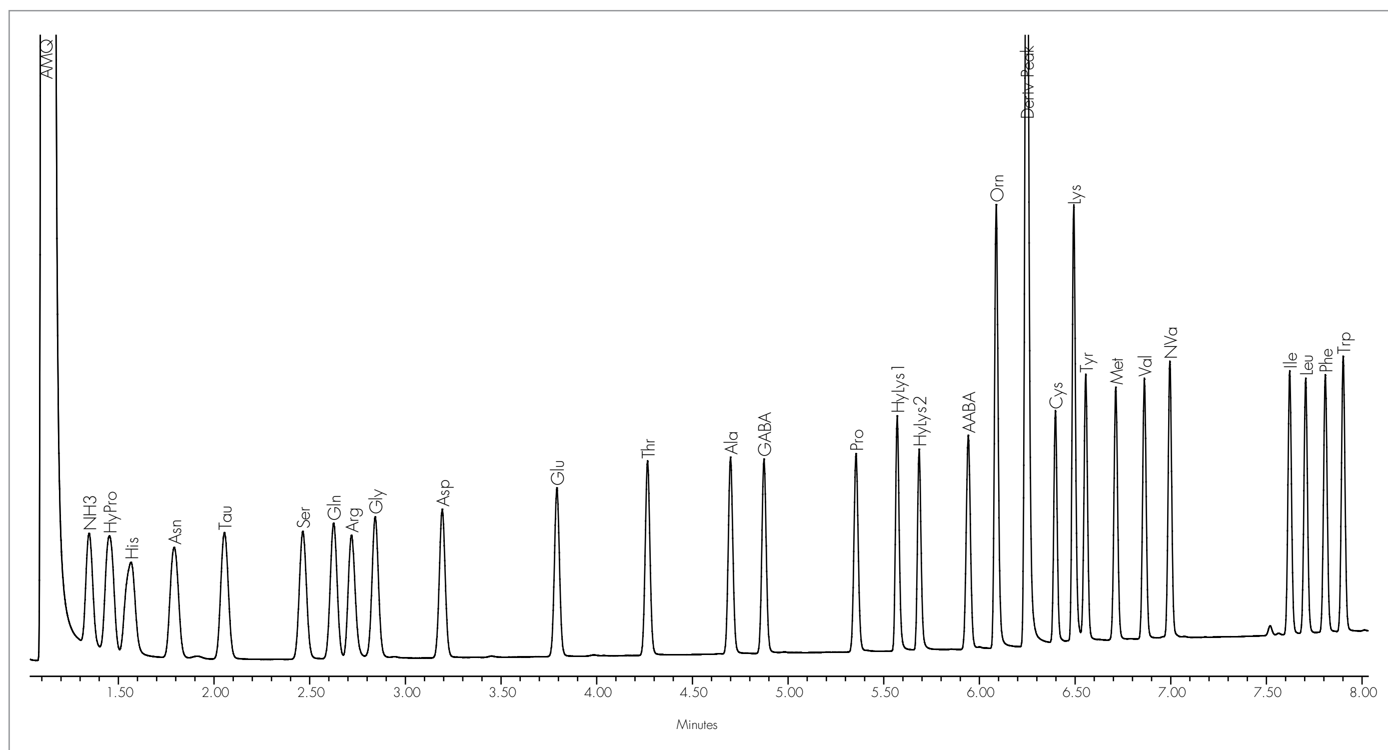

Figure 2. Analysis of standards of amino acids commonly found in cell culture media.

RESULTS

An analysis of amino acid standards representing the compounds commonly found in cell culture media is shown in Figure 2. This separation is obtained using the mobile phases and separation conditions that are part of the standard UPLC Amino Acid Analysis Solution. No adjustment of mobile phase pH or changes in composition are required. The resolution and reproducibility are sufficient for unambiguous peak identification and for reliable quantitation.

This method was applied to samples taken from an active bioreactor at daily intervals. These results are overlaid in Figure 3, and a second overlay in Figure 4 magnifies the region of the chromatogram which includes the amino acids that change most significantly during this growth experiment. The chromatographic characteristics observed with the standards are preserved with the authentic samples. The significant amino acids are readily identified and are sufficiently well-resolved for quantitation. There are a few small unidentified peaks that do not interfere with the amino acids.

The comparison of the 1, 3, and 6 day samples clearly shows the decline in concentration for some amino acids, notably glutamine, and the increase in others, such as alanine. These changes can be expressed quantitatively as plotted in Figure 5. All the amino acids can be quantitated, but only a few are shown as examples, including glutamine which increases in concentration with feeding.

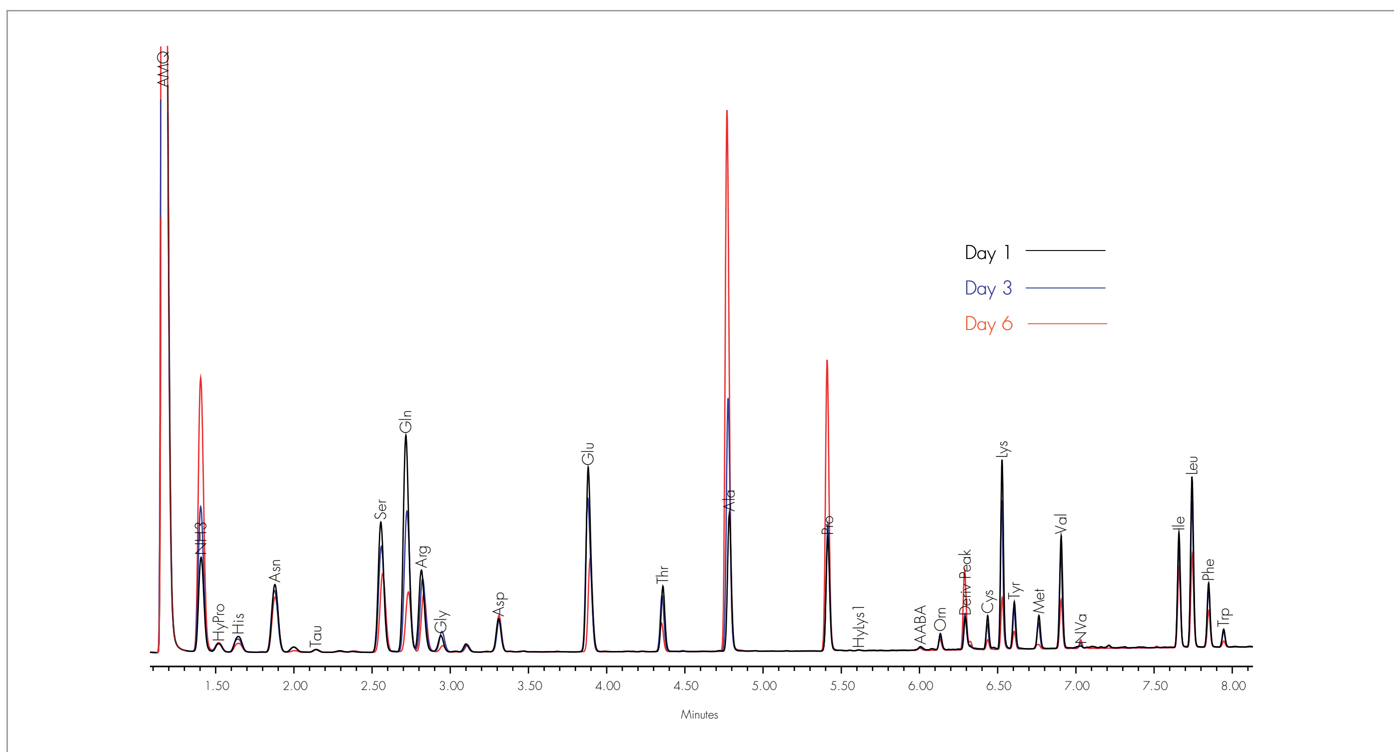

Figure 3. Analysis of amino acids in cell culture media after 1, 3, and 6 days of culture.

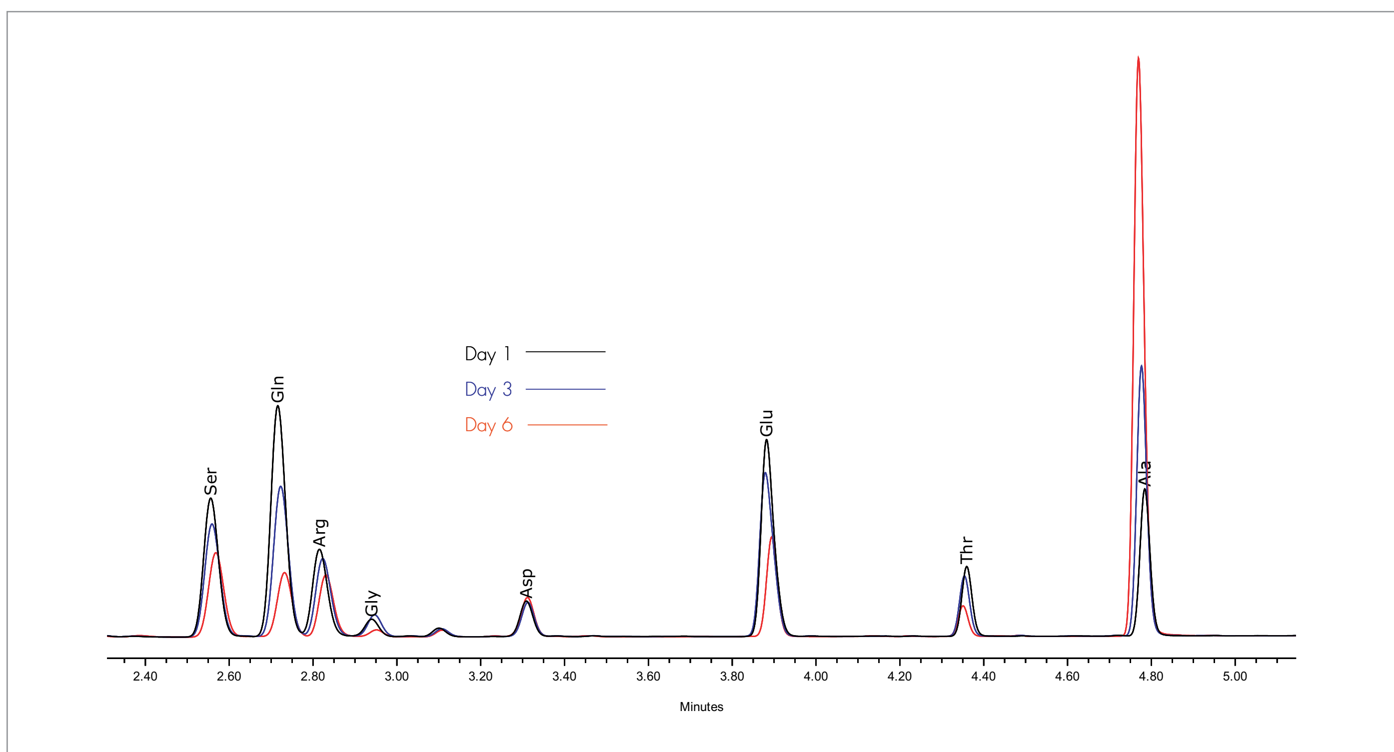

Figure 4. Analysis of critical amino acids in cell culture media after 1, 3, and 6 days of culture.

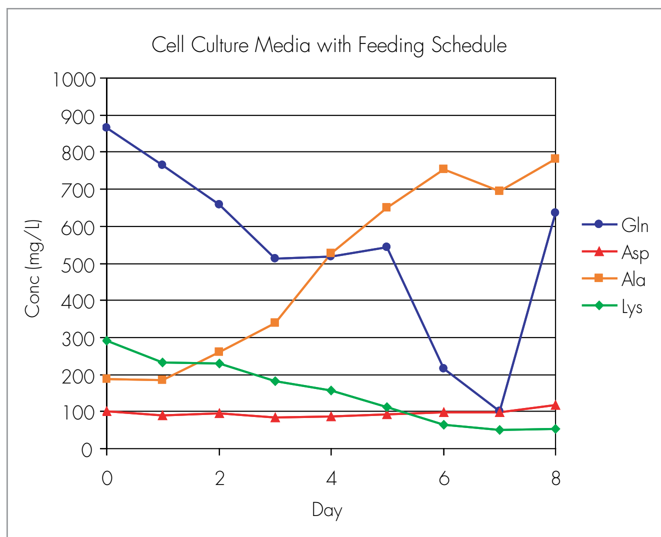

Figure 5. Quantitative trends in amino acid concentration during cell culture.

CONCLUSION

The Waters UPLC Amino Acid Analysis Solution has been used for the analysis of mammalian cell culture media. The standard method provides the chromatographic resolution required for peak identification. No sample preparation beyond simple dilution is required.

The analysis proves rugged and reproducible over a series of samples. No interferences are observed. The quantitative analysis is suitable for monitoring changes in concentration over time and for recognizing the proper time for a scheduled feeding. These analytical results are obtained with a short run time compatible with the high throughput requirements for optimizing growth conditions.

The Waters UPLC Amino Acid Analysis Solution provides a complete turnkey analytical method for monitoring amino acids in mammalian cell culture media. The pre-tested column, eluents and reagents ensure that the user will not spend time adjusting the method. The small amount of sample required for good analyses contributes to long column life and minimizes the chance of failure during a series of runs. The high resolution ensures reliable peak identification and quantitation so that runs need not be repeated. These analytical benefits are obtained with a short analysis time for the high throughput required for the optimization of cell culture conditions.

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34 Maple Street
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T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[TECHNICAL NOTE]

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Enhancement of the UPLC Amino Acid Analysis Solution with Flexible Detector Options

Hillary B. Hewitson, Thomas E. Wheat, and Diane M. Diehl
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The measurement of amino acids is important in many applications. Protein structure laboratories use it to confirm the identification and modification of proteins and peptides. Also, the sum of the amounts of amino acids gives the total concentration of the samples. Biopharmaceutical manufacturing facilities can optimize drug yield through careful monitoring and adjustments to the nutrient levels in cell cultures used in its production. In the animal feed industry, amino acid levels are measured as part of determining nutritional content. In each of these applications, it is essential to be able to quickly and accurately identify and quantitate amino acid levels. Incorrect results could result in poor batch yields, delay of product to market, or loss of product.

Waters provides a complete turnkey solution to meet the needs for each of these applications. The Waters UPLC® Amino Acid Analysis Solution was initially offered in 2006 as a total system solution that was available to users with a tunable UV (TUV) detector. Through the use of the application-specific quality tested columns, eluents, and derivatization chemistry, users can count on accurate results. Inclusion of pre-defined Empower™ software methods provides users with powerful data generation and handling capabilities and allows rapid analysis and reporting of sample results. Recently, photodiode array and fluorescence detection have been added as options in the defined system, providing the users with equipment flexibility to satisfy the requirements of their laboratories, while maintaining the same quality results regardless of which detection option is chosen.

Figure 1. Waters UPLC Amino Acid Analysis Solution

In this experiment, hydrolyzed samples of pure protein and of animal feed were analyzed using the Waters UPLC Amino Acid Analysis Solution with a TUV detector, photodiode array detector (PDA), and with fluorescence detection (FLR). Absolute amounts of amino acids as well as molar ratios were compared between TUV and FLR detection options for reproducibility, consistency, and accuracy as compared to expected values.

EXPERIMENTAL

Samples

Acid-hydrolyzed bovine serum albumin (BSA) and soybean meal samples were prepared in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl sealed under argon in ampoules. Samples were stored at -80 °C until analysis.

Sample Dilution and Derivatization

The supplied samples were diluted with 0.1 M HCl prior to derivatization, as necessary, to assure accurate pipetting and complete derivatization. The samples were derivatized in batches, and were stable for up to one week at room temperature when tightly capped. Conditions, including suggested neutralization, for pre-column derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Application System Guide. The following sequential modified derivatization conditions were used for these samples.

1. 60 µL AccQ•Tag™ Ultra Borate Buffer
2. 10 µL diluted sample
3. 10 µL 0.1 N NaOH
4. 20 µL reconstituted AccQ•Tag Ultra Reagent

Chromatographic Conditions

LC system: Waters ACQUITY UPLC® System
Column: AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm
Column temp.: 55 °C
Sample temp.: 20 °C
Flow rate: 700 µL/min
Mobile phase A: 1:20 Dilution of AccQ•Tag Ultra Eluent A Concentrate (prepared fresh daily)
Mobile phase B: AccQ•Tag Ultra Eluent B
Needle washes: Weak – 95:5 Water: Acetonitrile
Strong – 5:95 Water: Acetonitrile
Gradient: AccQ•Tag Ultra Hydrolysate Method (provided in the UPLC Amino Acid Analysis Solution)
Total run time: 9.5 min
Injection volume: 1 µL, Partial Loop with Needle Overfill (2 µL loop installed)
Detection: UV (TUV), 260nm
UV (PDA), 260nm, using 2D mode
Fluorescence (FLR),
λ_{Ex} 266 nm
λ_{Em} 473 nm

Acquisition and Processing Methods

The Waters UPLC Amino Acid Analysis Solution is provided with a CD that contains all the Empower methods necessary for acquisition and processing of the samples, as well as reporting of results. Details of the methods can be found in the Waters UPLC Amino Acid Analysis System Guide.

RESULTS AND DISCUSSION

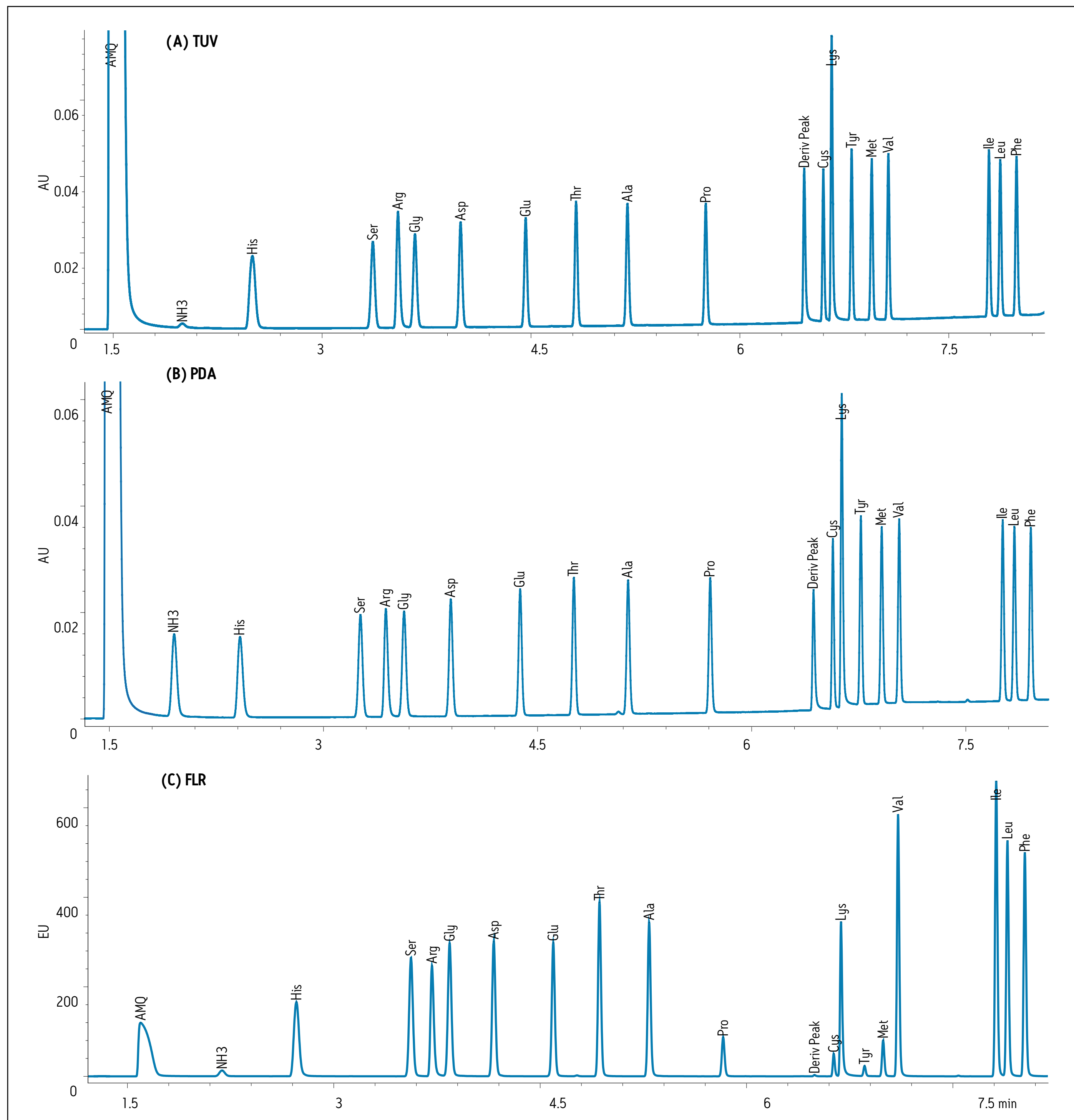

Figure 2. Analysis of Hydrolysate Standard, 10 pmoles of each amino acid on column (except Cystine at 5 pmoles), with (A) TUV detector, (B) PDA detector, and (C) FLR detector.

The operating conditions were optimized for each of the three detectors to give the highest signal-to-noise ratio. The results were compared, and a representative chromatogram of an amino acid hydrolysate standard is shown for each detector in Figure 2.

It has been widely accepted that a TUV is more sensitive than a PDA, and that a FLR is much more sensitive than UV detection. In addition, it is also believed in general that a FLR detector will give more selectivity, while a PDA can give UV spectral information to confirm peak identity and purity. The data was analyzed with these assumptions in mind, to see if they were true in this application solution.

The response for 10 pmoles on column is almost identical for the TUV and PDA detectors, while the FLR gives quite a different response. The TUV has lower noise than the PDA detector by approximately a factor of two, so the sensitivity as signal-to-noise is higher for the TUV by about the same factor.

With the FLR detector, we observe that the derivatives of the different amino acids have different fluorescence yields, and thus different sized peaks. The excitation and emission spectra are identical for all the amino acids. The differences do not seem to be related to spectral shifts. Tyrosine is the smallest peak in the fluorescence chromatogram, and, therefore, dictates the limit of quantitation. The usable range for both the TUV and FLR detectors in the application is 50 fmoles to 50 pmoles on column.

Peak identity and purity are often assessed based on spectral properties using a PDA detector. Figure 3 shows the UV spectra for five examples of AccQ•Tag derivatized amino acids, including acids, bases, neutrals, and doubly-derivatized molecules. The chemical distinctions between amino acids do not yield any useful spectral differences that could be used for peak identification. Therefore, the major value of using a PDA detector in the UPLC Amino Acid Analysis Solution is in the instrument flexibility created for other applications that require its use.

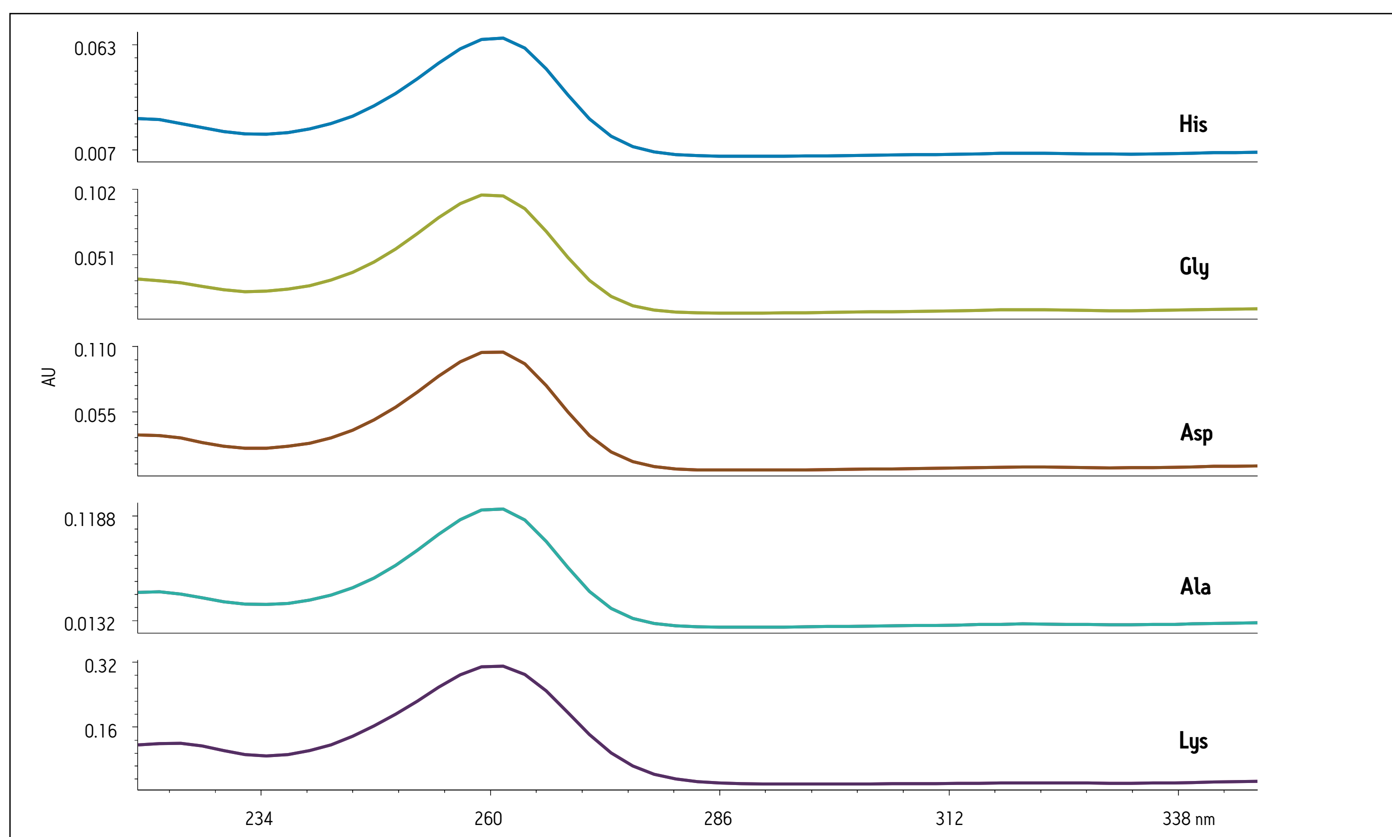

Figure 3. UV Spectra for various AccQ•Tag derivatized amino acids.

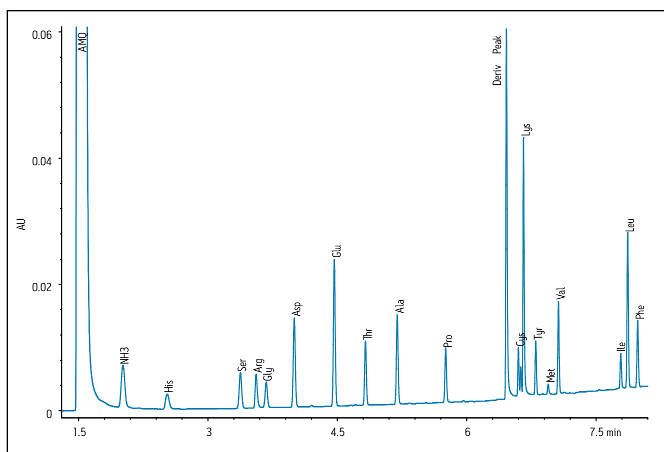

Figure 4. Analysis of BSA hydrolysate sample with UV detection, approximately 9 ng on column.

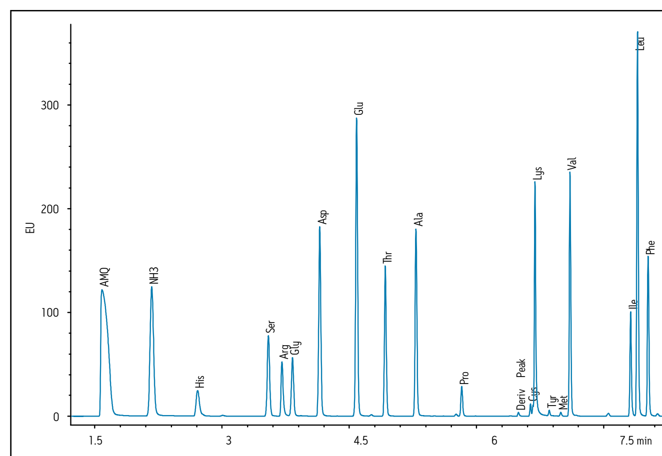

Figure 5. Analysis of BSA hydrolysate sample with fluorescence detection, approximately 9 ng on column.

Figures 4 and 5 show chromatograms with the same load of BSA hydrolysate on the column. Again, there is a difference in response for the amino acid peaks between the UV and fluorescence detectors. However, since the sample analysis is calibrated against a standard analyzed under the same conditions, no differences in the final result should be expected.

The accuracy of the results for both detectors is demonstrated by the quantitative results seen in Table 1. For all sample types, the 75 data points represent five days of analysis, each with independent sample dilutions, fresh mobile phase preparation, and each diluted sample derivatized five separate times, and injected in triplicate. The amino acid composition is expressed as residues per mole of BSA. Tryptophan and cysteine/cystine are excluded from the calculations because they are destroyed by the acid hydrolysis. The measured results for each detector match each other very well in addition to agreeing with the expected composition values.

Amino Acid	Expected Residues	*Observed Residues	
		TUV	FLR
His	17	15.36 ± 0.19	15.73 ± 0.16
Ser	28	26.00 ± 0.08	25.90 ± 0.41
Arg	23	22.37 ± 0.08	22.39 ± 0.20
Gly	16	17.68 ± 0.20	16.65 ± 0.42
Asp	54	55.47 ± 0.21	55.18 ± 0.32
Glu	79	80.68 ± 0.20	80.27 ± 0.44
Thr	33	31.92 ± 0.06	32.01 ± 0.07
Ala	47	47.51 ± 0.15	47.40 ± 0.16
Pro	28	28.35 ± 0.14	28.92 ± 0.13
Lys	59	57.78 ± 0.38	57.83 ± 0.99
Tyr	20	20.19 ± 0.08	20.67 ± 0.34
Met	4	4.16 ± 0.15	4.04 ± 0.05
Val	36	35.67 ± 0.13	35.38 ± 0.13
Ile	14	13.15 ± 0.16	13.44 ± 0.16
Leu	61	63.13 ± 0.28	63.18 ± 0.28
Phe	27	26.57 ± 0.13	27.00 ± 0.33

Table 1. Comparison of observed with expected composition derived from known sequence of BSA for both UV and fluorescence detection.

*Average of 75 data points (25 derivatizations, each injected in triplicate)

The analysis of complex animal feed hydrolysate samples with both UV and fluorescence detection is shown in Figures 6 and 7. As with the analysis of the BSA hydrolysate, the difference in response for amino acids between the detectors does not mean that one detector is more suitable for quantitation than the other. This fact is further supported by the comparison of measured absolute amounts of the same samples with both detectors. Table 2 shows the mean weight % values for both TUV and FLR for the 75 data points. The ratio of amount of each amino acid to amount of feed hydrolysate was expressed using the residue molecular weights of the amino acids. Since each analysis was calibrated relative to a standard with the same detector, the quantitative results are the same.

The reliability of the method is demonstrated with the reproducibility of the results over a large number of determinations that intentionally includes the variability that would be possible in routine analysis. These variations include multiple columns, eluents, and derivatizations. The largest contribution to variability in the method is due to the pipetting steps in the sample preparation. The addition of an internal standard to the sample to be hydrolyzed will correct for pipetting variability. Norvaline is the preferred internal standard for this purpose.

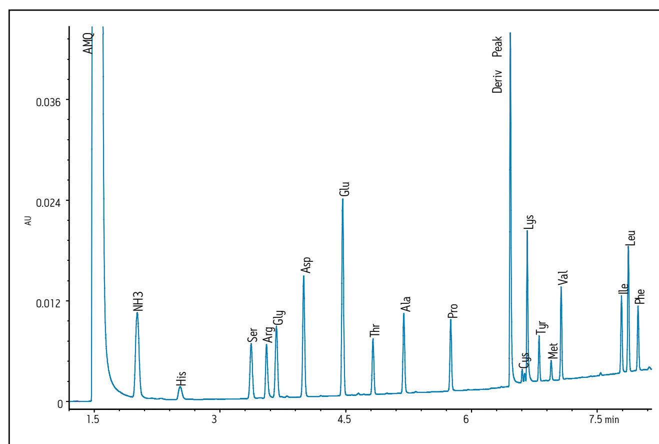

Figure 6. Analysis of soybean meal hydrolysate sample with UV detection, approximately 6 ng on column.

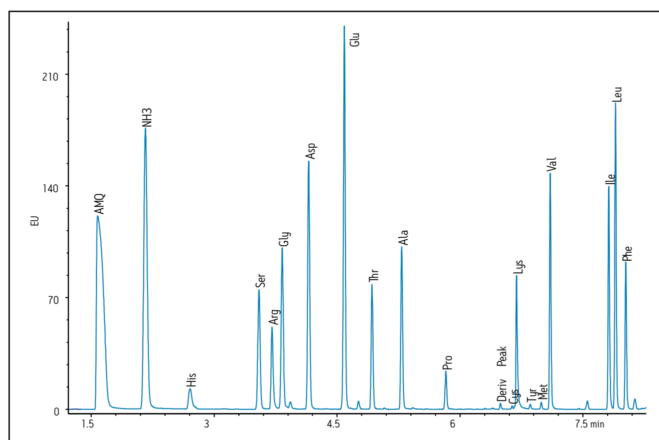

Figure 7. Analysis of soybean meal hydrolysate sample with fluorescence detection, approximately 6 ng on column.

	*Combined Mean	
Amino Acid	TUV	FLR
His	1.87 ± 0.06	1.98 ± 0.13
Ser	3.65 ± 0.07	3.58 ± 0.21
Arg	5.82 ± 0.13	5.74 ± 0.35
Gly	2.98 ± 0.07	2.87 ± 0.16
Asp	9.06 ± 0.17	8.92 ± 0.54
Glu	14.49 ± 0.28	14.36 ± 0.87
Thr	2.92 ± 0.06	2.90 ± 0.17
Ala	3.17 ± 0.08	3.15 ± 0.18
Pro	3.86 ± 0.08	3.87 ± 0.23
Lys	4.76 ± 0.11	4.80 ± 0.32
Tyr	2.90 ± 0.07	3.06 ± 0.21
Met	1.08 ± 0.03	1.08 ± 0.06
Val	3.66 ± 0.07	3.67 ± 0.21
Ile	3.45 ± 0.07	3.48 ± 0.20
Leu	6.12 ± 0.13	6.10 ± 0.35
Phe	3.92 ± 0.09	3.94 ± 0.22

Table 2. Weight/Weight % Comparison of TUV and FLR results for soybean meal hydrolysate; approximately 6 ng hydrolysate injected on column.

*Average of 75 data points (25 derivatizations, each injected in triplicate) ± Standard Deviation

CONCLUSION

The Waters UPLC Amino Acid Analysis is extended to three detector choices: TUV, PDA, and FLR. All three detectors give the same qualitative and quantitative result.

Historically, fluorescence detection has often been desired in amino acid analysis to provide enhanced sensitivity and to give specificity in the analysis of complex samples. The low variable fluorescence yield for the amino acids means that sensitivity is limited to the least responsive amino acid, specifically tyrosine. The analyses of pure protein and complex animal feed hydrolysates in this experiment shows that fluorescence and UV detectors both give accurate and consistent results with the Waters UPLC Amino Acid Analysis Solution.

It is generally true that cleanliness limits the usable sensitivity in any amino acid analysis method. Both the UV and fluorescence detectors give good analytical results well below the typical background limits. The Waters UPLC Amino Acid Analysis Solution provides a complete turnkey analytical method for the analysis of hydrolysate samples that allows the selection of a detector that not only meets the needs of the application, but also that of other assays in the laboratory as well. Regardless of the detector option chosen for the application, the ruggedness of the total system solution ensures highly reliable and rapid identification and quantitation of amino acids, with no interference or ambiguity.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Characterizing Polyethylene Glycol (PEG) by SYNAPT High Definition Mass Spectrometry (HDMS)

Petra Olivova, Weibin Chen, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Polyethylene glycol (PEG) is a polymer composed of repeating subunits of ethylene oxide. PEG and its functionalized derivatives can be formed in linear or branched shapes with different molecular masses, resulting in significant material complexity and diversity.

Due to the many unique properties of PEG materials – highly water soluble, non-toxic – PEG is often attached to biopharmaceuticals (i.e., PEGylation) to improve pharmacological properties. It is critically important to determine the quality of a batch of PEG prior to attaching it to a biopharmaceutical. Attaching a low-quality batch of PEG to a biopharmaceutical leads to poor end product performance, and increases costs because the final product does not meet specifications.

Because of the complexity associated with PEG materials, PEG characterization by conventional methods has been extremely challenging. In this application note, we present a method to characterize PEG using the Waters SYNAPT™ High Definition MS™ (HDMS™) System, a novel instrument that combines high-efficiency ion mobility (IMS) based measurements and separations with high performance tandem mass spectrometry. The additional ion mobility based gas-phase separation of the system provides a unique method to examine – in great detail – the composition of PEG materials. This better enables analysts to identify potential contaminants contained in the material and thus assess the quality of the material, providing for more confidence in the release of a PEGylated biopharmaceutical product.

EXPERIMENTAL

PEG 4450 was obtained from a Waters GPC molecular weight standard kit (part number WAT035711). PEG 20000 was purchased from Sigma [20% (w/v)]. The polymers were prepared at a concentration of 0.5% (w/v) in 50:50 acetonitrile/H_2O for mass spectrometric analysis. Samples were introduced to MS directly by infusion, using a syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 5 µL/min.

MS conditions

MS system:	Waters SYNAPT HDMS System
IMS gas:	N_2 gas
IMS gas pressure:	0.8 mbar
Pulse height:	Variable, 7 to 15 V
Ionization mode:	ESI positive
Capillary voltage:	3200 V
Cone voltage:	40 V
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Source temp.:	150 °C
Acquisition range:	100 to 4000 m/z
Trap collision energies:	8 V

RESULTS

An electrospray ionization time-of-flight (ESI-TOF) mass spectrum of PEG 4450 [0.5% (w/v) in 50:50 H_2O/ACN solution] results in a distribution of several charge envelopes (Figure 1, left panel). Each charge envelope contains multiple peaks representing a molecular weight distribution of the material. The overlap between each of the charge states, the polydiperse nature of the material, and the presences of low molecular weight PEGs/contaminants all make the complete characterizations of the material via conventional ESI-TOF a formidable task to undertake, even for a medium-size PEG.

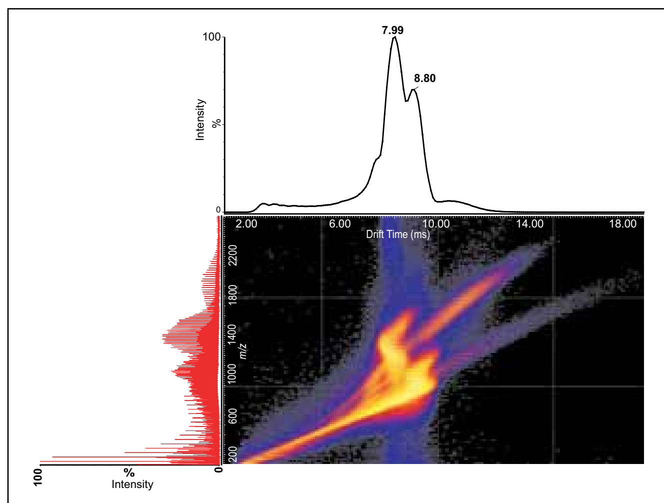

Figure 1. Data for analysis of PEG 4450 using the SYNAPT HDMS System, displayed in DriftScope™ Software. Each pixel represents an ion, with color representing its intensity (blue-low, to orange-high). To the left of the plot is the ESI-TOF spectrum without IMS separation. On the top of the DriftScope plot is the composite IMS spectrum from the projection of DriftScope on the drift time axis.

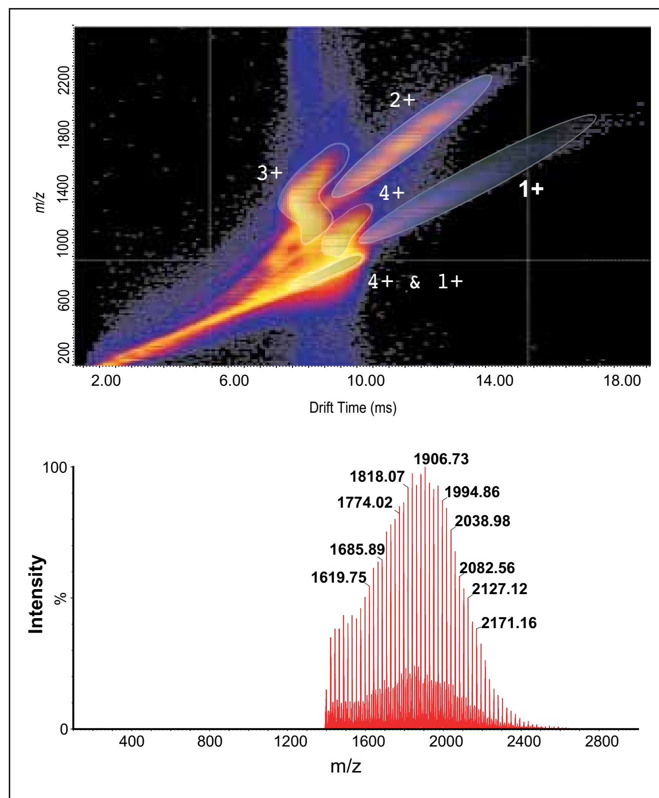

Figure 2. Analysis of PEG 4450 using HDMS. Top panel: HDMS data show the gas-phase separation power of the SYNAPT HDMS System in the analysis of PEG 4450. Components with different charge states are separated via ion mobility, thus enabling the examinations of different (minor) components in the PEG materials. Bottom panel: Mass spectrum showing the ions with +2 charge state.

By analyzing the sample in HDMS mode (IMS-MS), Triwave™ Technology can be used to rapidly separate components in complex mixtures in tens of milliseconds. Here, we have taken advantage of this capability to separate complex PEG ions formed during the ionization process. In these experiments, the time required for IMS separations is <20 ms. Ions with different charge states, or different conformers of the same m/z ions, were readily resolved by IMS (Figure 2). The separation greatly simplifies the complexity of the spectrum such that some of the minor components in the samples that cannot be observed otherwise can be easily identified from the sample (Figure 3).

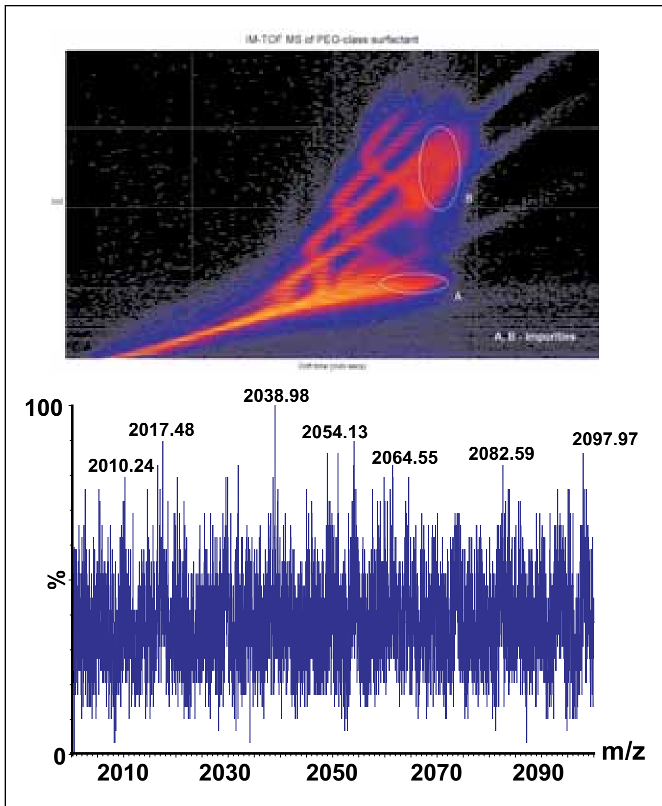

Figure 3. Analysis of PEG 20,000 using HDMS. Top panel: Using DriftScope Software, the HDMS data exhibit the separation of potential impurities (labeled as circle A and B) contained in the PEG materials from the rest of PEG components. These impurities would not be readily discovered without the gas-phase separations. Bottom panel: Zoomed mass spectrum showing the ions in circled region B. The mass difference between neighboring peaks indicated that they are not pure PEG material.

CONCLUSION

By employing IMS separations in HDMS mode with the SYNAPT HDMS System, the general molecular weight distribution of PEG material used in biopharmaceuticals can be rapidly assessed and potential contaminants in the materials can be quickly identified. Fast, more detailed characterizations of PEG are readily achieved. With the level of analytical detail provided by the SYNAPT HDMS System, analysts can be more confident that their PEGylated biopharmaceutical product will pass quality control tests towards product release.

The consequence of attaching a low-quality batch of PEG to a therapeutic protein is failure of the bioactivity test and the need to scrap a batch of very expensive product.

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

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RapiGest SF Surfactant: An Enabling Tool for In-Solution Enzymatic Protein Digestions

Ying Qing Yu and Martin Gilar
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

In this application note, we provide an overview of the physical and chemical characteristics of Waters' patented RapiGest™ SF and illustrate selected application areas. First introduced in 2002 as an enzyme-friendly surfactant to assist in-solution protein digestion, RapiGest SF is an innovative product created to improve protein solubility during sample preparation.

The mechanism by which RapiGest SF improves the speed and completeness of digestion is illustrated in Figure 1. Mild protein denaturation opens protein structure and exposes the proteolytic sites to enzymatic cleavage. Enzymes are more resistant to denaturation than common proteins and remain active in RapiGest SF solutions. More complete denaturation of globular proteins can be accomplished by heating in a RapiGest SF solution at elevated temperatures prior to the addition of enzymes and incubating the sample with the enzyme at 37 °C.[1,2]

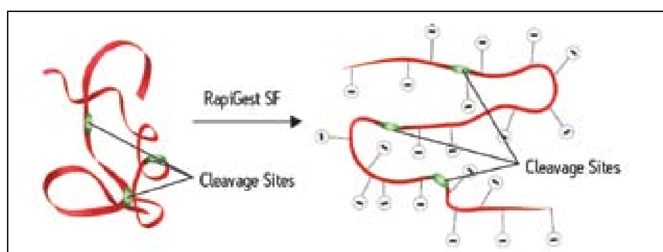

Figure 1. The protein substrate unfolds in RapiGest SF solution and becomes more amenable to proteolytic cleavage.

More than 200 peer-reviewed journals have cited the use of RapiGest SF for general sample solubilization purposes, mostly for proteomic applications. Recently, many pharmaceutical laboratories have adopted RapiGest SF for biotherapeutic protein characterization. Because of improved digestion and easy surfactant removal prior to LC and MS analysis, RapiGest SF has become widely accepted for many applications, including high sequence coverage LC-UV/MS peptide mapping of therapeutic proteins.

DISCUSSION

What is RapiGest SF?

RapiGest SF is an acid labile surfactant that undergoes hydrolysis in acidic conditions.[1] This unique feature can be utilized to remove the surfactant from solutions when desired. The structure of RapiGest SF and its byproducts from the acid hydrolysis are shown in Figure 2. The acid labile property facilitates a nearly complete surfactant degradation of within about 45 minutes at pH 2.[1]

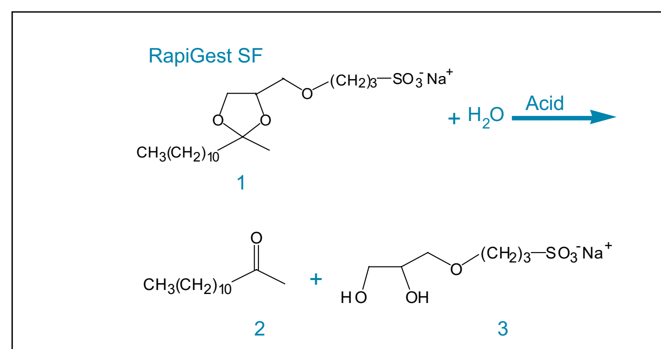

Figure 2. RapiGest SF (1) degrades in acidic solution to (2) and (3). The $t_{1/2}$ is 7.6 minutes at pH 2.

The surfactant decomposes into two products, dodeca-2-one and sodium-3-(2,3-dihydroxypropoxy) propanesulfonate. The first compound is water immiscible, and can be removed by centrifugation. The second degradant is highly soluble in aqueous solutions and practically unretained in reversed phase LC mode. The aqueous fraction of enzymatic digest can be directly analyzed by HPLC, LC/MS, or by MALDI-TOF MS.

Removal after digestion

No additional detergent removal steps are required (e.g., dialysis) prior to sample analysis. The enzymatic digestions are typically acidified with acids such as formic acid, trifluoroacetic acid (TFA), or hydrochloric acid (HCl) to degrade RapiGest SF prior to further analysis. The recommended pH for degradation is ≤ 2.

Compatibility with tryptic digestion

Trypsin is the most common proteolytic enzyme used for peptide mapping and proteomic applications. We have investigated trypsin activity in the presence of RapiGest SF and compared it to most common denaturants cited in literature. The assay was based on trypsin induced hydrolysis of N-α-benzoyl-L-arginine ethyl ester (BAEE) in 50 mM ammonium bicarbonate (pH 7.9) at room temperature. Changes in trypsin activity were calculated by measuring the rate of BAEE hydrolysis at UV 253 nm. Trypsin activity in the selected denaturant solution was compared against the control sample (no denaturant). The results are shown in Table 1.

Trypsin solution[A]	Trypsin activity[B] (%)	Trypsin solution[A]	Trypsin activity[B] (%)
No additive	100	50% Methanol	31
0.1% RapiGest	100	50% Acetonitrile	92
0.5% RapiGest	87	1 M Urea	97
0.1% SDS	20	2 M Urea	83
0.5 SDS	1	0.5 M Guanidine HCl	21
0.1 RapiGest/0.1% SDS	58	1 M Guanidine HCl	8

Table 1. Trypsin activity measured in the presence of selected denaturants.
A. 0.5 μg of trypsin was added to 1 mL of 50 mM ammonium bicarbonate, pH 7.9, containing 0.2 mM of BAEE.
B. Measured as delta BAEE absorbance at 253 nm (slope within 5 minutes).

The data in Table 1 suggest that at low concentrations (0.1%), RapiGest SF does not inhibit trypsin activity. This contrasts with structurally-similar surfactant SDS, which appears to be a strong denaturant and inactivates the trypsin. Urea, acetonitrile, or guanidine-HCl were also proposed as denaturants for tryptic digestions. However, acetonitrile is a strong eluent and interferes with reversed phase LC analysis of digested sample. Urea is known to cause covalent modification of proteins, and Guanidine-HCl inactivates enzyme, similarly to SDS.

The implication from this experiment is that enzyme proteolysis activity can be affected by the denaturant used to solubilize the protein samples. Using RapiGest SF at low to high concentrations does not alter the enzyme activity; therefore, optimum proteolysis digestion is achieved without using an excess of enzyme.

Fast proteolytic digestions

Proteins that are resistant to proteolysis can be digested within minutes using RapiGest SF. A complete protein digestion for a globular protein, horse myoglobin, was achieved within 5 minutes. The comparison of results for surfactant-aided and control digestion is shown in Figure 3. Due to its globular nature, myoglobin is known to be difficult to digest without the use of any denaturant. In the control reaction, only a small fraction of protein is digested after 9 hours of incubation with trypsin. The overall digestion efficiency significantly improved when using RapiGest SF.

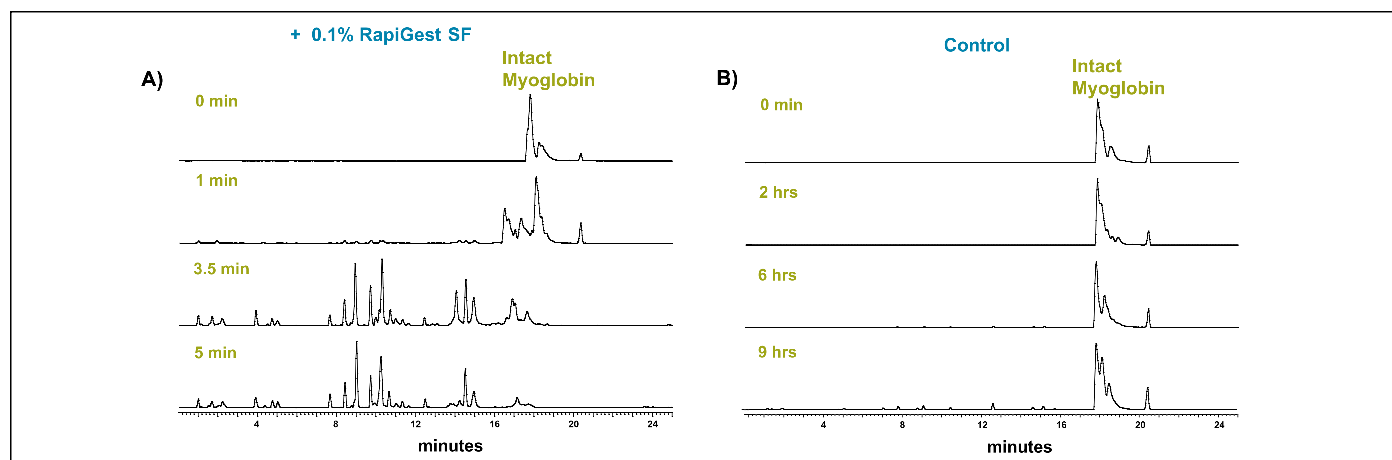

Figure 3. LC/MS total ion chromatograms of tryptic digest of horse myoglobin, (A) solubilized with 0.1% RapiGest SF, and (B) control digestion (no denaturant). Myoglobin digestion in 0.1% RapiGest SF solution provided complete tryptic digestion within 5 minutes, while control digestion remains incomplete even after 9 hours.

Improved sequence coverage in peptide mapping of therapeutic proteins

RapiGest SF has been widely used in proteomics sample preparation as an effective denaturant for protein solubilization. Recently, more biopharmaceutical labs have adopted RapiGest SF in their peptide mapping protocols. Several publications document the benefits of using RapiGest SF for therapeutic protein digestion.[4,5] The reported RapiGest SF concentration used ranges from 0.05 to 1% depending on the protein hydrophobicity and concentration.

We have found that a 0.05 to 0.1% concentration of RapiGest SF is sufficient to denature various sizes of proteins; higher concentration of RapiGest SF may be suited for a whole cell protein extraction type of experiment.

Peptide mapping of monoclonal antibodies (mAbs) is challenging due to the difficulty of digesting these large and hydrophobic proteins. The goal of peptide mapping analysis is to confirm the protein sequence and identify all present post-translational modifications (PTMs). Figure 4 shows an example of RapiGest SF-assisted digestion of humanized mAb. The parameters of sample preparation and analysis by UPLC® and quadrupole time-of-flight MS are listed as guidelines.

The overall sequence coverage in the experiment shown in Figure 4 was 98%. Data analysis was performed with BiopharmaLynx™ Software, v.1.2. The high sequence coverage (98%) indicates a complete digestion of mAb. No intact protein or large miscleaved peptides were detected in LC/MS analysis. The remaining 2% of unaccounted sequence belong to a few two amino-acid-long peptides or to a single amino acid (R or K) that are unretainable on the reversed phase column.

Sample preparation

Humanized mAb sample (10 µL, 21 mg/mL) was solubilized in 50 µL 50 mM ammonium bicarbonate containing 0.1% (w/v) RapiGest SF. 2 µL of 0.1 M dithiothreitol (DTT) was added to the sample, and the sample was heated at 50 °C for 30 minutes. 4 µL of 0.1 M iodoacetamide was added to the sample, after it was cooled to room temperature, and the sample was placed in the dark for 40 minutes. 8 µg of trypsin was added to the sample (trypsin conc. = 1 µg/µL) and the sample was incubated at 37 °C overnight. The digested sample was mixed with 1% formic acid in 10% acetonitrile (1:1, v:v). The sample was diluted to 5 pmol/µL with Milli-Q water (Millipore) prior to LC/MS analysis.

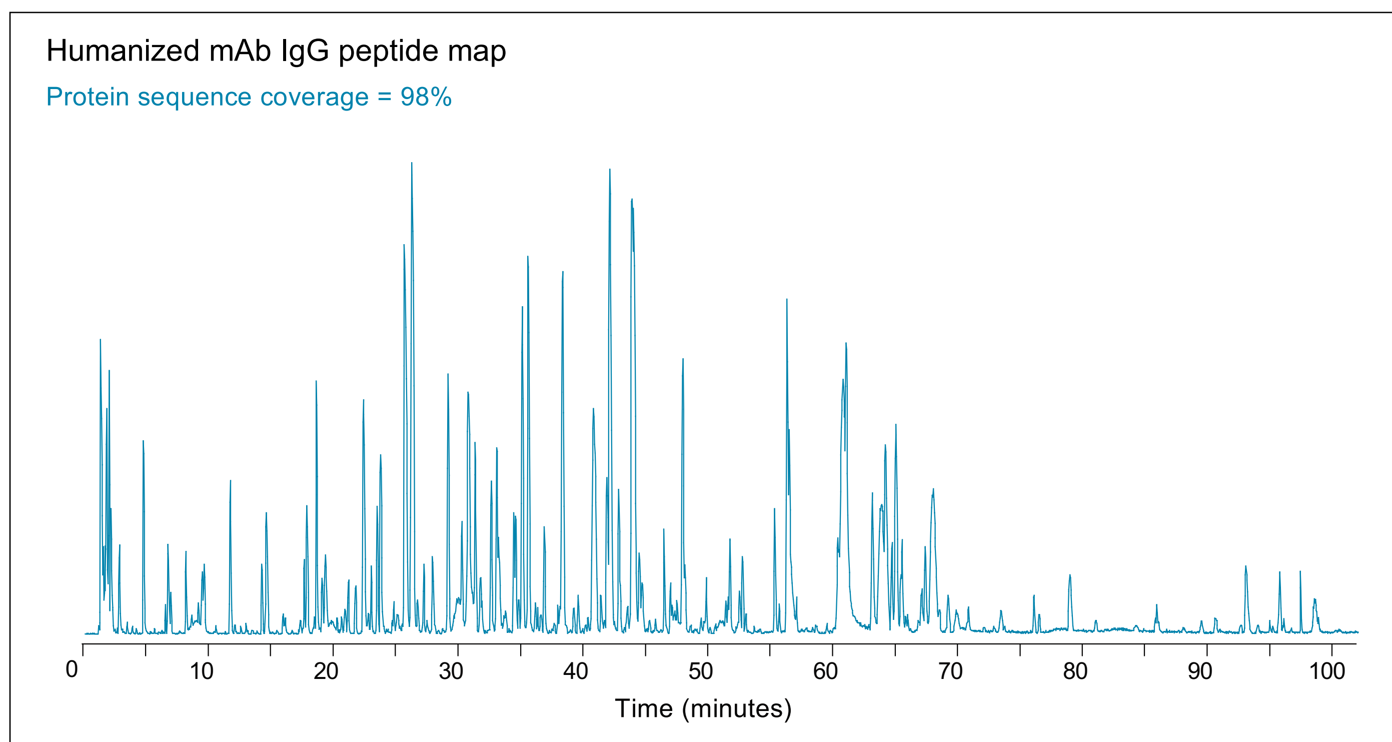

Figure 4. LC/MS analysis of a tryptic digest of humanized mAb. The sample preparation complexity was significantly reduced; no post-digestion cleanup is required. A total of 10 pmol of tryptic mAb was injected.

LC conditions

LC system: Waters ACQUITY UPLC® System
Column: ACQUITY UPLC BEH 300 C_{18} Peptide Separation Technology Column, 2.1 x 100 mm (P/N = 186003686)
Column temp.: 40 °C
Sample injected: 2 µL (10 pmol)
Solvent A: 0.1% formic acid in water
Solvent B: 0.1% formic acid in acetonitrile
Flow rate: 200 µL/min
Gradient: 0 to 2 min: 2% B
2 to 92 min: 2 to 35% B
92 to 102 min: 35 to 50% B
102.1 to 105 min: 90% B
105.1 to 110 min: 2% B

MS conditions

MS system: Waters SYNAPT™ MS (V mode)
Capillary voltage: 3.2 kV
Source temp.: 120 °C
Desolvation temp.: 350 °C
Desolvation gas: 700 L/hr
MS scan rate: 1 sec/scan
Lock Mass channel: 100 fmol/µL Glu-Fib peptide (m/z 785.8426, z = 2), flow rate 20 µL/min

Use with additional proteolytic enzymes

We tested RapiGest SF compatibility with multiple proteolytic enzymes, for example, Asp-N, Lys-C, and Glu-C. Efficient digest results were obtained using RapiGest SF to denature the protein prior to proteolysis (Figure 5).

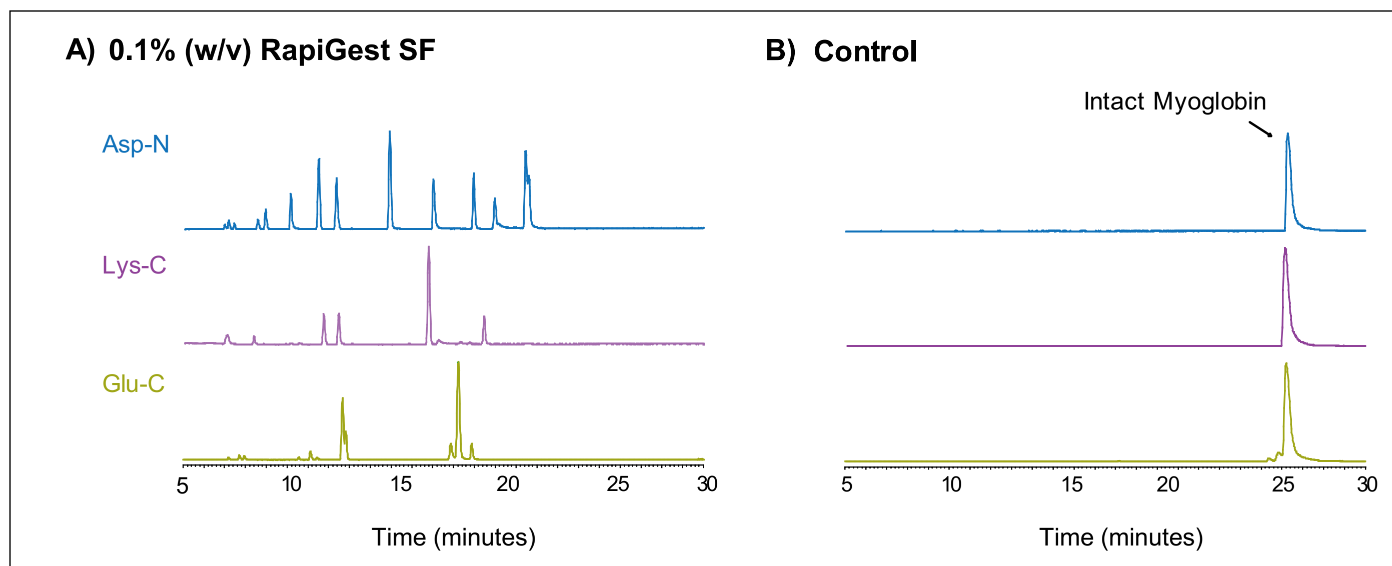

Figure 5. Horse myoglobin (50 pmol/µL) digestion with Asp-N, Lys-C, and Glu-C, with or without 0.1% (w/v) RapiGest SF.
A. LC/MS analysis after 1 hour incubation at 37 °C with 0.1% RapiGest SF; no intact protein was left undigested.
B. The control experiment (no surfactant) showed that majority of the myoglobin remains undigested.

Use for protein deglycosylation

RapiGest SF was also tested with other enzymes such as PNGase F, which is used to cleave N-linked glycans from glycoproteins.[2] Figure 6 illustrates the deglycosylation of chicken ovalbumin. Complete deglycosylation was observed after 2 hours in RapiGest SF-mediated digestion with PNGase F.

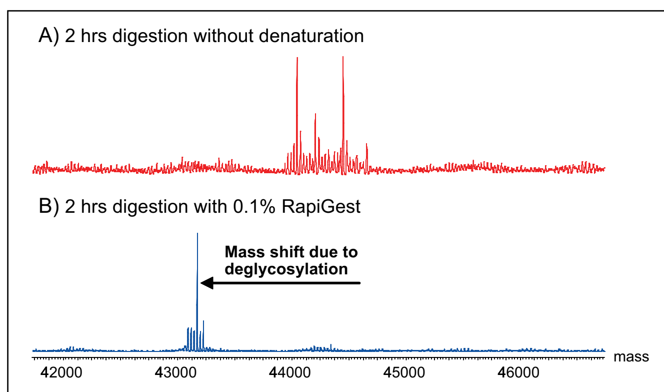

Figure 6. Chicken ovalbumin was deglycosylated with PNGase F without denaturation (A) and with 0.1% of RapiGest SF (B). The main signal in the deconvoluted LC/MS spectrum (B) represents ovalbumin without glycans (MW is consistent with amino acid composition). The heterogeneous MS signals in deconvoluted spectrum (A) indicates the presence of several glycoforms. The majority of glycans were not released from protein even after 2 hours of digestion. For details, see reference 3.

CONCLUSIONS

- RapiGest SF improves the speed and completeness of protein enzymatic digestions, enabling high-sequence-coverage peptide mapping of therapeutic proteins.
- RapiGest SF is a proven denaturant suitable for proteomic, glycomic, and biotherapeutic application areas.
- Minimal or no post-digestion sample preparation is required. Simple acidification of the sample is sufficient to remove RapiGest SF from solutions. In many cases, a simple dilution is acceptable prior to LC/MS analysis.
- RapiGest SF simplifies preparation protocols and improves throughput of analyses: its use improves laboratory productivity and overall data quality.

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

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UPLC for the Analysis of Synthetic Peptides and for the Development of Isolation Strategies

Joomi Ahn, Ziling Lu, Thomas E. Wheat, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Peptides are used in many areas of basic research as well as in biopharmaceutical development.

- A peptide representing a structural feature of a protein may be used to probe specific functional properties.
- An antigenic determinant may be represented by a peptide for developing vaccines or for studying the immune response.
- Peptides may be substrates for enzymes.
- Many peptides play important roles in the physiology of organisms.

In all these applications, it is common and practical to use synthetic peptides rather than isolating natural products. The use of synthetic peptides is preferred because they can be obtained in any required quantity. It is easy to modify the sequence to analyze function, and the identity and purity can be assured. The last point is particularly important since an impure peptide, heterogeneous at even a trace level, can make the results of other biological or chemical studies ambiguous.

Because the range of contaminating deletion sequences and side reaction products can be large, it is very important to use the highest possible chromatographic resolution to ensure that no contaminant coelutes with the main peak. It is, therefore, very useful to apply UltraPerformance LC® (UPLC®) to obtain the best resolution and sensitivity in the minimum runtime.

The enhanced chromatographic resolution associated with UPLC has been demonstrated for peptide mapping (Mazzeo, et al., *BioPharm.*). The technique improves resolution by a factor of three or more. In addition, the surface chemistry of the BEH Technology™ used in UPLC has proven especially advantageous for peptide mapping. Good retention and peak shape are observed with either TFA or formic acid as a modifier.

These chromatographic characteristics will be useful for synthetic peptides, but there are additional, special requirements. With peptide mapping, the relevant sample components represent a very wide range of chemical and physical properties. Since the map must accommodate all of these components, long, shallow gradients are used. In contrast, synthetic peptides involve a single structure with the contaminants all being closely related structures. It is, therefore, reasonable for the analytical method to be a segmented gradient that is very shallow at the point where the product elutes.

Steep gradients can be used to reduce runtime during regeneration and re-equilibration. UPLC should be especially useful for providing the high resolution and high throughput analyses desired for assessing the purity of synthetic peptides. The analytical conditions used in these experiments could also predict the conditions for purifying the peptide if the chemistry was matched in a larger scale column. These experiments test and demonstrate this process with a 23-residue synthetic peptide.

MATERIALS AND METHODS

Sample preparation

A synthetic peptide was purchased as a custom product. The target sequence was: YPIVSIEDPFAEDDWEAWSHFFK. The expected molecular weight is 2827.3. For analytical experiments, it was dissolved in 0.1% TFA containing 10% acetonitrile to a concentration of 0.2 mg/mL. For isolation, it was dissolved in 0.05% TFA containing 10% acetonitrile to a concentration of 9 mg/mL. For fraction analysis, the collected samples were directly injected.

Analytical chromatography

Instrument: Waters ACQUITY UPLC® System
Waters TUV Detector
Waters ZQ™ 2000 MS Detector

Column: Waters ACQUITY UPLC BEH 130 C_{18}
1.7 µm, 2.1 x 100 mm

Flow rate: 0.2 mL/min

Column temp.: 40 °C

Mobile phase: (A) H_2O with 0.02% TFA
(B) ACN with 0.018% TFA

Gradient Table (Vary Segment Start; 3.0%/col. vol.)		
Time	%A	%B
0	100	0
2	72*	28*
6.6	64*	36*
8.6	10	90
11.6	10	90
12.1	100	0
29.4	100	0

(*28-36;*30-38;*33-41)

Gradient Table (Vary Slope)		
Time	%A	%B
0	100	0
2	72	28
6.6	64	36
8.6	10	90
11.6	10	90
12.1	100	0
29.4	100	0

0.25%/col. vol. – 55.3 min
0.75%/col. vol. – 18.45 min
1.50%/col. vol. – 9.2 min
3.00%/col. vol. – 4.6 min

Isolation

Instrument: Waters 600 Multisolvent Delivery System
2767 Sample Manager
2996 PDA with Flow Cell
AutoPurification

Column: Waters Peptide Separation Technology
XBridge™ BEH 130 Prep C_{18}
5 µm, OBD™ 19 x 150 mm

Flow rate: 17.0 mL/min

Column temp.: Ambient

Mobile phase: (A) H_2O
(B) ACN
(C) 1% TFA in Water

Gradient Table			
Time	%A	%B	%C
0	93	5	2
2	70	28	2
15	62	36	2
19	48	50	2
19.1	8	90	2
21	8	90	2
21.1	93	5	2
30	93	5	2

RESULTS

Development of analytical method

In developing a rapid analytical method for synthetic peptides, two parameters must be defined: the range of the shallow gradient segment and the slope of the shallow gradient segment. Estimating acetonitrile concentration required to elute a given peptide, the samples are screened with a fast gradient. The known offset between the time observed at the detector and the gradient table defines the elution range (Jablonski and Wheat; Waters Application Note 720000920EN).

This peptide elutes near 35% acetonitrile. A window of ±4% is often useful. To define more closely the exact conditions, 8% segments starting at different points were compared (Figure 1). When the separation segment begins at a lower concentration, the main peak elutes later, as expected, and there are changes in the patterns of the surrounding contaminants.

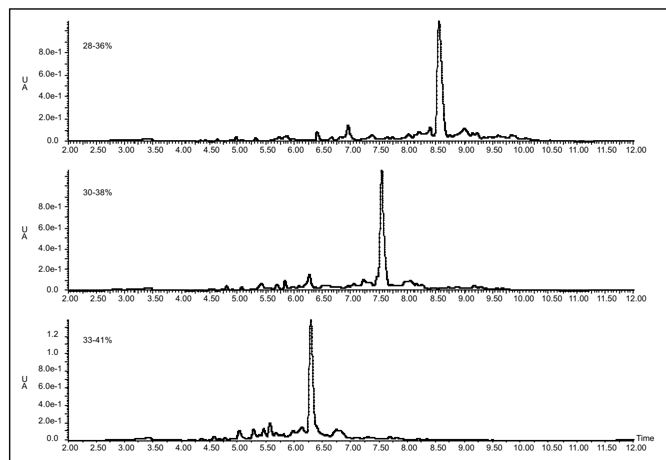

Figure 1. Optimizing the gradient segment for peptide analysis. The synthetic peptide was separated with UPLC at a gradient slope of 3%/column volume over 8% segments beginning at 28, 30, and 33% acetonitrile. Separations were monitored with UV at 214 nm.

To ensure that the product peptide is correctly identified and is well-separated from all the contaminants, the separation was monitored with MS in addition to UV (Figure 2). Significant changes in selectivity can be recognized in this way. Most important, the elution position of one contaminant, m/z 929, varies relative to the desired product peak with starting point of the shallow segment. The most useful analytical gradient runs from 28 to 36% acetonitrile, and that range is used in all subsequent experiments.

The development of the analytical method also requires definition of gradient slope over the narrow segment. It is generally recognized that peptides are best resolved with relatively shallow gradients with a useful range from 0.25%/column volume to 3%/column volume. Better resolution is obtained with shallower gradients, but the run times are longer and elution volumes larger.

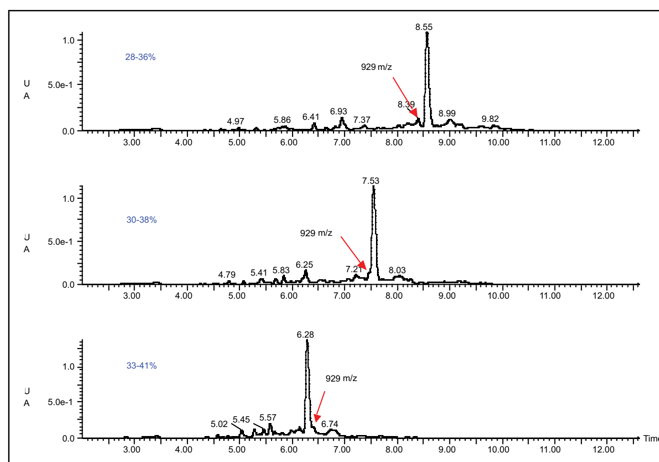

Figure 2. Optimizing the gradient segment for peptide analysis with peak tracking. The same experiments shown in Figure 1 were monitored with ESI/MS detection. Specific selectivity changes are shown with the change in elution position of the m/z 929 contaminant.

Using the segment range determined above, gradient slopes from 0.25% to 3%/column volume were compared (Figure 3). The expected relationship among slope, resolution, and peak volume holds for most components of the mixture. There is, however, a contaminant, m/z 978, that elutes before the target peak at 0.25%/column volume and after at 1.5%/column volume. That contaminant would be unobserved at 0.75%/column volume, directly coeluting with the intended product.

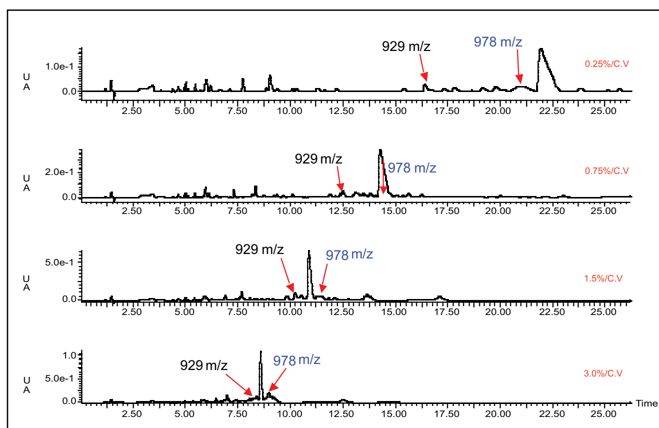

Figure 3. Optimizing the gradient slope for peptide analysis with peak tracking. Various gradient slopes were tested over the range of 28 to 36% acetonitrile. Monitoring the separations with ESI/MS demonstrates the change in selectivity for the m/z 978 contaminant relative to the main product. Note that the separation at 0.75%/column volume is not acceptable while the steeper and shallower gradients give useful resolution.

Isolation of the synthetic peptide

Purification of the synthetic peptide will usually be done on a relatively large column. Successful isolation can be ensured by basing the separation on the conditions developed for the analytical method. This approach requires matching the chemistry of the isolation-scale column to that used for the UPLC analysis.

The column selected here is a Peptide Separation Technology XBridge BEH 130 Prep C_{18} 5 µm OBD™ 19 x 150 mm because it is the same base particle as the UPLC column and is also tested with a peptide separation. The conditions selected for the separation, a gradient from 28 to 36% acetonitrile with a slope of 1.5%/ column volume, were selected to best separate the product from the contaminants, particularly the m/z 929 and m/z 978, as well as minimizing the collected volume to facilitate recovery. This separation, on a heavily overloaded column, is shown in Figure 4. Fractions were collected at 15 sec intervals across the main peak.

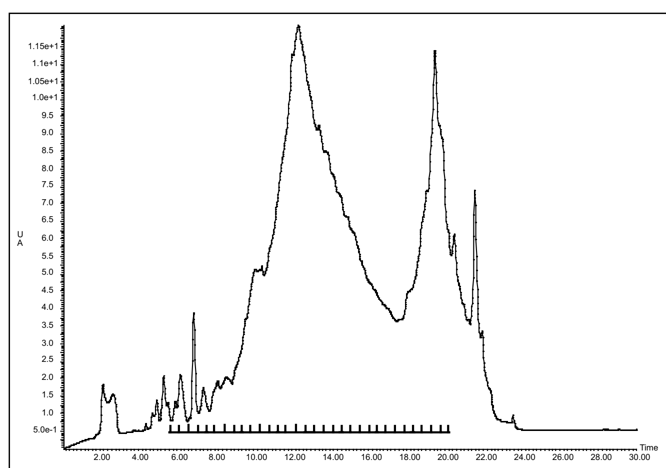

Figure 4. Peptide isolation. The UV trace of an overloaded separation of the synthetic peptide is shown. Because the detector is not a useful guide to collection, time-based fractions were taken at 15 second intervals as shown with the hashmarks.

Analysis of isolated peptide

Each collected fraction was assayed using the analytical method developed above. Results are shown for selected fractions in Figure 5. The examples highlight the early- and late-eluting fractions that contain contaminating material. These observations are consistent with the successful prediction of conditions for larger-scale isolation from the small-scale UPLC peptide analysis. Side fractions were set aside and re-pooled for inclusion in future isolations. The pure fractions were pooled and assayed as shown in Figure 6. The isolated material is greater than 95% pure and contains the intended product, recognized in mass spectrum as the doubly- and triply-charged species. This result confirms the successful isolation.

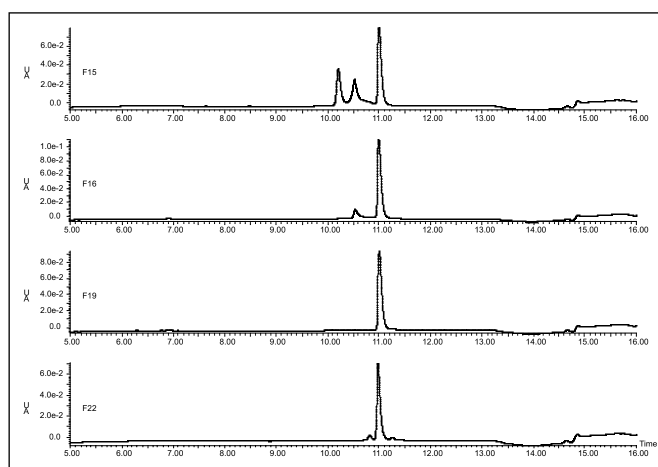

Figure 5. Fraction assay. Fractions across the overloaded isolation peak were assayed with the UPLC separation method. The early- and late-eluting F15, 16, and 22 show the contaminating materials. Fractions 17 to 21 were pooled.

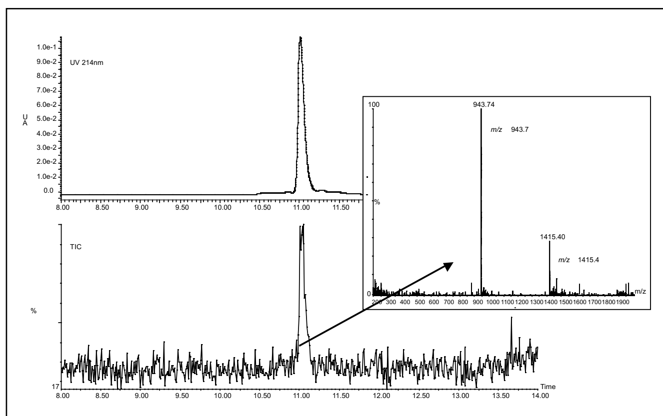

Figure 6. Assay of final product. The pooled fractions were dried and reconstituted for assay with the UPLC Peptide Analysis Application Solution. Both the UV trace at 214 nm and the MS TIC show a single component. The mass spectrum of this peak (inset) shows the doubly- and triply-charged masses for the intended product.

CONCLUSION

The Waters UPLC Peptide Analysis Solution was first described as a tool for protein characterization via peptide mapping. In these experiments, the technique is proven useful for synthetic peptides.

The inherently high resolution of UPLC ensures that contaminants can be quickly detected. Since high resolution is obtained in relatively short run times, it is practical to optimize conditions to eliminate coelution of contaminants. These optimized conditions can be used to predict the best separation conditions for isolation of purified peptide because the same Peptide Separation Technology column chemistry is available in both UPLC and larger formats.

The combination of these techniques should accelerate the production of high purity synthetic peptides for use in a variety of applications.

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

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Optimized Chromatography for Mass-Directed Purification of Peptides

Jo-Ann M. Jablonski and Thomas E. Wheat
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Peptides play important roles in the physiology of organisms. They do, therefore, provide a major class of compounds for novel therapeutic products. Pure peptides are required to effectively study biochemical interactions in organisms as well as to understand structure-activity relationships in the development of peptide therapeutics.

Traditionally, preparative peptide separations are monitored using UV detection with the collection of many fractions over the course of the entire chromatographic program, necessitating labor-intensive post-run analysis and processing. Mass spectrometry (MS) has proven useful in quickly assessing peak identity and homogeneity in complex chromatograms, increasing sample throughput. Mass-directed isolation of compounds uses the mass spectrometer to recognize the target peak and deposit it in a fraction collector tube, reducing the number of processing steps.

The chemistry of peptides poses unique chromatographic challenges, including the selection of the appropriate column chemistry, the selection and adaptation of the mobile phase, and the optimization of the mode of detection.

In this study, we illustrate the development of general instrumental parameters and chemical conditions for mass-directed purification of peptides in the range of 8 to 25 residues. A refined protocol for translating a rapid pilot separation to an optimal preparative separation is demonstrated. Peptide compound isolation is more efficiently and effectively obtained using optimized chromatography and mass-directed purification.

EXPERIMENTAL

The purification of peptides poses specific challenges to the chromatographer. The first challenge is achieving high throughput while maintaining the shallow gradients necessary for peptide separation. Variable impurities, such as side products, deletions, and remaining reagents, can complicate and interfere with separations. Finally, the multiple charging of peptides often makes it difficult to predict the most abundant species in a given MS experiment.

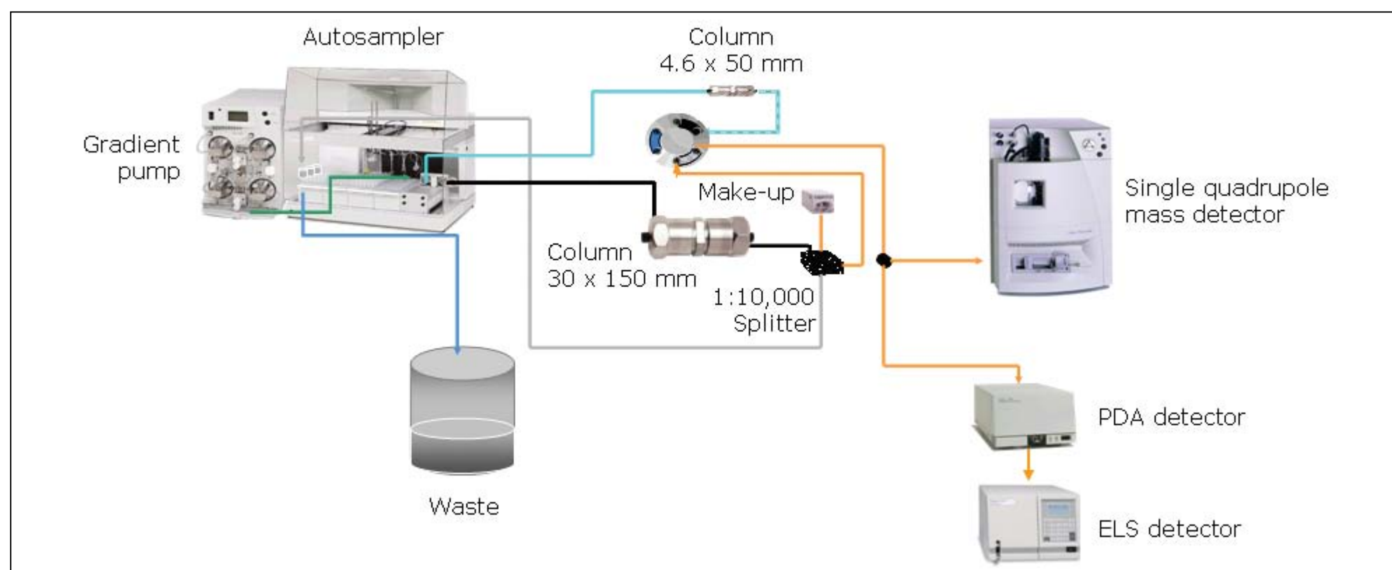

Figure 1. Configuration of the Waters Preparative Chromatography System used in these studies.

Waters Preparative Chromatography System

Throughout the experiments, a Waters Preparative Chromatography System consisting of a 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, ZQ™ Mass Spectrometer, 2996 Photodiode Array Detector, 2420 Evaporative Light Scattering Detector, two 515 Pumps (for makeup/buffer delivery), LC Packings Flow Splitter (20 to 100 mL, 1:10,000 split), MassLynx™ Software and its FractionLynx™ Application Manager was used.

The analytical and preparative flow capabilities of the 2525 Binary Gradient Module allow the system to be used for both pilot and preparative-scale experiments.

- For pilot-scale studies, a 4.6 x 50 mm Waters Symmetry® 300 C_{18} Column with 5 µm particles is used.
- For the preparative experiments, a 30 x 150 mm Waters Symmetry 300 C_{18} Column with 7 µm particles is used.

Special consideration must be given to detection in preparative chromatography. The highly concentrated peaks exceed the linear range of common detectors. In addition, the high flow rates are not tolerated by detector hardware. Finally, some detectors used in this study (MS and evaporative light scattering) are destructive. It is, therefore, necessary to use a technique that reduces the flow and concentration reaching the detectors while minimizing loss of desirable material. A passive splitter is commonly used in the high ratio split and dilute technique, as shown in Figure 2.

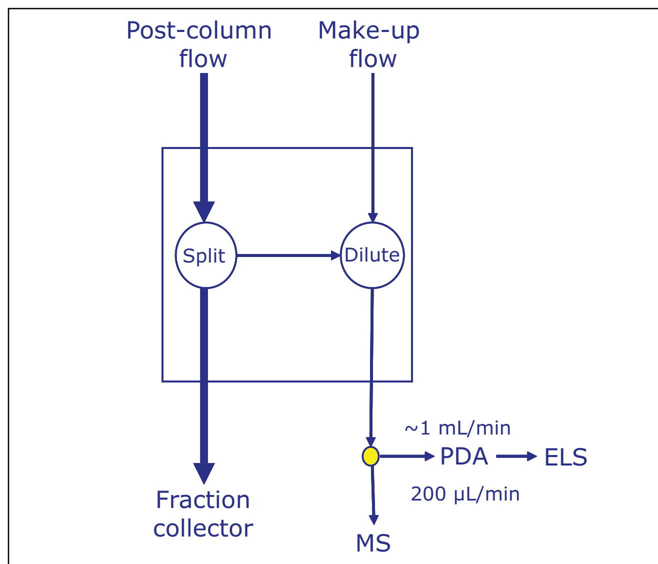

Figure 2. Flow path for the post-column sample flow. The passive flow splitter divides the prep flow 10,000:1, with 99.99% of the flow going to the fraction collector. The remaining 0.01% of the flow is transported to the detectors using the make-up flow.

Samples

The crude synthetic peptides were provided by Dr. Kelly Wasmund of Research Genetics, Inc. (Huntsville, AL, U.S.). The two peptides were:

- NH_2-ISQAVHAAHAEINEAGR-COOH (abbreviated as ISQA)
- NH_2-SIINFEKL-COOH (abbreviated as SIIN)

Purification strategy

The strategy for the purification of the peptides is based on the use of pilot runs to define preparative conditions. From the retention time of the pilot runs, the percent organic mobile phase (%B) needed to elute each peptide is estimated. Assuming that the pilot scale separation is satisfactory, the conditions for the preparative chromatography are scaled based upon the ratio of column volumes. A shallow preparative gradient is then defined, ranging from 5% below to 3% above the calculated %B needed for peptide elution. The masses of the expected ions for possible charge states are used as mass triggers. The fractions are analyzed for purity using the same chromatographic conditions as the pilot run, with multiple detection modes to ensure purity.

System calibration

The relationship between time and actual organic mobile phase delivered to the column on the pilot-scale system is established using uracil (an unretained, UV-absorbing compound) in the organic mobile phase (B). The conditions for the system calibration are given below:

Solvent A: 100% water
Solvent B: 100% acetonitrile with 0.01 mg/mL uracil
Column: 4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 1. Calibration gradient. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

The absorbance trace resulting from the calibration run is given in Figure 3. From this run, the observed percentage of organic mobile phase (%B) is calculated using the following equation:[1]

$$\text{Observed \% B} = \frac{\text{Observed Absorbance}}{\text{Absorbance at 100\% Acetonitrile}} \times 95 + 5$$

Where:

- The absorbance at 100% acetonitrile is 600,000
- The observed absorbance is obtained from the absorbance trace at 258 nm
- Table 2 summarizes the results of the calibration.

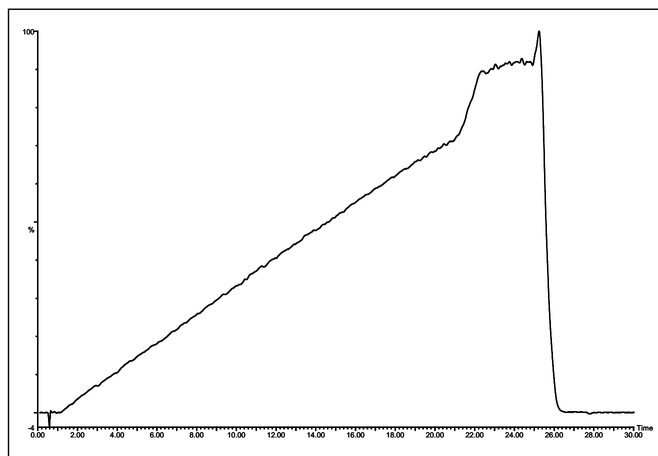

Figure 3. Absorbance trace for the system calibration.

Time	Observed Absorbance	Observed %B
0	1	
1	1	
2	24019	8.80
3	48966	12.75
4	70822	16.21
5	97858	20.49
6	121924	24.30
7	146816	28.25
8	172784	32.36
9	198040	36.36
10	221936	40.14
11	249464	44.50
12	272560	48.16
13	299896	52.48
14	320792	55.79
15	344572	59.59
16	370616	63.68
17	394568	67.47
18	416016	70.87
19	441728	74.94
20	460624	77.83

Table 2. Observed absorbance and calculated percentage of organic mobile phase with time.

Pilot-scale separations

Each peptide is individually wetted in 0.5 mL DMF and then diluted to 4.5 mL with water. The concentration of ISQA is estimated at 5 to 10 mg/mL and SIIN is estimated at 10 mg/mL. The conditions for the pilot-scale separations of each of the peptides are given in Table 3; the chromatographic results are presented in Figure 4.

Solvent A: 100% water
Solvent B: 100% acetonitrile
Injection vol.: 40 µL
Column: 4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 3. Gradient conditions used in the pilot-scale separations of ISQA and SIIN. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

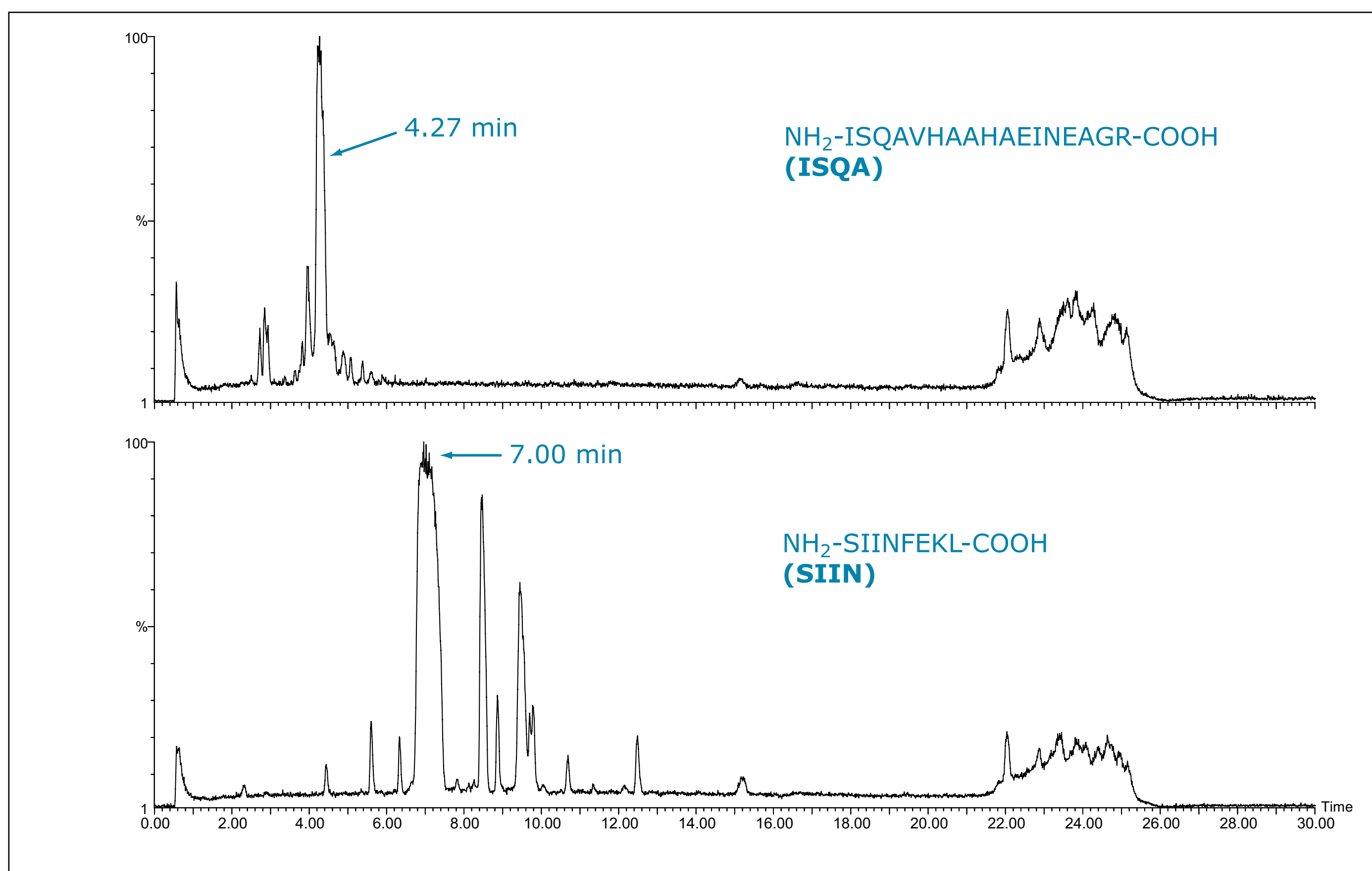

Figure 4. Total Ion Chromatograms (TIC) (ES+) for the pilot-scale separations of ISQA and SIIN.

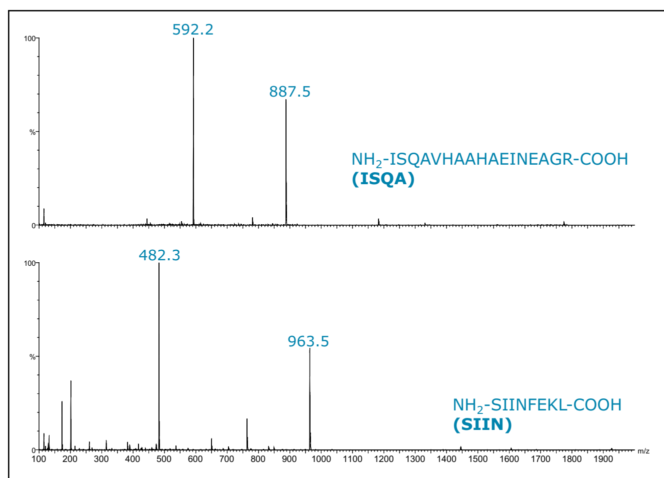

Figure 5. Mass spectra for ISQA and SIIN resulting from the pilot runs.

NH_2-ISQAVHAAHAEINEAGR-OOOH (ISQA)	
Monoisotopic Mass	1772.9 Da
Target Ions for Charge States	m/z
$[M+H]^+$	1773.9
$[M+2H]^{2+}$	887.5
$[M+3H]^{3+}$	592.2
$[M+4H]^{4+}$	447.2
NH_2-SIINFEKL-COOH (SIIN)	
Monoisotopic Mass	962.5 Da
Target Ions for Charge States	m/z
$[M+H]^+$	963.3
$[M+2H]^{2+}$	482.3
$[M+3H]^{3+}$	321.9

Table 4. Calculated target ions for various charge states of ISQA and SIIN.

Defining the preparative gradient

Shallow preparative gradients create enhanced separations of the peptides from closely-eluting contaminants. In this case, a shallow gradient that ranges from 5% below to 3% above the calculated %B for peptide elution is used. The 4.27-minute retention time for ISQA indicates that it elutes near 17%B. This suggests that the preparative gradient for ISQA should start at 12%B and go to 20%B. Similarly, the retention time of SIIN at 7.00 minutes indicates that it elutes near 28%B. The shallow preparative gradient for this peptide should run from 23%B to 31%B.

Mass triggers

Target masses are selected to include the expected multiply-charged ions, Table 4.

Preparative chromatography

Purification of the ISQA and SIIN peptides is performed using shallow preparative gradients (Tables 5 and 6) for separation and fraction collection based on multiple target ions. The conditions for the preparative run of ISQA and SIIN are given in Tables 5 and 6, and the resulting chromatograms are shown in Figures 6 and 7, respectively.

ISQA

Solvent A: 100% water
Solvent B: 100% acetonitrile
Injection vol.: 5 mL
Column: 30 x 150 mm Symmetry 300, C_{18}, 7 μm
Target masses: $[M+H]^+$ = 1773.9, $[M+2H]^{2+}$ = 887.5, $[M+3H]^{3+}$ = 592.2, $[M+4H]^{4+}$ = 447.2

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	57.50	95	5
2.00	57.50	95	5
3.00	57.50	88.4	11.6
36.50	57.50	80.4	19.6
37.50	57.50	20	80
39.50	57.50	20	80
40.00	57.50	95	5
55.00	57.50	95	5

Table 5. Shallow preparative gradient used for the purification of ISQA. A constant 6.30 mL/min of aqueous 1% (TFA) is added to the gradient stream to make a total flow rate of 63.8 mL/min and final concentration of 0.1% TFA.

SIIN

Solvent A: 100% water
Solvent B: 100% acetonitrile
Injection vol.: 5 mL
Column: 30 x 150 mm Symmetry 300, C_{18}, 7 μm
Target masses: $[M+H]^+$ = 963.5, $[M+2H]^{2+}$ = 482.3, $[M+3H]^{3+}$ = 321.9

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	57.50	95	5
2.00	57.50	95	5
3.00	57.50	77.1	22.9
36.50	57.50	69.1	30.9
37.50	57.50	20	80
39.50	57.50	20	80
40.00	57.50	95	5
55.00	57.50	95	5

Table 6. Shallow preparative gradient used for the purification of SIIN. A constant 6.30 mL/min of aqueous 1% (TFA) is added to the gradient stream to make a total flow rate of 63.8 mL/min and final concentration of 0.1% TFA.

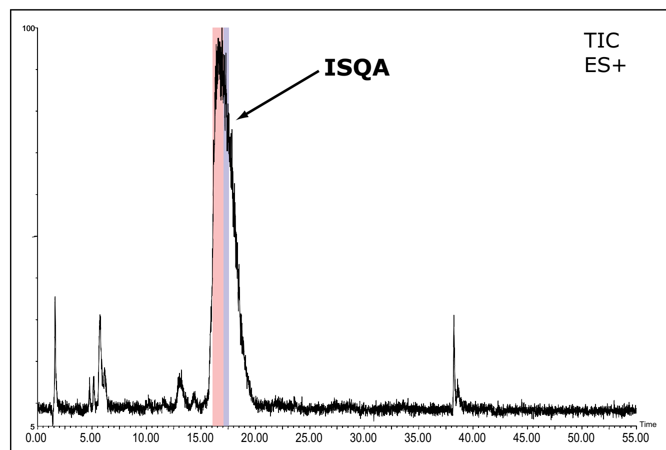

Figure 6. Preparative MS chromatogram for ISQA. The shaded bars represent fraction collection on m/z 592.2 in positive ion mode.

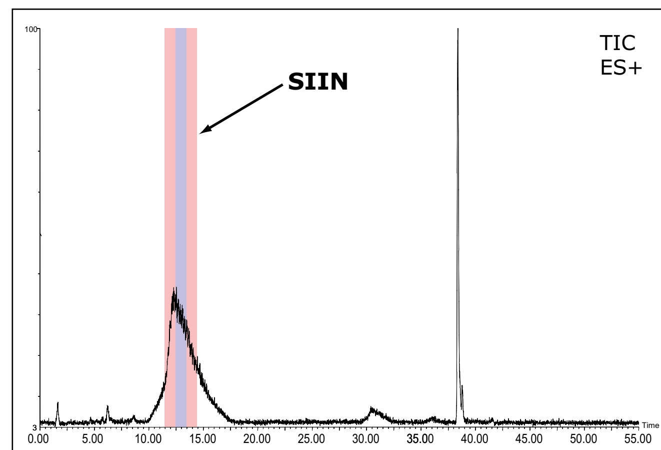

Figure 7. Preparative MS chromatogram for SIIN. The shaded bars represent fraction collection on m/z 482.3 in positive ion mode.

Fraction analysis

The fractions resulting from the preparative runs are analyzed to assess purity. Multiple detection channels are used to monitor the analysis to provide more complete characterization of the fractions. The fraction analysis of both ISQA and SIIN peptides use the separation conditions in Table 7. The results of the fraction analysis are shown for ISQA and SIIN in Figures 8 and 9, respectively.

Solvent A: 100% water
Solvent B: 100% acetonitrile
Column: 4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 7. Gradient conditions for re-analysis of the ISQA and SIIN fractions. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

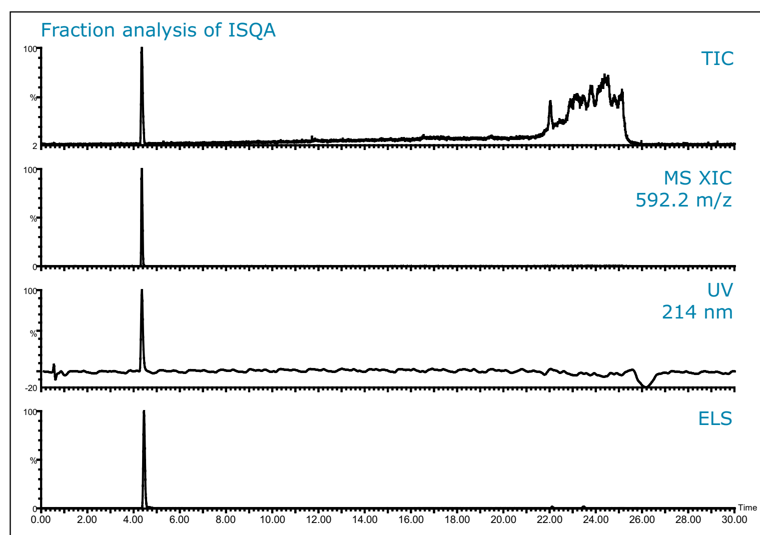

Figure 8. Fraction analysis of ISQA. The total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the UV trace at 214 nm, and the evaporative light scattering chromatogram are each shown.

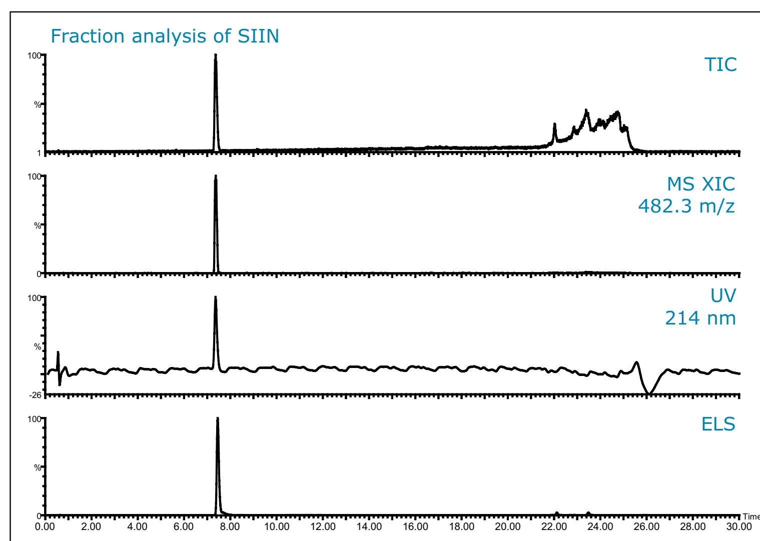

Figure 9. Fraction analysis of SIIN. The total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the UV trace at 214 nm, and the evaporative light scattering chromatogram are each shown.

CONCLUSION

The use of fast, pilot-scale runs provides a useful prediction of conditions for shallow-gradient, high-resolution preparative chromatography.

Notes

1. The calibration gradient occurs over the 5% acetonitrile to 100% acetonitrile range. Five percent acetonitrile has an absorbance of zero because the UV detector zeroes at the start of the gradient.

   At the end of the gradient, where 100%B is reaching the detector, the observed absorbance is 600,000.

   The interval between 5% acetonitrile and 100% acetonitrile is 95%. This corresponds to the range between 0 and 600,000 on the absorbance scale.

   To calculate the observed percentage of B in the middle of the 5% to 100% range as a percentage of the total range (0 %B to 100%B), the observed absorbance divided by the absorbance at 100% acetonitrile is multiplied by 95. Five percent is added to the value to account for the offset of the curve at the start of the run.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Preparative Scale Chromatography of a Hydrophilic Peptide using Hydrophilic Interaction Chromatography

Jo-Ann M. Jablonski, Christopher J. Hudalla, and Kenneth J. Fountain
Waters Corporation, 34 Maple Street, Milford, MA, USA

APPLICATION BENEFITS

BEH Amide columns are specifically designed to enhance the retention of polar compounds, making analysis, scaling, and isolation easier.

- Hydrophilic peptides that cannot be retained by reversed phase may be retained in hydrophilic interaction chromatography using the BEH Amide column. Longer retention allows separation to occur.
- The BEH Amide bonded phase is more compatible with slightly higher aqueous content in mobile phases and sample diluents than unbonded HILIC stationary phases, making the BEH Amide column a more viable alternative for the purification of hydrophilic peptides.
- Improved mass loading of polar peptides on BEH Amide columns reduces the number of injections required to isolate the product, promoting process efficiency.
- Reproducible chromatograms at the analytical and preparative scales reduce ambiguity in the identification of target peptides and demonstrate BEH Amide column scalability.

WATERS SOLUTIONS

AutoPurification™ System

XBridge BEH Amide Columns

KEY WORDS

Polar compound isolation, BEH Amide columns, preparative LC, hydrophilic interaction chromatography, HILIC, hydrophilic peptides

INTRODUCTION

Reversed phase columns are typically used for the analysis and isolation of peptides, however some hydrophilic peptides have little or no retention on C_{18} stationary phases. Insufficient interaction with the stationary phase leads to difficulties in the peptide isolation. Hydrophilic Interaction Chromatography (HILIC) is an alternative chromatographic technique useful in the isolation of compounds where analytes are separated based on a unique combination of liquid-liquid partitioning, adsorption, ionic interaction, and hydrophobic retention mechanisms. Compounds elute from the column as the gradient transitions from low aqueous to high aqueous mobile-phase composition.

The BEH Amide column, with a trifunctionally-bonded amide phase, was first introduced in 2009 with 1.7 µm particles for the analysis of polar compounds using the ACQUITY UPLC® System. Demand for a column capable of analyzing compounds such as hydrophilic synthetic peptides, saccharides, synthetic sugars, glycopeptides, and polar compounds from natural products has driven the development of a larger 5 µm particle for use in analytical and preparative HPLC applications. In this application note, we demonstrate the utility of the BEH Amide particle for the analysis and isolation of a hydrophilic peptide.

EXPERIMENTAL

LC Conditions

System:	Waters 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, 2996 Photodiode Array Detector, ZQ 2000 Mass Spectrometer, and 2420 ELSD Mass Detector
Columns:	XBridge BEH Amide, 5 µm, 4.6 x 150 mm, part number 186006595 XBridge BEH Amide, 5 µm, 19 x 150 mm, part number 186006605
Column Temp.:	40 °C
Mobile Phase A:	20/80 acetonitrile/ 10 mM ammonium formate pH 3
Mobile Phase B:	90/10 acetonitrile/ 10 mM ammonium formate pH 3
Weak Needle Wash:	90/10 acetonitrile/water
Strong Needle Wash:	20/80 acetonitrile/water
Seal Wash:	50/50 acetonitrile/water
Sample Diluent:	15/5/3 acetonitrile/ methanol/water
Flow Rate:	Reported in figures
Gradient:	Reported in figures
Injection Volume:	Reported in figures

SAMPLE PREPARATION

Analytical Scale

2.0 mg of polar peptide comprised of the following 20 residues: 4 basic, 11 polar and uncharged, 3 nonpolar, and 2 acidic, were dissolved in 1.15 mL of sample diluent, producing a concentration of 1.77 mg/mL peptide solution. The sample diluent was a mixture of 15/5/3 acetonitrile, methanol, and water. The crude peptide solution was vortexed and filtered through a 13 mm, 0.45 µm GHP syringe filter, part number WAT200516.

Preparative Scale

31 mg of polar peptide were dissolved in 2.3 mL of the sample diluent for a final concentration of 13.5 mg/mL. The sample mixture was vortexed and filtered.

RESULTS AND DISCUSSION

The analysis and isolation of polar peptides is often challenging because of the difficulty in ensuring the retention of very hydrophilic sequences on a reversed phase column. Hydrophilic Interaction Chromatography (HILIC), is an orthogonal chromatographic separation technique which separates hydrophilic compounds by their interaction with a polar stationary phase. Liquid-liquid partitioning, adsorption, ion exchange, and hydrogen bonding mechanisms all contribute to the retention of the sample. Analytes are eluted from the column by increasing the polarity of the mobile phase. The selectivity and retentivity of compounds on different stationary phases is dependent upon the specific properties of the column packing. As shown in Figure 1, the elution profile of the analytes is unique for each of the three HILIC stationary phases when the column dimensions and the chromatographic method are held constant. The BEH Amide column shows the most retention for the various types of compounds and a different selectivity compared to the other two columns. Better retention of similar compounds often improves the resolution between them.

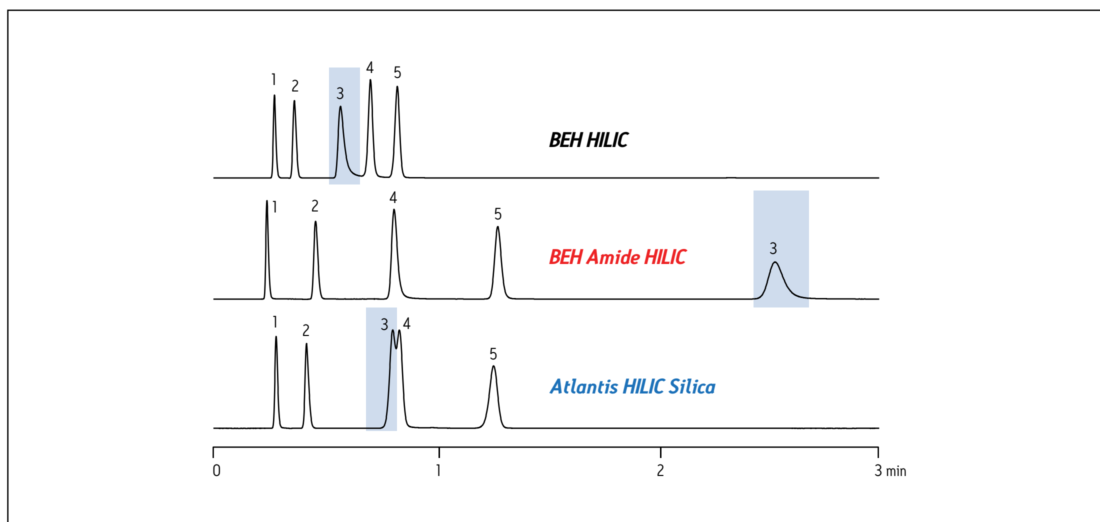


Figure 1. Here is a chromatographic representation of the differences in retentivity and selectivity of the 3 HILIC stationary phases. The Atlantis® HILIC Silica provides more retention and a different selectivity compared to the BEH HILIC (due to its higher surface polarity). The amide column exhibits the most retention for various types of compounds and a different selectivity compared to the other two columns. Conditions: Isocratic, 12 mM ammonium formate (pH 3) with 90% acetonitrile; UV at 254 nm. Compounds: (1) acenaphthene, (2) thymine, (3) 5-fluoroorotic acid, (4) adenine, (5) cytosine.

Although retention is crucial for effective separations, the nature of the target molecule must also be considered for a successful isolation of the compound. Deleted and failure sequences, adducts, and residual cleavage cocktail components contribute to the complexity of the crude sample mixture and complicate the isolation of the target peptide. The sample diluent also plays a role in retention, influencing solubility and peak shape. Traditional unbonded HILIC stationary phases usually require diluents and mobile phases with high organic concentration which limit the solubility of polar compounds at the high sample concentrations used in prep chromatography. Small amounts of water, even 10-20%, make the injection solvent incompatible with initial HILIC conditions on unbonded phases. Since the BEH Amide bonded phase tolerates mobile phases and injection solvents which are higher in aqueous content, polar peptides can be solubilized at concentrations amenable to preparative chromatography.

Because the amino acid sequence of the 20-mer in this study has no chromophores, peptide detection by UV is limited. Systems configured with alternate modes of detection identify target molecules with limited UV absorption or low ionization potential. Figure 2 shows the amino acid composition and the calculated monoisotopic mass and higher charge states for the peptide molecule used in this study. Since higher organic content mobile phases are typically used in HILIC, they are easily desolvated and provide an enhanced mass spectrometric response as well as faster fraction drying time. As shown in Figure 3, the peptide displays very little absorption at 220 nm due to the absence of chromophores in the amino acid sequence, but the ELSD and mass chromatograms have improved sensitivity, making isolation and analysis possible.

20 Amino Acids in Length

Amino acid composition:
4 basic
11 polar (uncharged)
2 acidic
3 nonpolar

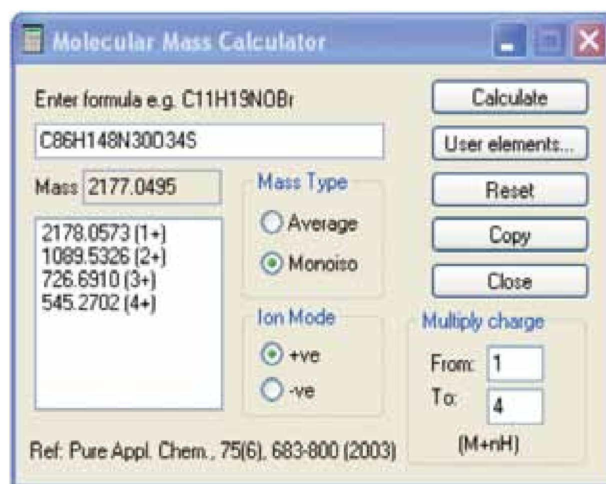
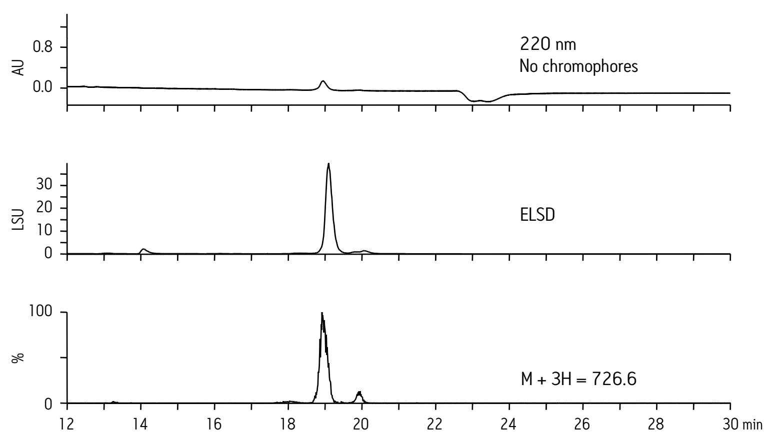


Figure 2. Amino acid composition and calculated monoisotopic and higher charge state masses for the hydrophilic peptide.



100% B-100% A in 18 min, hold 3 min, return to 100% B in 0.20 min, equilibrate 8.8 min.

Flow Rate: 1.46 mL/min
Mobile Phase A: 20 acetonitrile/80 10 mM ammonium formate pH 3
Mobile Phase B: 90 acetonitrile/10 10 mM ammonium formate pH 3
Strong Needle Wash: 20 acetonitrile/80 water
Weak Needle Wash: 90 acetonitrile/10 water
Sample Diluent: 75 acetonitrile/25 methanol with 150 µL water
Sample Concentration: 13.5 mg/mL

Figure 3. Comparison of the three modes of detection for the hydrophilic peptide on the 4.6 x 150 mm BEH Amide column.

Scaling separations requires matching column chemistry as well as appropriately scaled gradients. As laboratories explore options for increasing throughput in the purification process, fast screening gradients using UPLC® reduce the amount of time required for synthetic crude product analysis and, in some cases, fraction analysis. Since the BEH Amide column is available in sub-2-µm configurations, the synthetic crude peptide was analyzed using the ACQUITY UPLC. Maintaining the resolution between the UPLC and preparative scales requires the ratio of the length of the column to the diameter of the particle (or L/dp) remain constant. The UPLC, 2.1 x 50 mm, 1.7 µm column has an L/dp of about 29,400. The preparative, 19 x 150 mm, 5 µm column L/dp is 30,000, essentially equal to the L/dp ratio for the UPLC column. As expected, geometric scaling of the injection volume and chromatographic conditions produced a preparative chromatogram which is directly comparable to the sub-2-µm screening analysis done on the ACQUITY UPLC. Figure 4 illustrates the BEH Amide column scalability by comparing the chromatography using the fast screening gradient on UPLC and the larger scale chromatography used for the isolation.

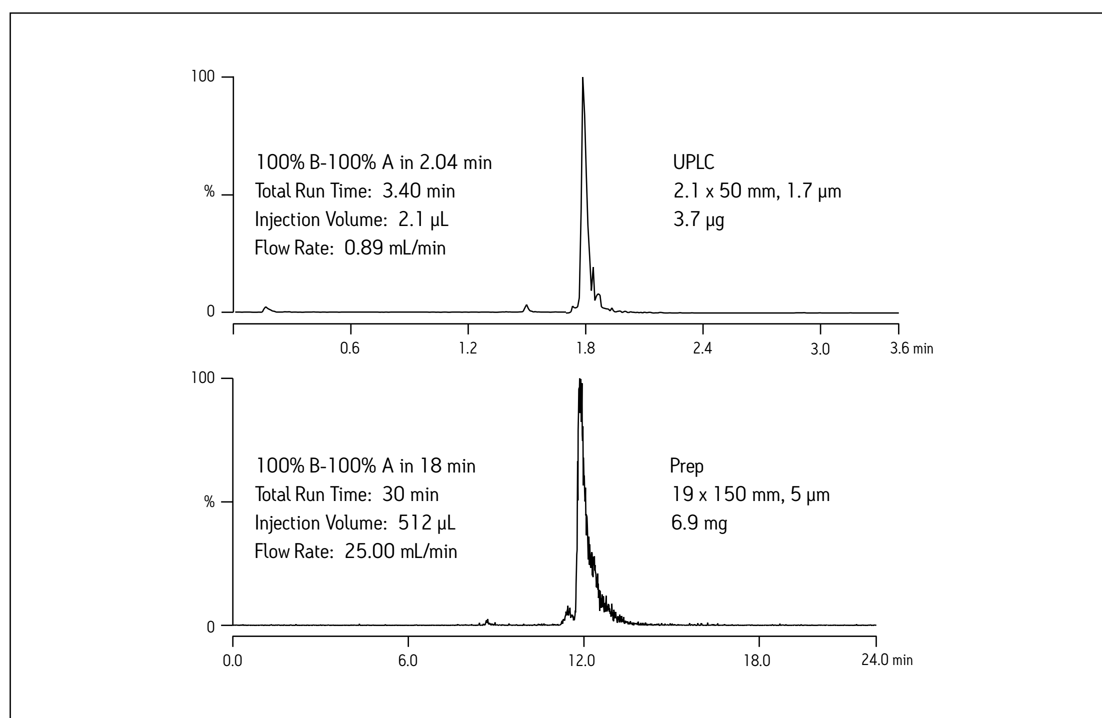


Figure 4. Hydrophilic peptide chromatograms at the UPLC and preparative scales.

CONCLUSIONS

- Hydrophilic peptides that cannot be retained by reversed phase may be retained in hydrophilic interaction chromatography using the BEH Amide column. Longer retention allows separation to occur.
- The BEH Amide bonded phase is more compatible with slightly higher aqueous content in mobile phases and sample diluents than unbonded HILIC stationary phases, making the BEH Amide column a more viable alternative for the purification of hydrophilic peptides.
- Reproducible chromatograms at the analytical and preparative scales reduce ambiguity in the identification of target peptides and demonstrate BEH Amide column scalability.

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

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Impurity Evaluation of Heparin Sodium by Anion Exchange Chromatography

Catalin E. Doneanu, Weibin Chen, and John C. Gebler
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Heparin is a blood thinning drug that is primarily used to prevent the development of blood clots. Heparin and its derivative, low-molecular-weight heparin (LMWH), have been widely used as anticoagulant drugs for decades during surgery and kidney dialysis. Heparin belongs to the group of linear polysaccharides called glycosaminoglycan (GAG), and consists of alternating glucosamine and hexuronic acid residues. Heparin has tremendous heterogeneity, due to N-acetylation, various sulphation patterns and chain lengths, making analytical characterizations extremely challenging.

Raw heparin material is extracted from mammalian tissues, such as pig intestines. The heparin material requires many treatment and purification steps before it can be used in a drug formula. Stringent quality control in the purification steps is essential to ensure the quality of heparin as a final active pharmaceutical ingredient (API) of the drug.

Recent incidents, including severe allergic reactions and several deaths have been attributed to heparin adulteration, resulting in a massive recall of heparin drugs by the manufacturer.[1] Oversulfated chondroitin sulfate (OSCS) is a contaminant in heparin associated with the adverse clinical events.[2]

Because heparin is a drug commonly used in clinics, these adverse events have created a worldwide crisis and a call for an analytical method that can readily monitor the purity of heparin API before formulation of the drug.

This application note presents a simple method to separate and quantify oversulfated chondroitin sulfate (OSCS) in the presence of heparin. The method uses anion exchange chromatography to achieve complete resolution between heparin and OSCS, and UV absorption to quantify the concentrations of heparin and OSCS. The results demonstrate the method not only generates reproducible, fast separations (10 minutes) but also detect OSCS at a concentration of less than 1% of overall content. The ability to quickly and unambiguously analyze the purity of heparin drugs can improve and accelerate the quality control of raw API materials in pharmaceutical industry. The sensitive testing method can be used to screen for heparin quality and OSCS adulteration in order to protect patient health.

EXPERIMENTAL

Sample preparation

Heparin Sodium Identification RS (part no. 1304038) and Heparin Sodium System Suitability RS (part no. 1304049) were purchased from U.S. Pharmacopeia. The Heparin Sodium System Suitability RS is a mixture that contains approximately 80% Heparin and 20% OSCS.

Stock solutions (10 mg/mL) of Heparin Sodium standard or Heparin Sodium System Suitability standard were prepared by reconstituting the samples in Milli-Q water. Samples with diluted concentrations were prepared by diluting the stock solutions to the desired concentration using Milli-Q water.

LC conditions

LC system:	Alliance® HPLC Bioseparation (Alliance HPLC Bio) System
Column:	Spherisorb® 5 µm SAX Column, 4.0 x 250 mm
Column temp.:	40 °C
Flow rate:	0.5 mL/min
Mobile phase:	
Eluent A:	50 mM NaH_2PO_4 (pH 2.5)
Eluent B:	50 mM NaH_2PO_4+ 2.0 M $NaClO_4$ (pH 2.5)
Gradient:	10% to 90% B in 10 min
Sample inj. vol:	25 µL

UV detection

Detector:	2998 Photodiode Array (PDA) Detector
Wavelength:	190 nm to 400 nm
Sampling rate:	2 pts/s
Resolution:	1.2 nm

RESULTS AND DISCUSSION

Bioseparations using ion exchange chromatography typically involve the use of harsh salts and extreme pH conditions. To develop a robust and high-resolution anion exchange chromatography separation method for routine heparin analysis, a Waters® Alliance® HPLC Bioseparation (Alliance HPLC Bio) System, featuring a titanium/polymeric flow path was chosen to ensure high-precision, reproducible delivery of mobile phases with high salt concentrations.

Figure 1 shows an overlay elution profile for the Heparin Sodium System Suitability RS and the Heparin Sodium Identification RS from U.S. Pharmacopeia. The extracted chromatogram for the system suitability sample (at 202 nm wavelength) showed two distinct peaks with retention times of 8.25 and 11.43 minutes, while the heparin sodium standard sample only gave one chromatographic peak at 8.25 minutes. Comparison between the two chromatographic traces indicates that heparin is eluted first at 8.25 minutes, followed by OSCS at 11.43 minutes. This figure shows that strong anion exchange (SAX) chromatography can be used to rapidly separate heparin from OSCS with a 10-minute linear gradient.

The chromatographic repeatability of the separation from run to run was investigated using a 1.0 mg/mL solution of Heparin Sodium System Suitability RS. To determine the reproducibility of the separation, the retention times at the peak top for corresponding heparin and OSCS peaks were collected for 10 consecutive injections, and the retention time variations were calculated. Figure 2 shows an example of the overlay of UV chromatograms obtained from four injections of the sample. The retention time RSD (relative standard deviations) values for heparin and OSCS were 0.08% and 0.05%, respectively.

Capillary electrophoresis (CE) was previously employed to separate heparin and OSCS. The electropherogram generated from the same system suitability test sample is different from Figure 1. Only limited separation was achieved between heparin and OSCS during CE separation,[3] and the elution order of heparin and OSCS was also reversed in the electropherogram with over-sulfated chondroitin sulfate migrating faster than heparin sodium in the CE analysis. The retention time difference of heparin and OSCS between the two different separation techniques confirms that the SAX separation of heparin and OSCS is based on the negative charge density on the linear polysaccharide chain. Structurally, OSCS bears at least one extra sulfate group for every disaccharide repeat unit compared to heparin.

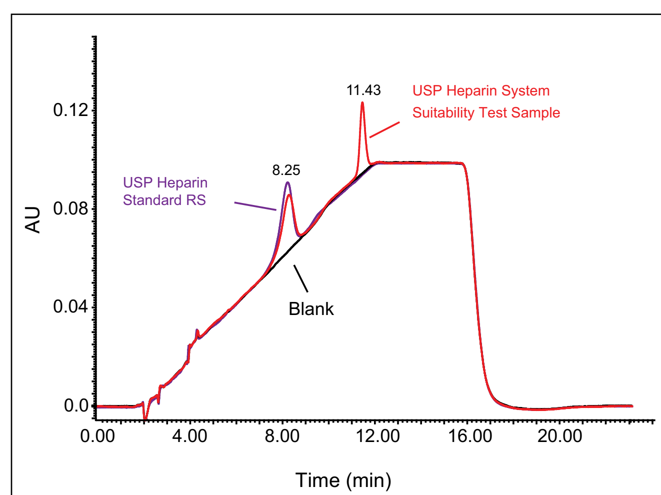

Figure 1. UV chromatograms (202 nm) of USP heparin standard RS (in purple) and USP heparin system suitability test sample RS (in red) using Spherisorb 5 µm SAX 4.0 x 250 mm. 25 µg of the materials were injected onto the column for each analysis.

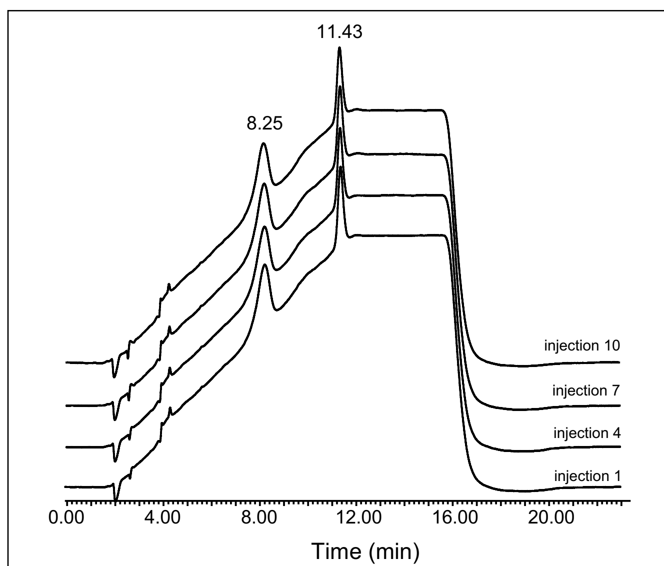

Figure 2. Overlay of UV chromatograms (202 nm) of four replicate injections of USP heparin system suitability test sample RS, showing the reproducibility of the separation by Spherisorb 5 µm SAX 4.0 x 250 mm. 25 µg of the materials was injected onto the column for each injection.

One of the basic requirements for developing analytical methods for quality control purposes lies in quantitative impurity analysis. The method should entail simultaneous analysis of a large amount of the parent compound and a low level of impurity. On the basis of successful separation of heparin and OSCS by SAX, the linear dynamic range of the method was investigated. A stock solution of the system suitability sample was prepared (10.0 mg/mL), and samples with a series of concentrations from 5.0 mg/mL to 0.1 mg/mL were prepared by sequential dilution of the stock solution. These solution standards were injected onto the SAX column in triplicate at an injection volume of 25 µL. Figure 3 shows the calibration curves generated from these injections. The calibration curves were generated by plotting the integrated respective peak areas of heparin and OSCS against the total concentrations of the two components. As shown in Figure 3, the calibration curves were linear over two orders of magnitude with R^2 values in excess of 0.999.

To test the applicability of the SAX method in impurity analysis, a heparin sample containing roughly 1% of OSCS was created by mixing the solution of heparin sodium standard (at 10.0 mg/mL) and the solution of heparin sodium system suitability RS (1.0 mg/mL) at a pre-calculated ratio. The calculation was based on the presumption that OSCS accounts for 20% of the total concentration in the solution of the heparin sodium system suitability RS. Figure 4 shows the chromatogram obtained from the mixture, where a small well-defined chromatographic peak for OSCS was observed. Integration of the chromatographic peaks for heparin and OSCS yielded peak areas of 87, 294, and 1889 respectively. Based on the calibration plot in Figure 3, the concentration of heparin and OSCS was calculated at 4.610 mg/mL and 0.048 mg/mL. This indicates that heparin was 96.5-fold more concentrated than OSCS in the synthetic mixture, implying that the method indeed can readily detect and quantify the concentration of OSCS with only 1% of heparin concentration.

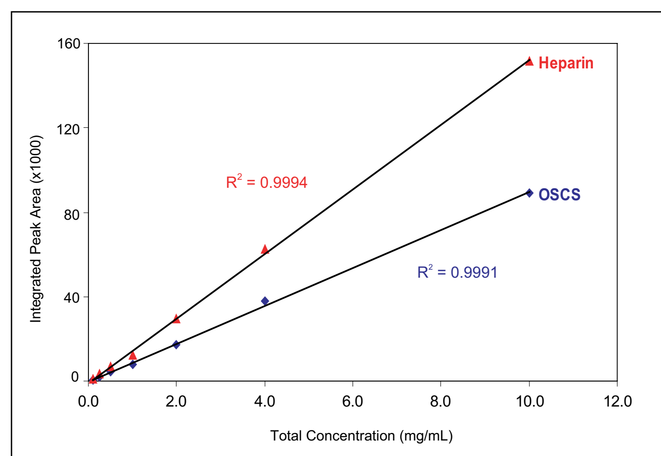

Figure 3. Calibration curves of heparin and oversulfated chondroitin sulfate (OSCS) over the concentration range from 0.1 mg/mL to 10 mg/mL. The concentrations are given as the sum concentration of the two components.

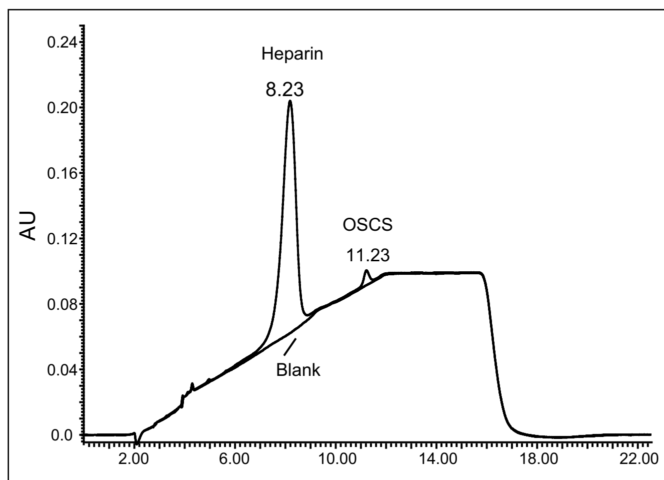

Figure 4. UV chromatograms showing the separation between heparin and oversulfated chondroitin sulfate (OSCS) where the concentration of OSCS is approximately 1% of heparin concentration. A UV trace of blank injection was also plotted in the graph to show the integrated peak areas of heparin and OSCS.

CONCLUSIONS

The combination of the Alliance Bioseparation System with the Spherisorb SAX Column is an ideal solution for the separation and quantification of heparin and OSCS. This method yields rapid, sensitive, and high-resolution separations, and generates quality data for the evaluation and determination of heparin purity. The wide linear dynamic range, in conjunction with the superior separation of the system, make it well-suited for quantitative impurity analysis. OSCS at 1% of heparin concentration is readily detected by the system. The results demonstrate that this system is a suitable method to determine whether OSCS exists as an adulterant to the heparin API .

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

[APPLICATION NOTE]

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A Generic 2D-UPLC/MS Assay for the Identification and Quantification of Host Cell Proteins in Biopharmaceuticals

Catalin Doneanu, Keith Fadgen, StJohn Skilton, Martha Stapels, and Weibin Chen
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

- An efficient and generic UPLC/MS^E assay is developed for identification and quantification of host cell proteins (HCPs) in biotherapeutics that provides over four to five orders of magnitude in concentration.
- This targeted, high-throughput quantitative assay for selected HCP proteins enables researchers to monitor their abundance changes.

WATERS SOLUTIONS

nanoACQUITY UPLC with 2D Technology

SYNAPT HDMS

XBridge and ACQUITY UPLC Columns

ACQUITY UPLC

Xevo TQ MS

KEY WORDS

Host cell proteins (HCPs), 2D chromatography, high pH, low pH, MS^E, multiple reaction monitoring (MRM)

INTRODUCTION

Most biotherapeutics today are produced by recombinant DNA technology using a well-selected host cell system. Host cells express a large number of their own proteins that can easily contaminate the recombinant protein drug. Even after sophisticated purifications steps, low levels (1 to 100 ppm) of host cell proteins (HCPs) may still remain in the final purified biopharmaceutical product. Because HCPs can sometimes trigger an unpredictable immunogenic response, regulatory guidelines stipulate that they need to be identified and quantified to protect patient safety.

The presence or absence of HCPs in protein drugs can determine whether or not a biopharmaceutical is accepted by the regulatory agencies. For example, in 2008, the European Medicines Agency (EMA) approved a recombinant form of human somatropin only after the manufacturer added additional purification steps to remove the HCPs responsible for immunogenic response in patients.[1] The same agency rejected an interferon biosimilar in 2006 because of insufficient validation for immunogenicity testing.

All analytical methods employed for measuring HCPs face significant challenges due to the wide dynamic range of the protein concentration (four to five orders of magnitude). Some widely used analytical methods, such as process-specific ELISAs and western blots,[2] require prior knowledge regarding the nature of HCP contaminants. In addition, process specific immunoassays are both time consuming (e.g., six months), and expensive to develop (more than $100K), and are not readily adapted to fully evaluate biopharmaceutical products from different cell types and purification schemes.

Two-dimensional gel electrophoresis coupled to fluorescent staining,[3,4] another popular method for HCP analysis, is only semi-quantitative, has limited dynamic range (two to three orders of magnitude) and requires additional, confirmatory techniques (e.g., mass spectrometry) for HCP identification. Although commercial ELISA kits are developed for generic application to the monitoring of HCPs, they are less specific than the process-specific immunoassays, and cannot offer a complete coverage for all the existing HCPs in the samples.[5,6]

An organization that can demonstrate that it is capable of accurately identifying and monitoring the HCPs in its biotherapeutics is more likely to overcome regulatory hurdles in the acceptance of its products.

EXPERIMENTAL

LC conditions

2D-UPLC system: nanoACQUITY UPLC® System with 2D Technology and on-line dilution

First LC dimension (Operating at pH 10.0)

Column: XBridge™ BEH300 C_{18}, 5 µm, 1 x 50 mm, (p/n 186003615)

Flow rate: 10 µL/min

Mobile phase A: 20 mM ammonium formate in water (pH 10)

Mobile phase B: Acetonitrile

Step-elution gradient: A 10-step elution gradient to fractionate the peptides in the first dimension at pH 10 is undertaken. The percentage of mobile phase B in each step is: 10.8, 12.4, 14.0, 15.0, 16.7, 18.6, 20.4, 25.0, 30.0, and 50.0%, respectively.

Diluting solution: 0.1% TFA in Milli-Q water, 100 µL/min flow rate

Trap column: Symmetry C_{18}, 0.5 x 20 mm, 5 µm (2.7 µL internal volume)

Second LC dimension (Operating at pH 2.4)

Column: ACQUITY UPLC® BEH C_{18}, 0.3 x 150 mm, 1.7 µm (p/n 186002605)

Column temp.: 65 °C

Flow rate: 12 µL/min

Mobile phase A: 0.1% FA in Milli-Q water (pH 2.4)

Mobile phase B: 0.1% FA in ACN

Gradient elution: 7 to 35% of mobile phase B in 30 min

MS conditions

HCP discovery platform

MS system: SYNAPT® HDMS™

Acquisition time: 0.5 s

m/z range: 50 to 1990

ESI spray voltage: 2.6 kV

Cone voltage: 37 V

Source temp.: 120 °C

Low-energy fragmentation: 5eV (fixed)

High-energy fragmentation: Collison Energy ramp between 15 and 35 eV

High-throughput MRM assay platform

LC system: ACQUITY UPLC®

Column: ACQUITY UPLC BEH300 C_{18}, 2.1 x 150 mm 1.7 µm packed (p/n 186003687)

Column temp.: 35 °C

MS system: Xevo® TQ MS

ESI spray voltage: 3.5 kV

Cone voltage: 37 V

Source temp.: 90 °C

MS1/MS2 isolation window: 0.75 Da (FWHM)

Dwell time: 20 to 30 ms

Informatics

ProteinLynx Global SERVER™ (PLGS) 2.4

In this application note, a generic UPLC®/MS assay that offers comprehensive HCP identification and quantification for biotherapeutic protein samples is described. The assay applies an on-line two-dimensional LC approach for peptide separations and a high-resolution and high-mass-accuracy mass spectrometer for protein identification and quantification.

In contrast to the traditional 2D-chromatography setup schemes that are based on strong cation exchange (SCX) and low pH reversed phase[7] separation, the 2D method employed here couples a high pH reversed phase (RP) separation to a low pH RP separation to achieve maximum chromatographic separation and to cope with the complexity and the wide dynamic range that are encountered in the HCP samples.

In addition, a multiplexed data acquisition method (MS^E) is employed in the mass spectrometric analysis so low-abundance HCP peptides can be reproducibly sampled and identified without bias.

Furthermore, a fast quantitative assay has been developed based on multiple reaction monitoring (MRM) principles to provide a high-throughput method for monitoring HCP variation in samples from a variety of manufacturing/purification conditions.

In this application note, we evaluate the performance of the assay using monoclonal antibody samples derived from different purification methods.

SAMPLE PREPARATION

MIX-4 protein digest standard

The MIX-4 protein digest standard was prepared by diluting stock solutions of the individual MassPREP™ protein digests of ADH (p/n 186002328), PHO (p/n 186002326), BSA (p/n 186002329), and ENL (p/n 186002325) in 20 mM ammonium formate, pH 10, to achieve a final concentration of 20 nM ADH, 4 nM PHO, 1 nM BSA, and 0.2 nM ENL.

HCP sample preparation

A chimeric anti-phosphotyrosine IgG1 monoclonal antibody (PTG1 mAb) was expressed in two different Chinese hamster ovary (CHO) cell lines and purified by Protein A chromatography using two different protocols following manufacturer recommendations.

Among the six samples analyzed, four samples labeled as A1, B1, A2, and B2 were expressed in DG-44 CHO cells, while two samples labeled C and D were expressed in CHO-S cells. Two different purification protocols were followed. Samples A1/A2, and B1/B2 were biological replicates, grown under identical conditions.

Five protein standards (LA, PHO, ADH, BSA, and ENL) were spiked in 250 µL of PTG1 (5 to 10 mg/mL) and the resulting protein mixture was denatured with 0.1% RapiGest™ for 15 min at 60 °C, reduced with 10 mM DTT for 30 min at 60 °C, alkylated with 20 mM IAM for 30 min (at RT) and digested overnight (37 °C) with porcine trypsin (Promega) using a 1:20 (w/w, enzyme:protein) ratio. After digestion, the RapiGest surfactant was decomposed by adding 5 µL of pure TFA and the samples were incubated for 30 min at 37 °C and centrifuged (10 min at 10,000 rpm) to separate the insoluble component of RapiGest by precipitation. After adjusting the pH of the supernatant solution to pH 10 using a solution of 2 M ammonium formate (pH 11), the digest volume was brought to 1 mL using 20 mM ammonium formate (pH 10). The amounts of spiked protein digests loaded on-column using a 100 µL sample loop were: 4000 fmoles of LA (bovine alpha-lactoglobulin), 800 fmoles of PHO, 320 fmoles of ADH, 80 fmoles of BSA, and 16 fmoles of ENL.

Internal standards for MRM analysis

For MRM quantification, three $^{13}C^{15}N$-isotopically labeled peptides (Sigma Aldrich) were spiked at a concentration of 20 nM into 250 µL of PTG1 digest that was prepared following the digestion protocol described above.

RESULTS AND DISCUSSION

Chromatographic performance of the 2D-UPLC system

A schematic diagram illustrating the operation of the 2D-UPLC system during sample loading, sample elution from the first dimension, and sample separation from the second dimension is shown in Figure 1A–C. Peptide samples are loaded under basic conditions (pH 10) on the first-dimension column (Figure 1A) and fractionated by RP chromatography using the 10-step elution with increasing acetonitrile concentrations (Figure 1B). Each peptide fraction is diluted (on-line) with a solution containing 0.1% TFA (pH 2.1) at a 1:10 ratio so the peptides eluted from the first dimension can be temporarily retained on a trapping column. The peptides are then separated on the second-dimension analytical column using a 30-min gradient under acidic conditions (Figure 1C).

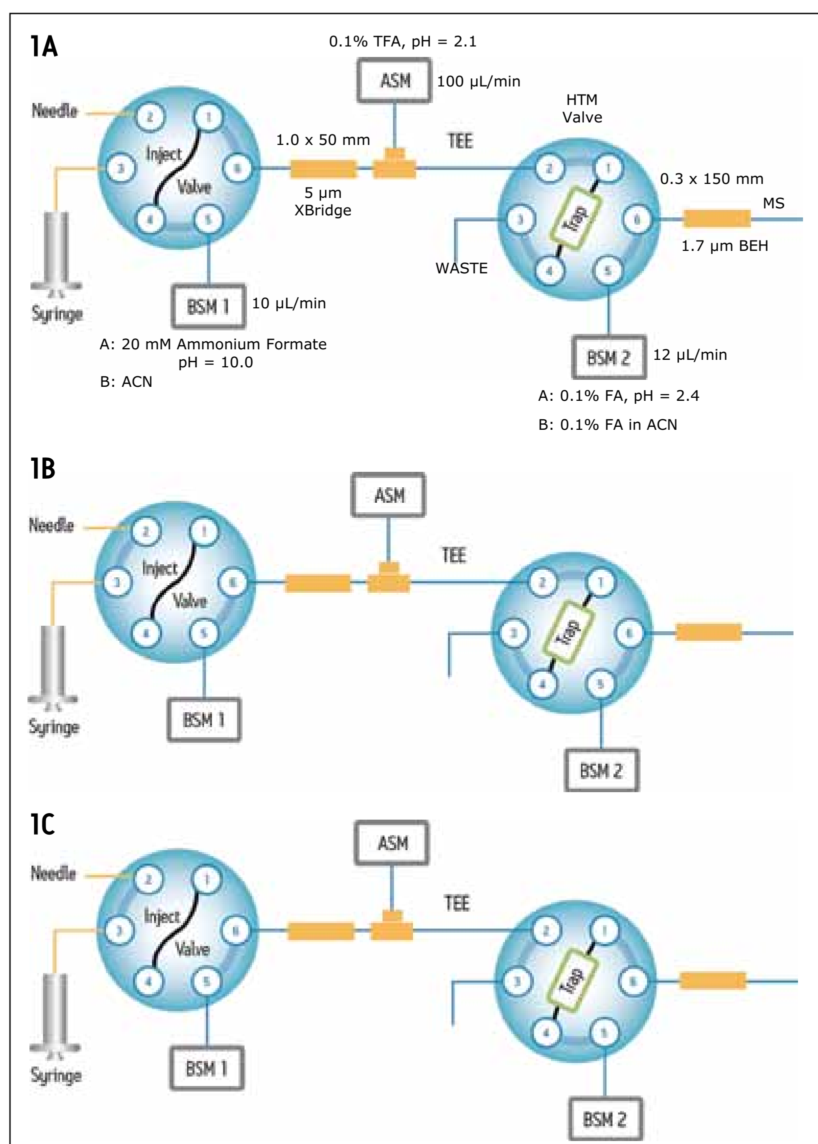

Figure 1. Fluidic configuration for 2D chromatography with on-line dilution: (A) sample loading; (B) peptide fractionation using the first chromatographic dimension (high pH reversed phase), and peptide trapping; (C) peptide separation in the second dimension (low pH reversed phase).

A critical chromatographic parameter in multidimensional chromatographic separations is the reproducibility of peptide fractionation during an extended period of operation. This is illustrated in Figure 2 using a MIX-4 protein digest, which contains 20 nM of ADH, 4 nM of PHO, 1 nM of BSA, and 0.2 nM of ENL in 20 mM ammonium formate (pH 10). Figure 2 shows the extracted mass chromatograms of the T43 peptide from ENL protein (VNQIGTLSESEIK, monoisotopic peak $[M+2H]^{2+}$ of 644.86), from the second dimension (low pH) separations in four consecutive injections (experiments). In each experiment, a five-step fractionation using 10.8, 12.4, 15.4, 18.6, and 50% Eluent B (100% ACN) was performed in the first dimension. T43 ENL peptide is eluted only in Fraction 4, demonstrating great reproducibility of the first dimension fractionation. In addition, the retention time reproducibility (0.05% RSD) for the same peptide over 48 hours of separation demonstrates the stability of the 2D-UPLC system.

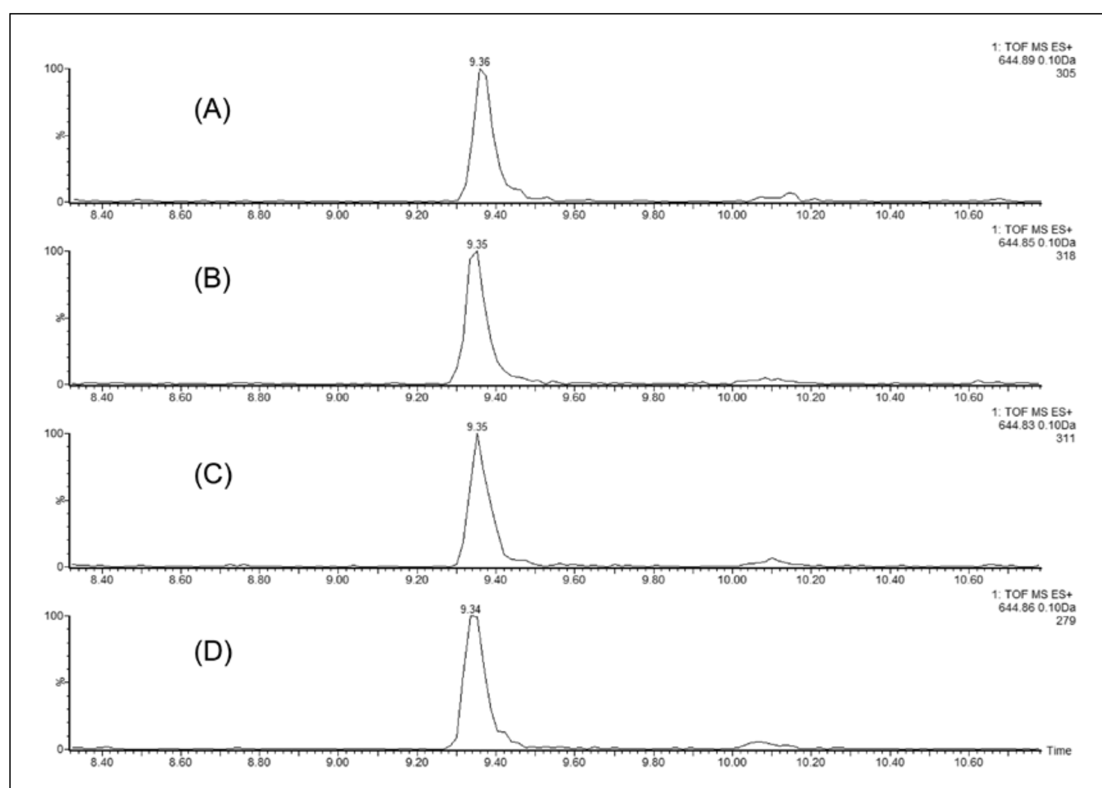

Figure 2. Reproducibility of 2D-UPLC for four consecutive experiments: extracted mass chromatograms of T43 peptide from ENL (VNQIGTLSESEIK, m/z for $[M+2H]^{2+}$: 644.86) eluted only in Fraction 4 out of 5 (using 18.6% ACN). Second dimension chromatography runs were performed at 12 µL/min using a 30-min gradient (7–35% ACN, 0.1% FA). Twenty femtomoles of ENL digest was loaded on column.

In the second experiment, we investigated the ability of the 2D-UPLC system to maintain good chromatographic performance, independent of the number of fractionation steps in the first dimension. Figure 3 displays the extracted mass chromatograms of T43 peptide generated from the second dimension separations under four fractionation schemes: "simulated 1D" (single step elution from 10.8 to 50% of Eluent B), 3-step, 5-step, and 10-step fractionations, respectively. The system demonstrates the highly reproducible retention time over the different operation schemes (0.15% RSD). The 10-step fractionation experiment revealed an important aspect regarding the high pH/low pH 2D separation of peptides: peptides can elute entirely within a single fraction (5/10) even with a relatively narrow elution step-gradient (1.9% B). As shown in Figure 4, no T43 peptide could be detected in the previous or the following fractions.

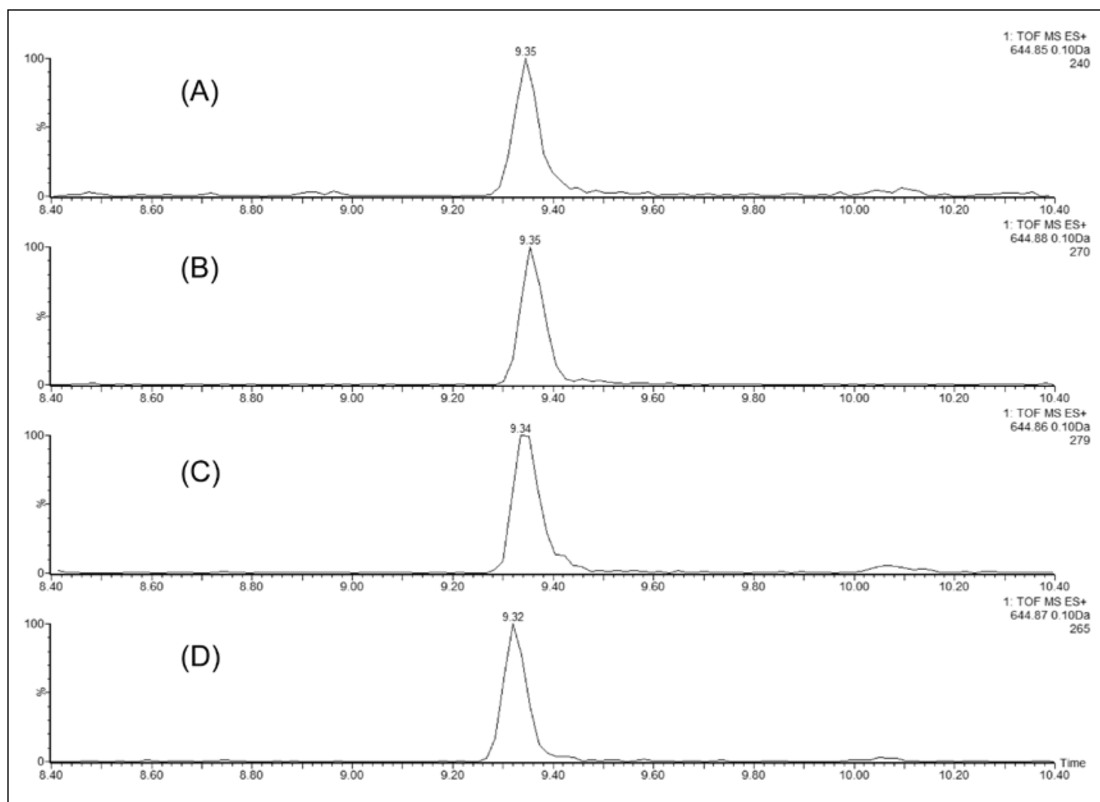

Figure 3. Chromatographic performance (e.g., RT reproducibility and peak width) is maintained during first-dimension fractionation: mass chromatograms of ENL T43 peptide obtained under four fractionation conditions: (A) "simulated" 1D run using a single elution step (from 10.8 to 50% ACN); (B) fraction 2 out of 3 (from 10.8 to 18.6% ACN); (C) fraction 4 out of 5 (from 15.4 to 18.6% ACN); (D) fraction 5 out of 10 (from 15.4 to 16.7% ACN). All separations used a 30 minute-gradient (7–35% ACN, 0.1% FA).

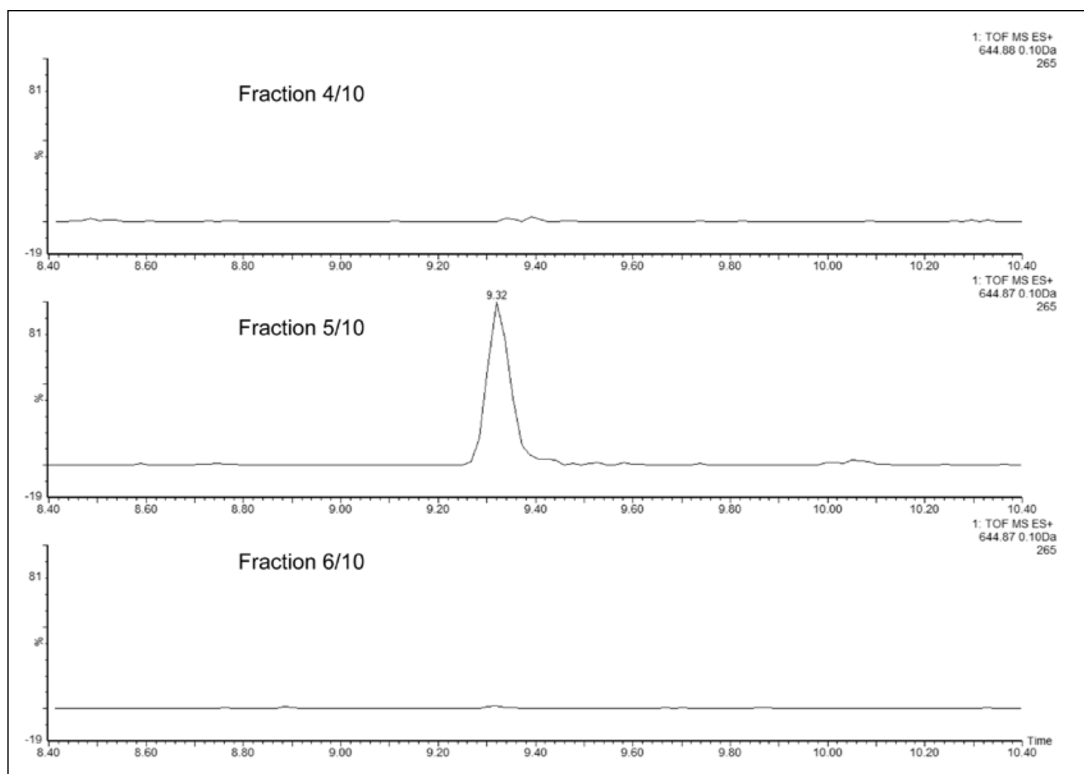

Figure 4. Performance of peptide fractionation at high pH conditions: mass chromatograms of ENL T43 peptide from three consecutive experiments using a 10-step fractionation in the first dimension: Fraction #4 corresponds to a step elution from 14.0 to 15.4% ACN; Fraction #5 was from 15.4 to 16.7% ACN; Fraction #6 was from 16.7 to 18.6% ACN. All separations were performed using a 30 min gradient (7–35% ACN, 0.1% FA). The amount loaded on-column was 20 fmoles of ENL digest.

The chromatographic performance of the 2D-UPLC setup can be maintained, regardless of the number of fractionation steps. The reproducibility of the 2D-UPLC setup with respect to the retention time, peak width/shape, and the intensity of the ESI-MS signal within four consecutive experiments demonstrates the absolute trust a laboratory studying HCPs can place in the system. Overall, the data demonstrates the ability of the high pH XBridge Column to retain peptides over the duration of the fractionation experiment (six to 10 hours) without measurable sample losses.

Identification of HCPs in mAb preparations

To test the analytical capability of the 2D-UPLC/MS^E technology for identification of low-abundance HCPs in biopharmaceuticals, we analyzed six mAb (PTG1) samples that were expressed by DG-44 (samples labeled A1, A2, B1, and B2) and CHO-S hamster cell lines (samples C and D).

Each of the samples was purified by two different protein-A chromatography columns. This single-step purification protocol was not designed to achieve fully optimized purification for the highest purity of mAb, rather it provided good test samples to examine the capability of the LC/MS assay to identify HCPs from relatively complex biological samples.

A relatively large number of CHO proteins (about 40) were co-purified (see Table 1) with the mAb target. Five proteins standards (originating from other species than the host hamster cells) were spiked in the PTG1 preparations before tryptic digestion. These protein standards serve as an internal control to probe the dynamic range of the assay and to provide internal references for quantification of HCPs using the summed signal of the three best responding peptides of each protein identified in the analysis.[8]

As shown in Table 1, a total of 37 HCPs across six PTG1 preparations were identified. Because the CHO protein database is not available in the public domain, these HCP proteins were identified using the mouse/hamster homology search. The measured HCP concentrations varied widely, from 10 to 3000 ppm. Four out of five spiked protein standards (except the lowest abundance ENL) were identified in all samples (two out of three replicates). Most of the HCPs identified in this study were high-abundance CHO proteins.

Table 1 indicates that the HCP composition/concentration significantly depends on the cell lines used for PTG1 expression. Total HCP concentrations for samples C and D produced by the CHO-S cell line are significantly lower than the total HCP concentrations measured for DG-44 (samples A1, A2, B1, and B2). In addition, the protocols used for Protein A purification of PTG1 also influences the HCP composition/concentration as suggested by Table 1.

		Protein concentration (ppm)					
Prot no	Protein Description	DG-44 cells				CHO-S cells	
		A1	A2	B1	B2	C	D
1	Nucleolin Mesocricetus auratus Golden hamster		191	1615	3034		
2	Heterogeneous nuclear ribonucleoprotein isoforms Mus musculus			1459	2413		
3	Elongation factor isoforms Mus musculus	304	745	1705	1354	142	532
4	Procollagen C endopeptidase enhancer 1 Mus musculus			1655	1265		
5	Actin cytoplasmic isoforms Mesocricetus auratus Golden hamster	907	956	813	877	287	45
6	Clusterin Mus musculus	1010	1068	658	855	537	185
7	Glycogen phosphorylase b rabbit - PHO	621	423	536	659	801	621
8	Plasminogen activator inhibitor 1 RNA binding protein Mus musculus			400	464		
9	Lipoprotein lipase Mesocricetus auratus Golden hamster	1168	481	669	464		
10	78 kDa glucose regulated protein Mesocricetus auratus Golden hamster	201	341	168	463		
11	Glyceraldehyde 3 phosphate dehydrogenase Golden hamster	448	771	573	442	104	31
12	Nascent polypeptide associated complex subunits Mus musculus			494	440		40
13	Nidogen 1 Mus musculus	38	263	42	300		
14	T complex protein 1 subunits Mus musculus	100	271	95	296		
15	Serine protease HTRA1 Mus musculus	565	265	471	286		
16	High mobility group protein isoforms Mus musculus			113	278		59
17	40S ribosomal protein S3 Mus musculus	354	323	268	278		
18	Alpha-lactalbumin bovine - LA	120	260	155	270	200	104
19	Lysosomal alpha glucosidase Mus musculus	40	385	152	266		
20	Nuclease sensitive element binding protein 1 Mus musculus		17	296	208		
21	Pyruvate kinase isozyme M2 Mus musculus	242	332	143	196		
22	Activated RNA polymerase II transcriptional coactivator p15			132	161		
23	Heat shock protein HSP 90 beta Mus musculus	76	114	121	154		
24	Nucleophosmin Mus musculus			96	145		
25	Insulin like growth factor binding protein 4 Mus musculus			150	129		51
26	Complement C1q tumor necrosis factor related protein 4	53	51	164	104		
27	Eukaryotic translation initiation factor isoforms Mus musculus	26	16	239	98		17
28	Alcohol dehydrogenase yeast - ADH	72	59	81	83	101	77
29	Interleukin enhancer binding factor 2 Mus musculus			71	81		
30	Laminin subunits Mus musculus			31	71		
31	Guanine nucleotide binding protein subunits Mus musculus	52	86	44	78		
32	Serum albumin precursor bovine - BSA	46	25	47	57	61	46
33	Cofilin Mus musculus	15	39	21	53		
34	Peroxiredoxin 1 Mus musculus	31	33	39	49		
35	Heat shock cognate 71 kDa protein Mus musculus	122	212				
36	Heat shock protein HSP 90 alpha Mus musculus	71	83				
37	Tubulin isoforms Mus musculus	122	147			81	60
	TOTAL ppm HCPs (without considering the spiked proteins)	5945	7190	12897	15302	1151	1020
	PTG1 purity	94.05	92.81	87.10	84.30	98.85	98.98

Table 1. HCP concentrations (expressed in ppm or ng HCP/mg protein) measured across six PTG1 mAB preparations using the three best responding peptides in ESI-MS. Samples labeled A1, A2 and C were purified on a ProSep-vA column, while B1, B2, and D were processed on a MabSelect Sure column. Samples A1/A2 and B1/B2 were biological replicates produced by DG-44 hamster cells, while samples C and D were expressed in CHO-S hamster cells grown under identical conditions. High abundance proteins are highlighted in red (> 1,000 ppm), medium abundance are either in yellow (500–1000 ppm) or green (100–500 ppm) and low-abundance HCPs are highlighted in grey (< 100 ppm). The mAb purity for each preparation is displayed at the bottom of each sample column.

High-throughput monitoring of HCPs by UPLC/tandem quadrupole MS

Once a purification process is established, organizations need to monitor the known HCPs to prove that their process is well-controlled. They may also need to demonstrate to the regulator that the claims made for the product are consistent throughout a number of batches.

UPLC/MRM-MS methodology can provide this information in a rigorous and objective manner that does not rely on operator interpretation. Furthermore, changes in process methodology may also mean that new ELISAs may take months to develop, whereas UPLC/MRM-MS methods can be changed within minutes to accommodate new proteins.

Therefore, a 20-min UPLC/MRM-MS method was developed on the basis of the discovery results from the 2D-UPLC/MS^E step. The UPLC/MRM-MS assay is developed to rapidly monitor the concentration changes of the previously identified HCPs prepared from a variety of experimental conditions. Twenty HCPs were selected from the list of identified proteins in the six samples.

In total, 58 transitions for 29 peptides representing the twenty HCPs were monitored in MRM experiments. The assay generated highly reproducible measurements with an average peak area RSD of 13.8% for the entire MRM dataset. The results demonstrate that UPLC/MRM-MS methodology offers an efficient method for high-throughput HCP monitoring during the late stage of biopharmaceutical purification. In addition, an MRM assay provides an easy method for absolute quantification of each individual HCP by using spiked-in isotopically labeled peptides.

It is also important to understand the correlation between the different UPLC/MS techniques used. Three HCPs (out of 20 HCPs monitored in MRM experiment) were quantified using spiked-in isotopically labeled peptides. This method matches the technique used in the industry today to obtain absolute quantification values. The MRM results correlate well with the MS^E quantification across all six samples, as shown in Figure 5A–C.

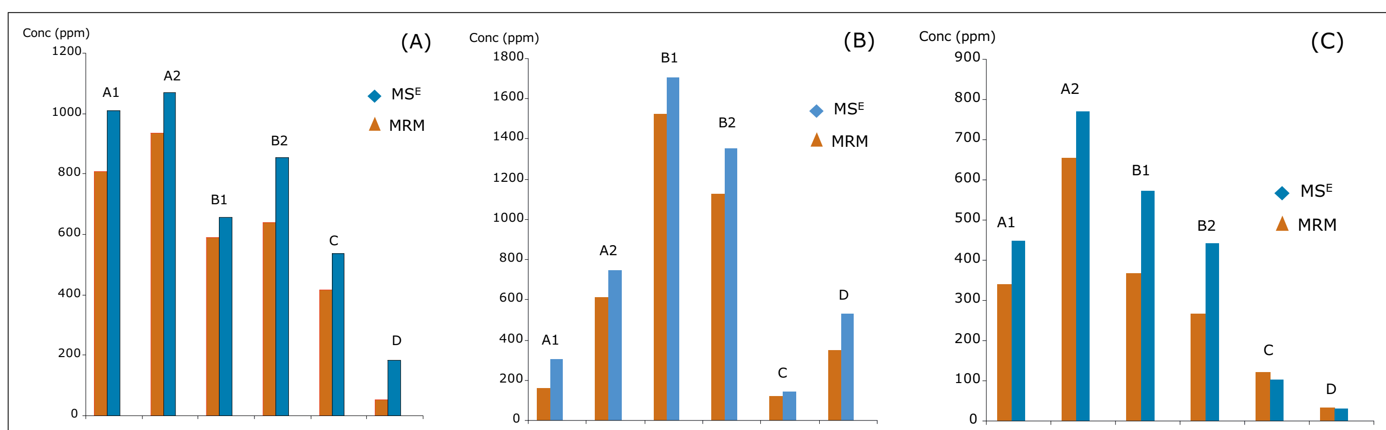

Figure 5. Comparison of HCP quantification between MS^E and MRM methods: TOF-based quantification (MS^E) is based on the precursor signals generated by the average intensity of the three best ionizing peptides against the signals produced by a spiked-in protein standard with a known concentration. MRM quantification is based on using the peak area from the signal of a spiked $^{13}C^{15}N$-isotopically labeled peptides with known concentration. Protein concentrations (ppm) measured in six mAb preparations are shown for (A) clusterin, (B) elongation factor 1-alpha, and (C) glyceraldehyde 3-phosphate dehydrogenase.

CONCLUSIONS

- The UPLC/MS^E assay allows the identification and quantification of low-abundance HCP contaminants in biopharmaceuticals over four to five orders of magnitude in concentration.
- Cell lines used for mAb purification can significantly affect the HCPs' identity and concentration.
- Protein A purification of mAbs using different purification protocols produces different HCP patterns.
- A high-throughput UPLC/MRM-MS assay for monitoring targeted HCPs using tandem quadrupole mass spectrometry can be readily implemented.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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[APPLICATION NOTE]

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UPLC/MS Monitoring of Water-Soluble Vitamin Bs in Cell Culture Media in Minutes

Catalin E. Doneanu, Weibin Chen, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

To improve a biopharmaceutical laboratory's efficiency and productivity by demonstrating a robust, specific, and rapid UPLC/MRM-MS assay for the simultaneous monitoring of 10 water-soluble B vitamins in fresh or spent cell culture media.

WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ MS

MassLynx™ Software

TargetLynx™ Application Manager

KEY WORDS

Water-soluble vitamins, vitamin B, protein production, cell culture media

INTRODUCTION

The increasing demand for protein biopharmaceuticals for therapy, diagnosis, and research has driven manufacturers to maximize protein production yield. Cell culture media is an important ingredient for increasing protein production in biomanufacturing. Therefore the biopharmaceutical industry is continually looking to optimize and efficiently monitor the composition of cell culture media.

All cell culture products contain a complex mixture of polar compounds, rich in amino acids, carbohydrates, inorganic salts and vitamins. Water-soluble vitamins are critical components because they act as catalysts or substrates to facilitate and/or control many metabolic functions during cell growth. Monitoring the concentration of B-complex vitamins during biomanufacturing allows for a better understanding of vitamin uptake by different cell lines. These measurements can be used to optimize protein production conditions in order to increase the yield of the final biopharmaceutical product.

Water-soluble vitamins from cell culture media are typically measured individually using microbiological, spectrophotometric, or titrimetric assays. A single, multi-component analysis for all B-complex water-soluble vitamins is highly desirable because this type of assay can save significant time and resources for the biopharmaceutical industry.

Simultaneous analysis of all water-soluble vitamins in cell culture media is challenging due to the complexity of the sample matrix. In addition, several vitamin B-complex components are generally very hydrophilic and are not retained well on traditional reversed phase HPLC columns. As a result, vitamin B-complex components are usually analyzed by ion-pairing reversed phase HPLC.

A UPLC® method coupled with multiple reaction monitoring (MRM)[1] and UV detection[2] was recently introduced for rapid analysis of water-soluble vitamins in foods and beverages. The method used a high-strength silica C_{18} column (HSS T3), a specially designed UPLC column for the retention of polar analytes, to overcome the weak retention of vitamins.

Here, we adapt this method for analysis of 10 water-soluble B vitamins in fresh or spent cell culture media.

EXPERIMENTAL

LC conditions

LC system: ACQUITY UPLC System

Column: ACQUITY UPLC HSS T3 C_{18} 2.1 x 150 mm, 1.8 µm

Flow rate: 300 µL/min

Column temp.: 30 °C

Mobile phase A: 10 mM ammonium formate, 0.1% formic acid in water (pH 3.1)

Mobile phase B: 10 mM ammonium formate, 0.1% formic acid in methanol

Gradient:

0.0 min	0% B
2.0 min	0% B
12.0 min	35% B
13.0 min	90% B
14.0 min	90% B
15.0 min	0% B

Injection vol.: 50 µL (partial loop injection mode)

MS conditions

MS system: Xevo TQ MS

Ionization mode: ESI+

Capillary voltage: 3.5 kV

Source temp.: 120 °C

Acquisition mode: MRM

MS1/MS2 mass window: 1 Da (unit mass resolution)

Dwell time: 20 ms

Data management

Target Lynx Application Manager for MassLynx Software

RESULTS AND DISCUSSION

Samples

Ten high-purity vitamin standards were obtained from Sigma-Aldrich (St. Louis, MO) for the following vitamins: B1 (thiamine), B2 (riboflavin), B2 phosphate (riboflavin-5'-phosphate), B3 (nicotinamide), B5 (calcium pantothenate), B6 (pyridoxal and pyridoxine), B7 (biotin), B9 (folic acid) and B12 (cyanocobalamin). Stock solutions of vitamins B1, B3, B5, B6s, B7, and B12 were prepared using deionized (Milli-Q) water. For vitamins B2s and B9 stock solutions were prepared in 100 mM ammonium formate, pH 10, to increase their solubility in water. Sample dilutions in the range of 0.1 to 10,000 ng/mL were prepared using mobile phase A. Throughout the preparation and analysis, all solutions were protected from light exposure and stored below 5 °C.

MRM method

MRM assays were performed on an ACQUITY UPLC System coupled to a Xevo TQ MS tandem quadrupole instrument. Table 1 shows the retention times, MRM transitions, optimized cone voltages, and collision energy values for vitamins analyzed in this study.

Vitamin compound	RT (min)	MRM transition	Cone voltage (V)	Collision energy (eV)
Thiamine (B1)	2.6	265.1 > 122.1	24	17
Riboflavin (B2)	13.8	377.1 > 243.1	42	22
Riboflavin-5'-phosphate (B2)	12.9	457.1 > 439.1	41	18
Nicotinamide (B3)	5.6	123.0 > 80.0	40	20
Calcium pantothenate (B5)	8.8	220.1 > 90.1	30	15
Pyridoxal (B6)	4.8	168.1 > 150.1	27	15
Pyridoxine (B6)	5.6	170.1 > 152.1	28	14
Biotin (B7)	13.3	245.1 > 227.1	28	13
Folic Acid (B9)	12.5	442.1 > 295.1	23	17
Cyanocobalamin (B12)	12.3	678.3 > 147.1	36	34

Table 1. Optimized experimental parameters for vitamins monitored.

UPLC separation of vitamins

Figure 1 shows that baseline separation of all 10 water-soluble B vitamins can be achieved in a single reversed phase UPLC run without ion-pairing reagents. In addition, the use of UPLC conditions allows the separation to be completed within a short 8-min gradient.

The chromatographic conditions reported in the experimental section were obtained after systematic optimization of gradient composition and slope, flow rate, column temperature, and injection volumes. The goal of the optimization process was to achieve the best separation for all 10 vitamin standards. Special attention was paid to the separation between vitamin B9 and B12 (see Figure 1) because they are the most challenging to be resolved at the baseline.

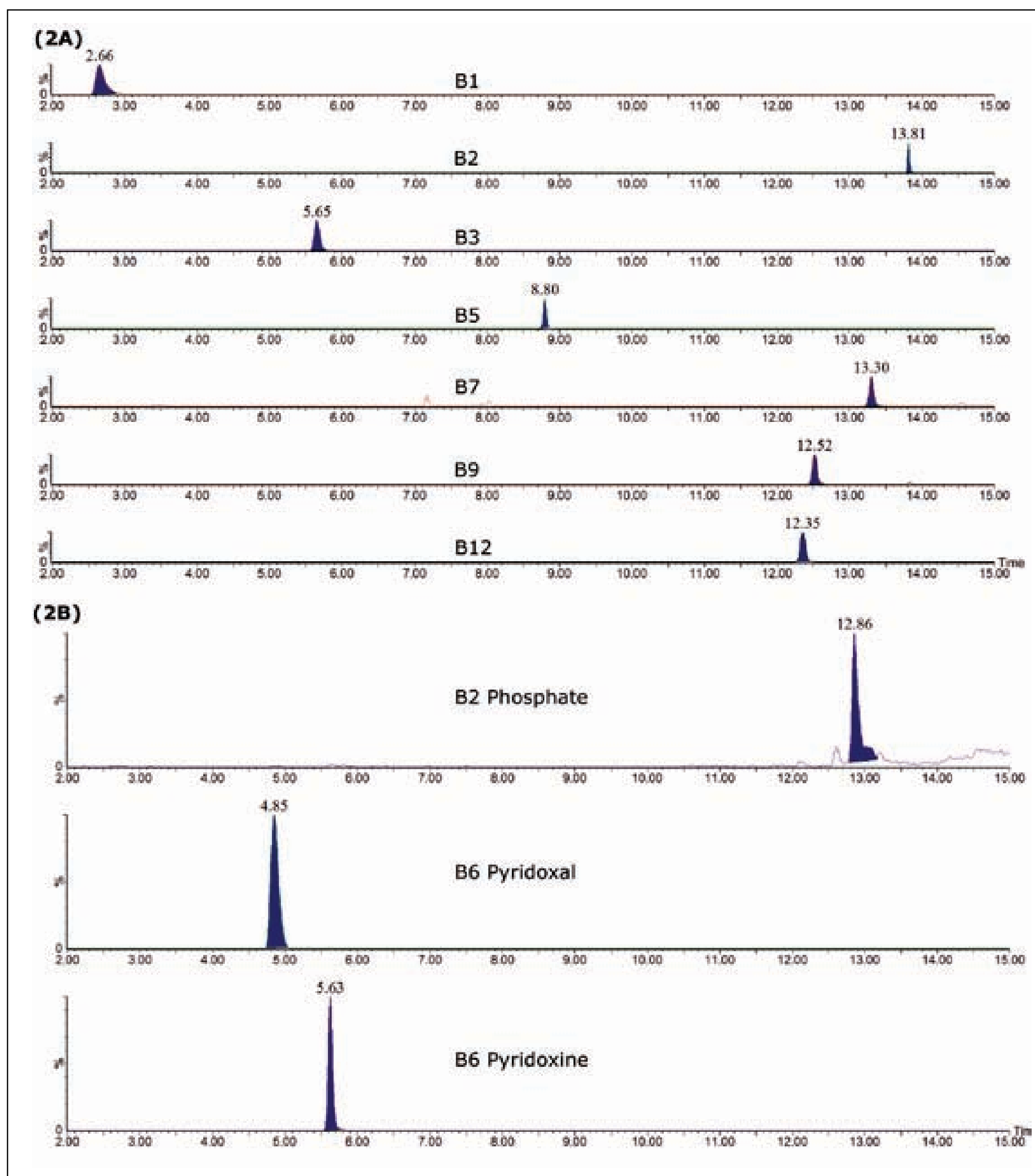

Figure 1. MRM chromatograms of 10 water-soluble B vitamins. (A) Seven B-complex vitamins detected in spent cell culture media; (B) three vitamin standards not detected in the spent media sample: riboflavin-5'-phosphate (13 ng/mL), pyridoxal (200 ng/mL), and pyridoxine (100 ng/mL).

Detection of vitamins by the MRM method

Detection of all vitamin analytes by UV absorbance was initially attempted in a spent cell culture media sample, at three different wavelengths (232, 260, and 278 nm). Figure 2 shows a UV chromatogram recorded at 260 nm, and clearly demonstrates that samples are highly complex. The presence of other interfering species in the spent cell culture media greatly hinders the utility of a UV-only detector method for monitoring vitamins in this chemical background. A more selective and sensitive detection method is clearly needed.

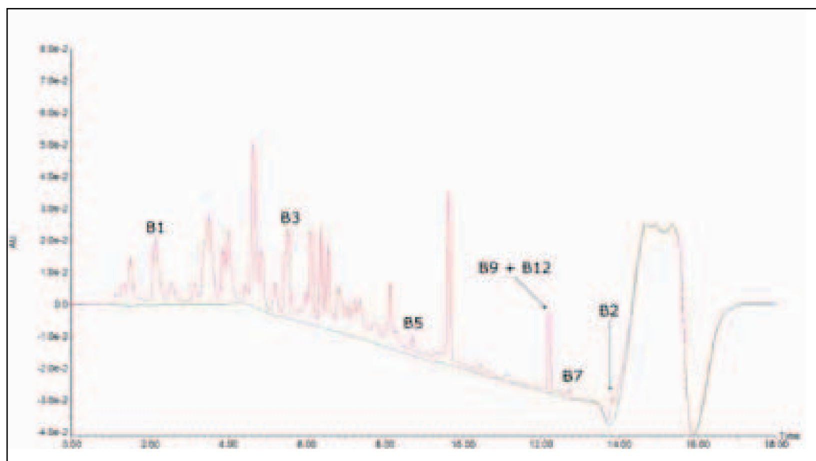

Figure 2. UPLC chromatogram (260 nm detection) acquired for a spent cell culture media sample after 1:50 sample dilution with eluent A. The green trace corresponds to a blank injection analyzed under the same conditions.

A triple quadrupole instrument, operated in MRM mode, is known to provide selectivity and sensitivity that cannot be readily matched by other detectors (e.g., UV, fluorescence), especially in the case of a complex sample matrix as shown here. With the developed MRM method, each of the 10 B-complex vitamins can be confidently identified (Figure 1).

In addition, the MRM method shows very good reproducibility and linearity as illustrated by the calibration curve of vitamin B2 presented in Figure 3. Even in the absence of an internal standard, the MRM response was linear over two orders of magnitude. For concentrations above 10 ng/mL, the peak area RSD (%) were better than 10% for all analytes, indicating the utility of this MRM assay for monitoring the vitamin uptake during protein biomanufacturing.

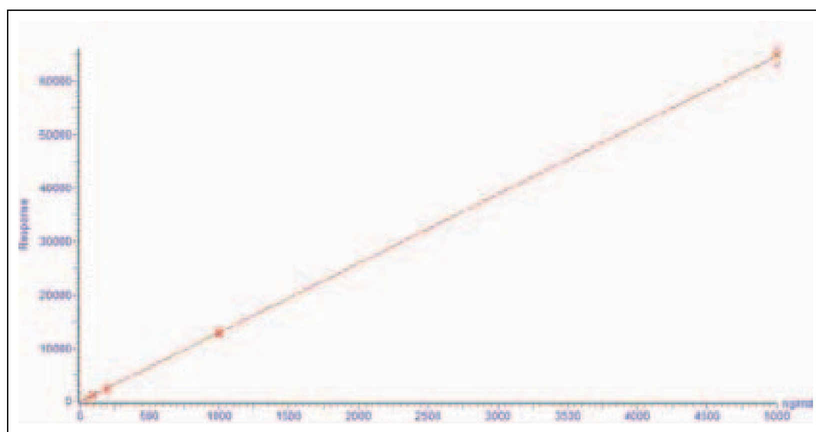

Figure 3. TargetLynx calibration curve obtained for the MRM assay of vitamin B2 in the concentration range of 50 to 5000 ng/mL. The average peak area RSD (%) for this concentration range was 5.6% (n=5).

The limits of quantification (LOQs) determined for all 10 vitamins are displayed in Table 2. These are the lowest analyte concentrations showing a residual plot deviation of 50% or lower.

Vitamin compound	LOQ (ng/mL)
Thiamine (B1)	20.0
Riboflavin (B2)	50.0
Riboflavin-5'-phosphate (B2)	13.0
Nicotinamide (B3)	20.0
Calcium pantothenate (B5)	20.0
Pyridoxal (B6)	40.0
Pyridoxine (B6)	20.0
Biotin (B7)	20.0
Folic Acid (B9)	50.0
Cyanocobalamin (B12)	20.0

Table 2. Limits of quantification (LOQ) found for MIX-10 water-soluble B vitamins assuming a residual plot value below 50%.

Vitamin analysis in spent cell culture media

Table 3 displays the calculated concentrations and peak area RSD (%) obtained for a sample of spent cell culture media when using the UPLC/MRM-MS method described here. These concentrations were measured using the standard addition method, after spiking 100 to 200 ng/mL of each vitamin standard into a spent cell culture media sample diluted 1:50 with mobile phase A.

Vitamin compound	Conc. (µg/mL)	Peak area RSD (%) (n=5)
Thiamine (B1)	7.6	2.5
Riboflavin (B2)	1.5	5.3
Riboflavin-5'-phosphate (B2)	ND	–
Nicotinamide (B3)	9.2	1.1
Calcium pantothenate (B5)	10.7	0.7
Pyridoxal (B6)	ND	–
Pyridoxine (B6)	ND	–
Biotin (B7)	0.2	2.6
Folic Acid (B9)	9.1	3.9
Cyanocobalamin (B12)	5.7	3.1

Table 3. Calculated concentrations and peak area RSD (%) values for 7 water soluble B vitamins measured in spent cell culture media

According to Table 3, the MRM assay generates very good RSD (%) values, better than 5% for all vitamins. It was found that dilution of spent media by 50–100 fold was one of the critical factors in maintaining the robustness of the separation column over hundreds of injections.

Table 4 shows the variance of peak area (RSD, %) for selected vitamins from a spent cell culture media. Each value was calculated based on the integrated peak areas that were continuously acquired over 24 hours for the same spent cell culture media sample (72 injections). The entire experiment was repeated in triplicate using freshly thawed/diluted samples each time. The RSD values observed in this study were less than 10%, demonstrating the robustness of the UPLC/MRM system solution for this application.

Vitamin compound	Experiment 1	Experiment 2	Experiment 3
Thiamine (B1)	4.0	7.2	5.1
Riboflavin (B2)	10.0	5.8	4.4
Nicotinamide (B3)	4.0	5.0	3.4
Calcium pantothenate (B5)	2.6	2.2	1.6
Biotin (B7)	3.9	3.2	3.1
Folic Acid (B9)	2.6	4.3	2.8
Cyanocobalamin (B12)	4.4	2.1	3.2
Biotin (B7)	0.2	2.6	
Folic Acid (B9)	9.1	3.9	
Cyanocobalamin (B12)	5.7	3.1	

Table 4. Peak area RSD (%) for seven water-soluble B vitamins monitored in spent cell culture media over 24 hours (72 injections) during three independent experiments.

CONCLUSIONS

A single MRM-based assay for simultaneously measuring 10 water-soluble B vitamins was developed. The assay can be applied for monitoring these analytes in cell culture media.

The assay meets a number of key performance criteria:

- The linearity is at least two orders of magnitude in concentration, with the LOQ lower than the vitamin concentration levels commonly found in cell culture media
- Minimal sample preparation/handling is required and the assay is very appealing for fast, continuous monitoring of vitamin uptake during biomanufacturing
- The UPLC/MRM-MS assay is robust, with peak areas with less than 10% RSD during three replicate experiments, each consisting of 72 injections performed over a 24-hour interval.

Biopharmaceutical organizations that adopts UPLC/MRM-MS methodology stands to benefit from gains in efficiency and productivity. Being able to rapidly react to cell culture changes or to prevent cell culture failures can save time and improve product yields, with a direct contribution to the profitability of the organization.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
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MRM Quantification of Low-Abundance Oxidation in an IgG1 Monoclonal Antibody

Hongwei Xie, Catalin E. Doneanu, Martin Gilar, and Jeffery R. Mazzeo
Waters Corporation, Milford, MA, U.S.

APPLICATION BENEFITS

This work evaluates the use of targeted LC/MS analysis using multiple reaction monitoring (MRM) for fast and sensitive monitoring and quantification of degradations in mAb digests. This approach, using UPLC® with a sensitive tandem quadrupole MS, can also be adapted for monitoring and quantification of other PTMs and degradations such as N-linked glycosylation. This solution offers a significant improvement in speed, specificity, and sensitivity of mAb analysis and has the potential to expedite the research and development of protein drugs.

WATERS SOLUTIONS

ACQUITY UPLC®

Xevo® TQ MS

KEY WORDS

Multiple reaction monitoring (MRM), quantification of degradation, monoclonal antibody digests

INTRODUCTION

Post-translational modifications (PTMs) and chemical degradations impact the efficacy and safety of therapeutic proteins and peptides. Therefore, they have to be effectively controlled and monitored during production, formulation, and storage. Liquid chromatography (LC)-based peptide mapping with ultra violet (UV) and/or mass spectrometry (MS) detection is a method of choice for characterization of PTMs and degradations.[1]

However, higher throughput and more sensitive methods are needed for routine monitoring and quantification of protein drugs during development and manufacturing. For example, in protein formulation development hundreds of samples are typically analyzed to insure purity and stability. Although intact protein mass measurement (including limited proteolysis followed by LC/MS analysis) can be used for fast evaluation of large mass-shifted PTMs such as glycosylation, it does not provide information on modification sites and has limitations for detection of modifications with small mass shifts,[2, 3] such as asparagine (N)-deamidation, aspartic acid (D)-isomerization, and methionine (M)-oxidation.

Monoclonal antibodies (mAbs) are an important class of biotherapeutics. They are susceptible to a number of PTMs and degradations common to other classes of therapeutic proteins. Peptide mapping studies[3, 4] demonstrate that PTMs and degradations, such as glycosylation, deamidation, and oxidation, only occur in specific motifs of a mAb. This application note evaluates the use of targeted UPLC/MS analysis using multiple reaction monitoring (MRM) for fast and sensitive monitoring and quantification of degradations in mAb digests.

As a proof of concept, we performed a UPLC/MRM study with the goal of confirmation and quantification of site-specific M-oxidations in a recombinant IgG1 tryptic digest previously identified by UPLC/MS^E peptide mapping studies.[3, 4]

Both oxidized and unmodified M-containing tryptic peptides were unambiguously determined by multiple MRM transitions, and relative quantification data were obtained for a low-abundance M-oxidized peptide.

The large dynamic range and linearity of the combined ACCQUITY UPLC/Xevo TQ MS System[5,6] used for this UPLC/MRM assay offers a significant improvement in speed, specificity, and sensitivity of mAb analysis and has the potential to expedite the research and development of protein drugs by saving both time and analytical resources.

EXPERIMENTAL

LC conditions

LC system: ACQUITY UPLC

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

Column temp.: 60 °C

Flow rate: 300 µL/min

Injection vol.: 10 µL (partial loop injection mode)

Mobile phase A: 0.1% FA in water

Mobile phase B: 0.1% FA in ACN

Gradient: 0-40% B in 60 min

MS conditions

MS system: Xevo TQ MS

Ionization mode: ESI+

Acquisition mode: MRM

Capillary voltage: 3.5 kV

Cone voltage: 35 V

Mass window: 1 Da (unit mass resolution)

Dwell time: 5 ms

Materials and reagents

Synthetic peptides DIQMTQSPSSLSASVGDR (purity 80%) and DIQMoxTQSPSSLSASVGDR (purity 87%, Mox-oxidized methionine) were purchased from Biomatik Corporation (Canada). The IgG1 antibody trastuzumab digest was prepared by a RapiGest™-assisted 4-h trypsin digestion protocol.[7,8]

RESULTS AND DISCUSSION

In previous LC/MS^E peptide mapping studies[3,4] we detected two primary M-oxidation sites from the antibody. One site is located in heavy chain tryptic peptide HT21 (DTLMISR, ~4% oxidation) and the other in light chain tryptic peptide LT1 (DIQMTQSPSSLSASVGDR, <0.5% oxidation). Because of the complexity of the mAb digest, low-abundance oxidized peptide peaks are often obscured by more dominant peptides in both LC/UV and LC/MS analysis.

Here, we demonstrate that LC/MRM analysis provides enhanced selectivity and can detect M-oxidation of peptides at low-abundance. The method is potentially applicable for monitoring other PTM in protein drugs as well.

Figure 1 contains the MRM chromatograms of unmodified and oxidized LT1 eluted from the antibody tryptic digest. As expected, oxidized LT1 (at 19.35 min) eluted earlier than unmodified LT1 (at 23.29 min) due to reduced hydrophobicity upon M-oxidation. The use of three MRM transitions (see Figure 1 for details) clearly confirms the identity of the peptides, demonstrating that the use of multiple MRM transitions in this manner has advantages over single ion monitoring (SIM) or extracted ion chromatogram (XIC) techniques for monitoring protein PTMs from complex samples such as protein tryptic digests. Very limited in-source M-oxidation[9] (<0.01%) was observed in this experiment (signal present at retention time identical to non-oxidized peptide peak).

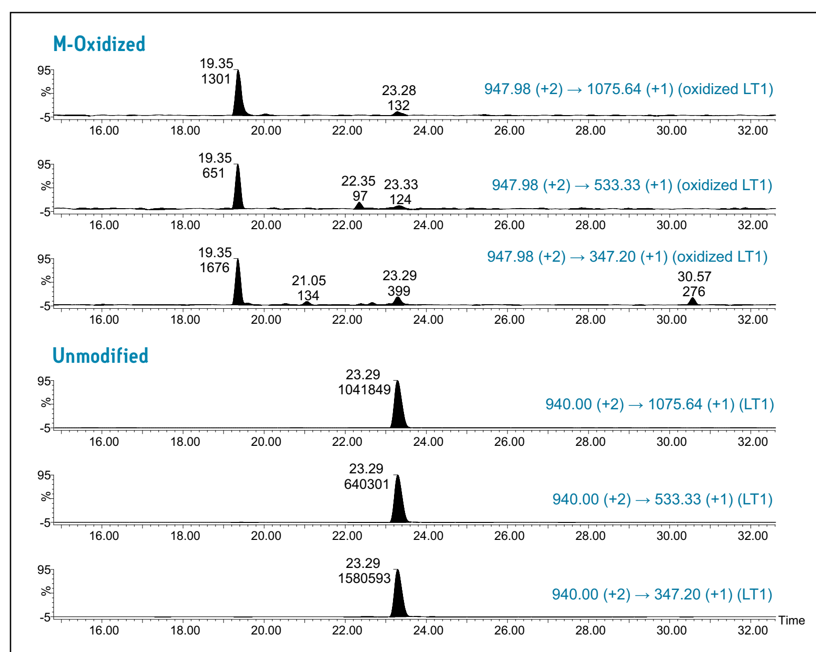

Figure 1. MRM chromatograms of unmodified and M-oxidized LT1 (DIQMTQSPSSLSASVGDR).

The same relative concentration (~0.1%) of oxidized LT1 was obtained when quantifying by integrated peak areas of either corresponding transition pair. The relative standard deviation (RSD in %) for integrated peak area of unmodified LT1 is 2.7%; the RSD for low-abundance oxidized LT1 is 23.9% (calculated from 3 repeated injections). The relatively large RSD for low-abundance oxidized LT1 is due to the MS response variation of the minor signal. The MRM transition of precursor to fragment y_3 (374.20 Da) has the highest intensity for both oxidized and unmodified LT1. Quantification using peak areas of this transition provides the highest sensitivity and accuracy.

Next, we tested the dynamic range and quantification linearity of the method using synthetic oxidized and unmodified LT1. The transitions 947.98 Da to 347.2 Da (for oxidized LT1) and 940.0 Da to 347.2 Da (for unmodified LT1) were selected for this study because of their high MS response.

Four orders of dynamic range and linear response (from low fmole to nmole) were observed for both unmodified and oxidized LT1. This indicates that M-oxidation with relative concentration as low as 0.01% could be detected and quantified by this method. The MRM response of LT1 at different dilutions and concentrations of the stock (with dilution factors of 0.001, 0.002, 0.01, 0.02, 1, 10 and 50) was plotted in Figure 2. The estimated limit of detection (LOD) and limit of quantification (LOQ) were below 50 fmole.

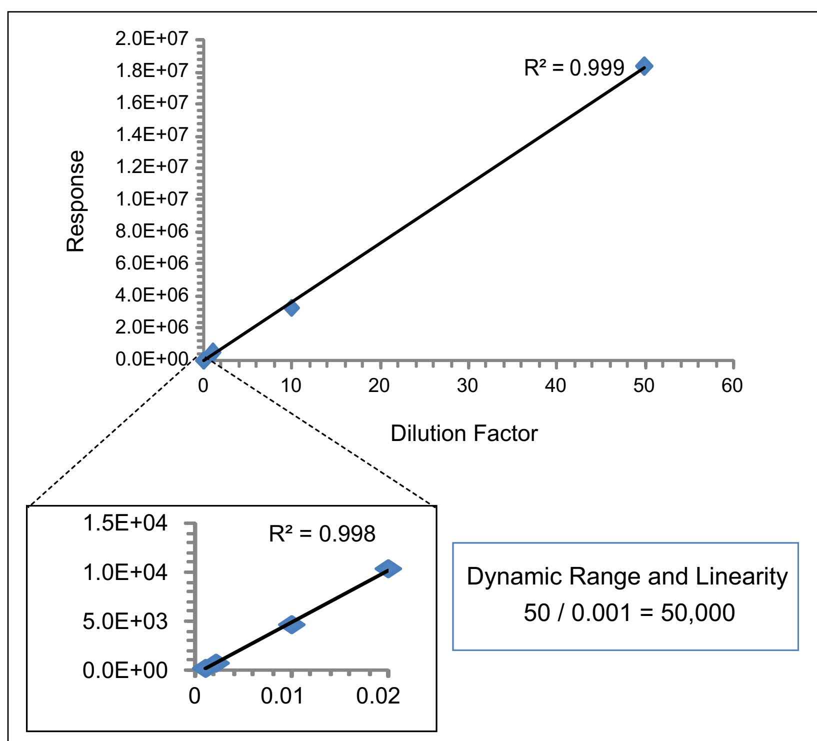

Figure 2. MRM responses of LT1 (DIQMTQSPSSLSASVGDR) at different dilutions.

CONCLUSIONS

A UPLC/MRM assay was developed for quantification of site-specific M-oxidization in a recombinant monoclonal antibody. The method offers the following benefits:

- Simultaneous confirmation and quantification of peptide modifications
- Sensitive quantification of low-abundance modifications in protein digests
- LOD and LOQ for M-oxidation as low as fmole level
- 10^4 order dynamic range and linearity, relative quantification to 0.01% level.

The technology demonstrated here can also be adapted for monitoring and quantification of other PTMs and degradations such as N-linked glycosylation. Unlike traditional MRM methods, no internal standards are required for relative quantification of PTMs and degradations in therapeutic proteins.

The technology could be targeted for high throughput monitoring of critical attributes in biotherapeutics from batch to batch.

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T: 1 508 478 2000
F: 1 508 872 1990
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