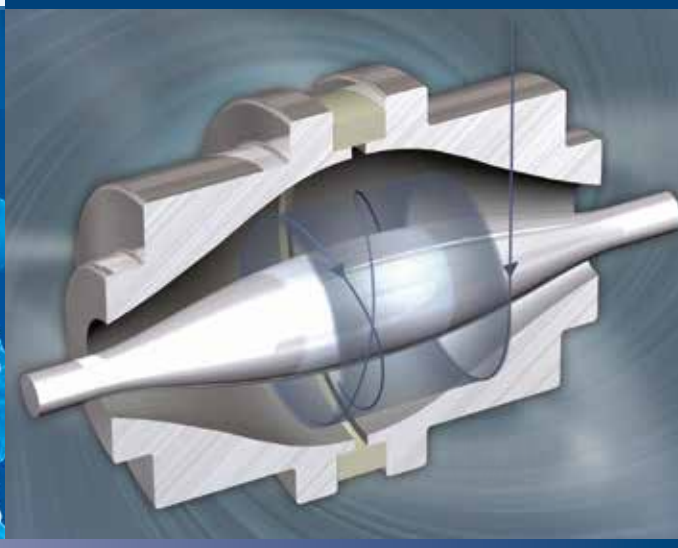
LCMS for Biopharm Analysis



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Improving Intact Antibody Characterization by Orbitrap Mass Spectrometry

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Thermo Fisher Scientific, Dreieich[1] and Bremen,[2] Germany

Introduction

Recombinant monoclonal antibodies have gained significant importance in diagnostic and therapeutic applications over the past years. In order to verify the correctness of the overall molecule to provide a reproducible, safe and effective biological drug compound, the correct protein sequence, as well as the presence and relative abundance of different glycoforms have to be confirmed.

Here we present an approach to analyze an intact monoclonal antibody in non-reduced and reduced condition by LC-MS using the Thermo Scientific™ Orbitrap Elite™ mass spectrometer. The intact antibody and the separated light and heavy chains were analyzed in Full MS experiments as well as with top-down experiments using in-source CID (SID), CID, HCD and ETD fragmentation techniques making use of the ultrahigh resolution of the mass spectrometer. For data evaluation ProSight software and Thermo Scientific™ Protein Deconvolution™ software version 1.0 packages were used.

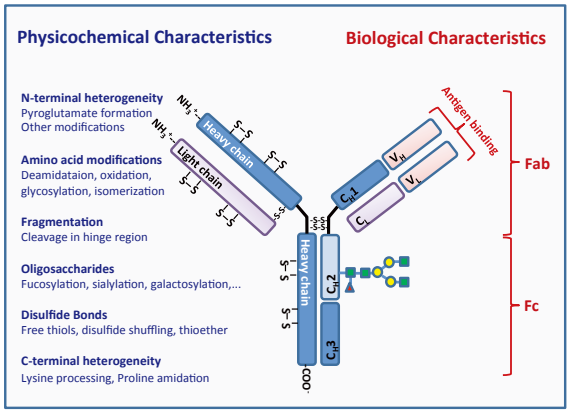


FIGURE 1. General structure of mAbs and their biological and physico-chemical characteristics.

Methods

Sample Preparation

AbbVie™ HUMIRA™ (adalimumab, Figure 2) [1]: The intact antibody (144 kDa) was dissolved in 0.1 % FA to 1 µg/µL; 5 µg HUMIRA were loaded onto the column.

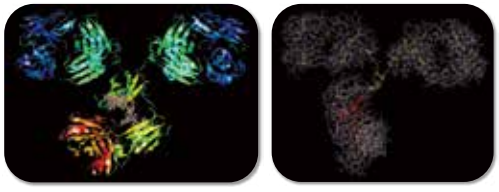


FIGURE 2. 3D structure of HUMIRA highlighting the attached glycans and cystein residues forming inter- and intra-chain disulfide bridges.

For analyzing HUMIRA light chain (24 kDa) and heavy chain (51 kDa) separately, 50 µg HUMIRA was reduced with DTT (20-fold molar excess, 56 ℃ for 1 h) and alkylated with iodoacetamide (50-fold molar excess, room temperature for 30 min in the dark).

Instrument

A Thermo Scientific™ Surveyor™ MS Pump Plus was coupled to an Orbitrap Elite mass spectrometer that was equipped with ETD (Figure 3).

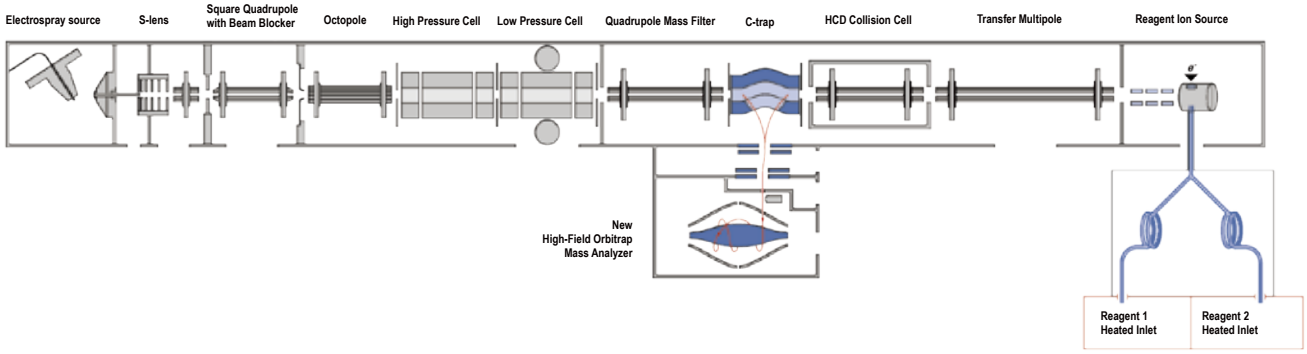


FIGURE 3. Schematics of the Orbitrap Elite hybrid ion trap-Orbitrap mass spectrometer equipped with an ETD source.

Samples were purified on a Thermo Scientific™ BioBasic™ C4 column (150 × 1 mm, 5 µm particles), solvent A: 0.1 % FA, 2 % ACN in H2O, solvent B: 0.1 % FA in ACN. The LC gradient was 7 min 20–40 % B, 3 min 40–80 % B at a flow rate of 100 µL/min.

Data analysis was done using Protein Deconvolution and ProSight software packages.

Results

The analysis of large proteins of the size of intact antibodies (~150 kDa) using Orbitrap mass spectrometers has been significantly improved over the past few years. Large molecules like mAbs show only very short transient lifetimes due to their relatively big cross section. Thus, the method of choice for intact antibodies is to use the shortest transient duration (48 ms) available on the Orbitrap Elite MS (Figure 4).

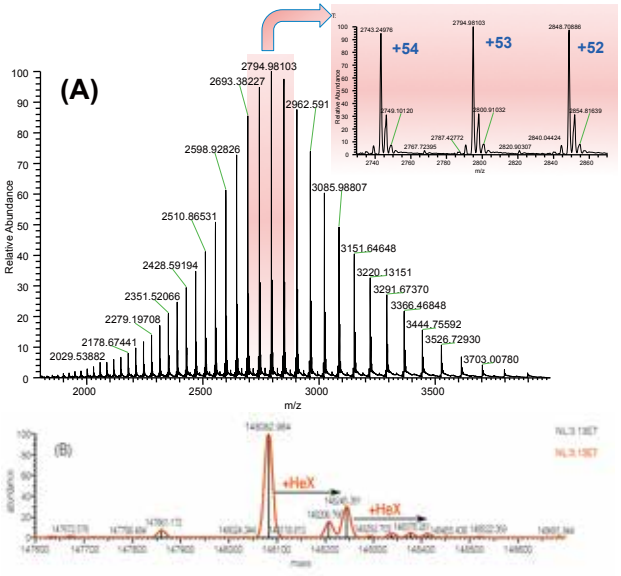


FIGURE 4. (A) Full MS spectrum of intact HUMIRA. The insert shows a zoom into the three most abundant charge states z=52,53,54. (B) Spectrum after deconvolution.

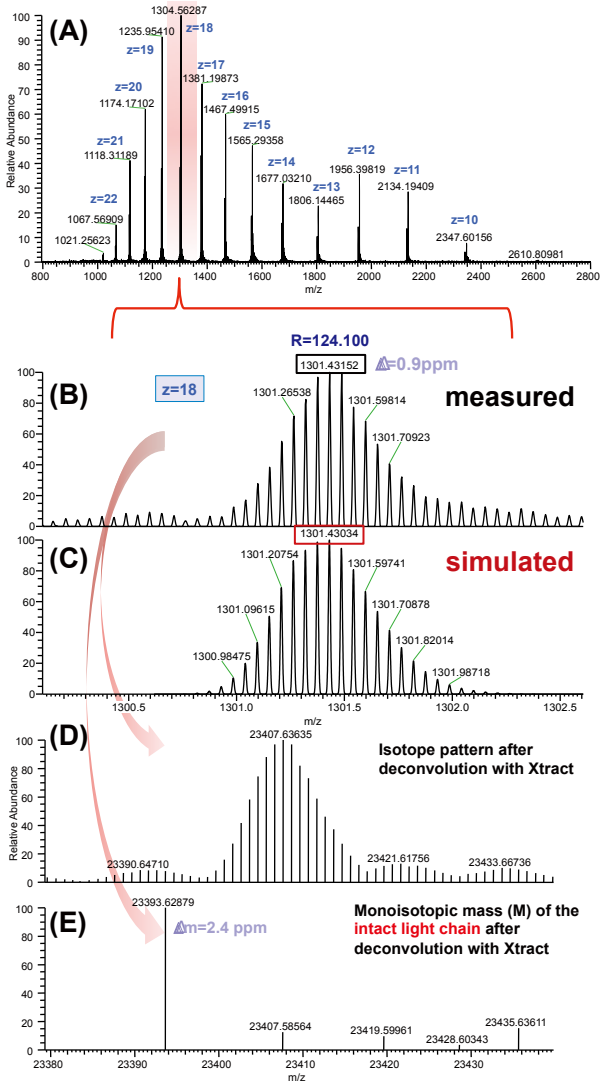


FIGURE 5. (A) Full MS spectrum of intact light chain of HUMIRA. (B) Zoom into +18 charge state of intact light chain. (C) Simulation of isotope pattern of +18 charge state. (D) Isotope pattern of intact light chain after deconvolution. (E) Monoisotopic mass (M) of the measured light chain of HUMIRA obtained after deconvolution with Xtract.

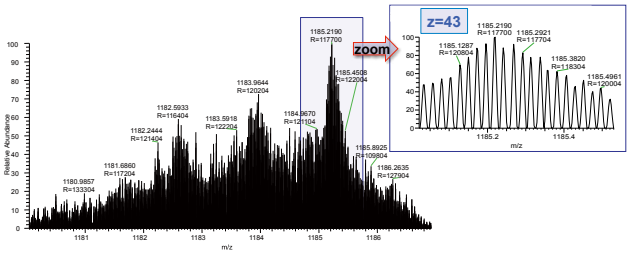


FIGURE 6. HUMIRA heavy chain acquired in SIM scan mode (z=43). 60 µscans were averaged. Deconvoluted mass: Mr 50,891.04317 Da. The inserts on the right demonstrate isotopic resolution of that charge state detected at m/z 1185 and masses obtained after deconvolution using Xtract.

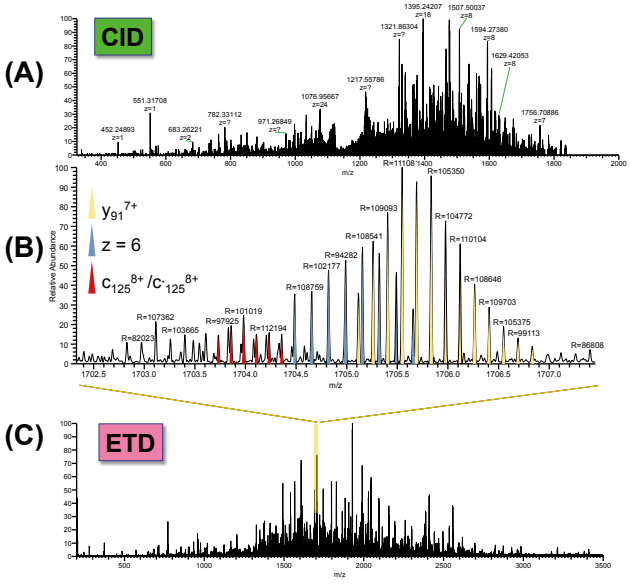


FIGURE 7. (A) CID spectrum and (C) ETD spectrum of intact HUMIRA antibody. (B) Zoom in into the ETD fragment ion spectrum of intact HUMIRA showing the need for highest resolution possible.

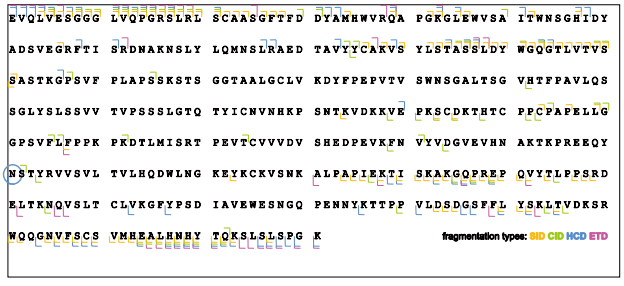
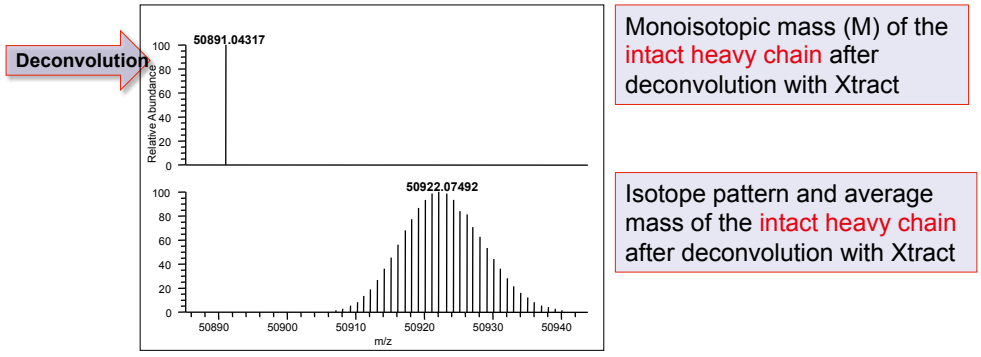


FIGURE 8. Summarized sequence coverage of the HUMIRA heavy chain using fragmentation techniques SID, CID, HCD, and ETD. Optimized conditions: trapping under high pressure settings. N: Putative glycosylation site.



Conclusion

- The analysis of intact and reduced antibodies on the Orbitrap Elite mass spectrometer provides the accurate molecular weight, as well as valuable information about the presence and abundance of glycoforms.
- Analysis of the reduced antibody provides isotopically resolved mass spectra for both light and heavy chain.
- The combination of multiple fragmentation techniques in top-down analysis (SID, CID, HCD and ETD) generates comprehensive sequence coverage and enables fast localization of modifications with minimum sample preparation.
- For measurements of intact light and heavy chain as well as for the detection of fragment ion spectra from top-down experiments ultrahigh resolution as provided by the Orbitrap Elite mass spectrometer is essential.

Abbreviations

ACN, acetonitrile; CID, collision-induced dissociation; C-trap, curved linear trap; DTT, dithiothreitol; ETD, electron transfer dissociation; FA, formic acid; HCD, higher energy collision-induced dissociation; mAb, monoclonal antibody; µS, micro-scan; SID, *in-source* decay; SIM, single ion monitoring.

References

1. Bondarenko, P.V., Second, T.P., Zabrouskov, V., Makarov, A. & Zhang, Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. *Journal of the American Society for Mass Spectrometry* **20**, 1415-24 (2009).
2. Michalski, A. *et al*. Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. *Molecular & cellular proteomics : MCP* (2011).doi:10.1074/mcp.O111.013698

Acknowledgements

We would like to thank Paul Thomas from Northwestern University (USA) for processing the HUMIRA ETD data.

Comprehensive Characterization of Intact Monoclonal Antibody Using High Resolution Benchtop Quadrupole-Orbitrap LC-MS/MS

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Overview

Purpose: A LC/MS-based workflow solution was developed for robust, accurate and comprehensive intact monoclonal antibody (mAb) characterization.

Methods: Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometers were used for intact mass measurement and top-down sequencing. Full MS spectra of intact or reduced mAb were analyzed using Thermo Scientific Protein Deconvolution 2.0 software that utilizes the ReSpect™ algorithm for molecular mass determination. The top-down msx HCD spectra were analyzed using Thermo Scientific ProSight PC 2.0.

Results: A mass error of less than 10 ppm was routinely achieved for intact mAb mass measurement. Low mass modifications, such as oxidation, can be confidently identified on substructure level such as intact Fab, or light chain. Using an on-line high resolution top-down MSMS approach, over 30% of the fragmentation site was covered for intact light chain as well as for Fab heavy chain. Sequence coverage from top-down approach also confirmed disulfide linkage on partially reduced samples.

Introduction

Monoclonal antibodies (mAbs) are increasingly developed and utilized for the diagnostic and therapeutic treatment of diseases including cancer. mAb can exhibit heterogeneity and thorough analytical characterization is required to obtain the approval to use mAb as a therapeutic product. Among the analytical tools used for the analysis of therapeutic mAb, mass spectrometry has become important in providing valuable information on various protein properties, such as intact mass, amino acid sequence, post-translational modification including glycosylation form distribution, minor impurities due to sample processing and handling and high order structure, etc. Characterization at intact protein level is usually the first step. In this study, a high resolution LC-MS based workflow solution was developed for robust, accurate and comprehensive mAb characterization at intact protein level. The fast chromatography, the superior resolution and mass accuracy provided by the Q Exactive Orbitrap™ MS, and accurate data analysis of this workflow provides high-confident screening tool to accelerate biopharmaceutical product development cycles.

Methods

Samples: Four intact mAbs were used in this study. To reduce intact mAb, the sample was incubated for one hour at 60 ℃ or 37 ℃ in 6 M guanidine-HCl containing 5 mM DTT for complete or partial reduction, respectively. Fab was generated using papain in 1mM EDTA, 10 mM Cys, 50 mM sodium phosphate buffer, pH 7,0. Before digestion, the enzyme suspension (10mg/ml) was activated for 15 min at 37C in the same buffer at an enzyme: buffer ratio of 1:9. The digestion was performed at 37C overnight using an enzyme: antibody ratio of 1:99 w/w.

HPLC: Thermo Scientific ProSwift RP-10R monolithic column (1 × 50mm) was used for desalting and separation of light and Fab heavy chain. LC solvents are 0.1%

formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80℃ during analysis. Flow rate was 60 µL/min. After injection of 1 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0min, 20%B; 1.0min, 35%B; 3.0min, 55%B; 4.0min, 98%B; 7.0min, 98% B; 7.1min, 20%B; 15.0min, 20%B).

Mass Spectrometry: Q Exactive Orbitrap instruments were used for this study. Intact and reduced mAbs were analyzed by ESI-MS for intact molecular mass. Top-down MSMS was performed using high energy collision dissociation with a unique spectrum multiplexing feature (msx HCD). In this data acquisition mode, fragment ions produced from several individual HCD events, each on a precursor of a different charge state of the reduced mAb, were detected together in the Orbitrap mass analyzer. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275℃ . S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb and intact Fab average mass measurement, or 140,000 for light chain and Fab heavy chain monoisotopic mass measurement. Resolution was set at 140,000 for top-down MSMS. The AGC target was set at 3E6 for full scan and 2E5 for MSMS. Maximum IT was set at 250 ms.

Data Processing: Full MS spectra were analyzed using Protein Deconvolution 2.0™ that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input m/z spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses with the various combinations of commonly found glycoforms. The top-down msx HCD spectra were analyzed using ProSightPC™ software under the single protein mode with a fragment ion tolerance of 5 ppm.

Results

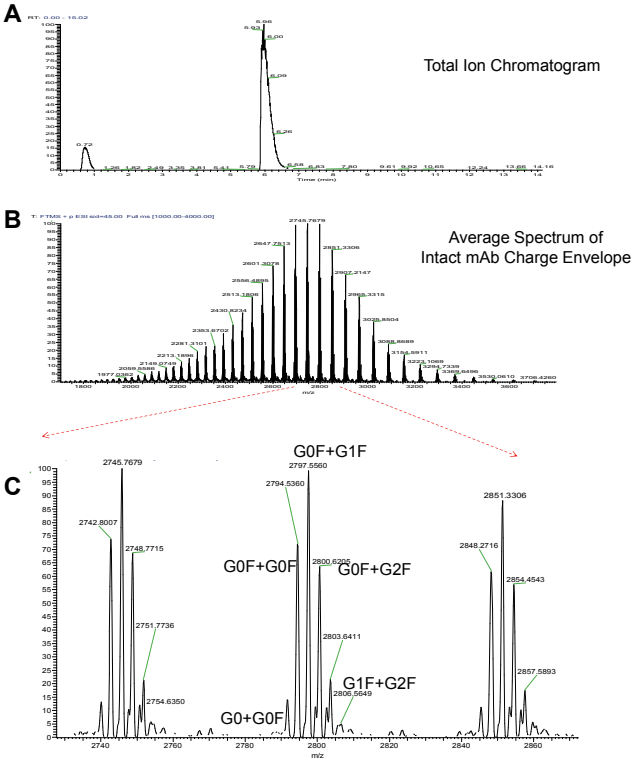

Figure 1. Intact mAb analysis using LC MS

One micrograms of mAb were desalted and eluted from a Dionex ProSwift RP-10R™ monolithic column using a 15min gradient and analyzed using ESI-MS on the Q-Exactive mass spectrometer. As shown in Figure 1., the mAb was eluted over one minute as shown in (A). The average spectrum over the elution time shows a nicely distributed complete charge envelope of the mAb (B). A zoom-in view of each charge state reveals five major glycosylation forms that are baseline separated (C).

After each of the mAb datasets were analyzed using the Protein Deconvolution software, the masses were compared to the masses expected for the known amino acid sequence with the various combinations of glycoforms commonly found on mAbs. One such result is shown below in Figure 2.

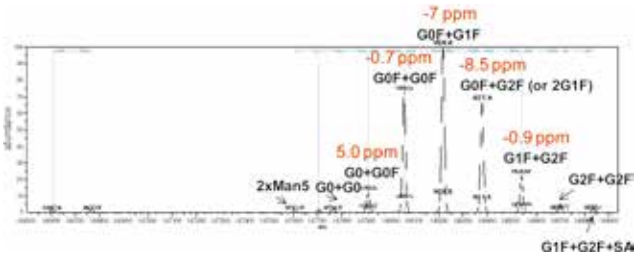

Figure 2. Deconvoluted spectrum for a mAb with known composition and mass errors of average molecular mass

To measure the mass accuracy and reproducibility of mAb samples on the Q Exactive mass spectrometer in conjunction with Protein Deconvolution, the mAb sample was analyzed several times using two different instruments over three different days. The results for ppm mass accuracy are shown in Table 1 and the results for relative abundance of the various glycoforms are shown in Table 2.

Table 1. ppm mass deviations from expected target masses for the 5 most abundant glycoforms

RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	-10.5	0.7	-10.5	-13.8	-18.0
2	1	-11.6	-1.1	-8.8	-11.2	-12.0
3	1	5.1	-5.0	-2.6	5.1	5.6
4	2	-14.3	3.0	-6.9	-5.4	-5.9
5	2	-8.6	-2.2	-12.2	-12.5	-12.9
6	2	-14.3	-6.6	-12.3	-14.8	-10.1

The average ppm error for all 34 measurements of four different mAbs on multiple instruments was 6.9 ppm with a standard deviation of 6.4 ppm (not all the data are shown here). This indicates that the Q Exactive is a very powerful platform for confirmation of protein primary structure.

Table 2. Relative abundance for the 5 most abundant glycoforms

RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	12.9	74.1	100.0	67.0	23.4
2	1	12.0	72.8	100.0	66.2	22.0
3	1	12.2	75.0	100.0	67.0	23.6
4	2	12.7	75.7	100.0	63.6	21.6
5	2	13.2	75.4	100.0	64.8	21.0
6	2	12.9	76.6	100.0	64.7	21.6
CV		3.4%	1.6%	N.A.	3.9%	4.4%

For the top 5 glycoforms, the relative intensity reproducibility is within a few percent.

Sub-structure	Resolution	Delta Mass from non-oxidized (Da)	Protein level mass error (ppm)
Fab	17.5K	16.3	6.4
Fab heavy	140K	16.0158	0.7
Fab light	140K	16.0152	0.7

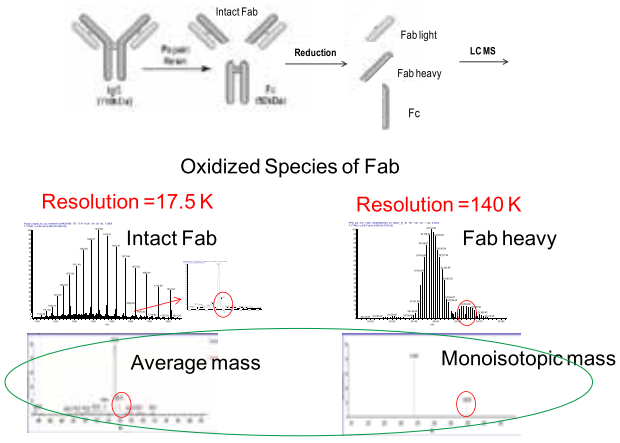

Figure 3. Identification of oxidation on intact Fab, light and Fab heavy chain

Further characterization at substructure level is shown in Figure 3 to Figure 5. Fab was generated using papain which cleaves this molecule at hinge. Fab was then reduced to generate light chain and Fab heavy chain (Figure 3, top). LC-MS of intact Fab, light chain and Fab heavy chain identified oxidation species as shown above (Figure 3, middle). The mass errors of the identification at protein level were 6.4 ppm at resolution 17,500 for Fab and less than 1 ppm at resolution 140,000 for light chain and Fab heavy chain.(Figure 3 bottom)

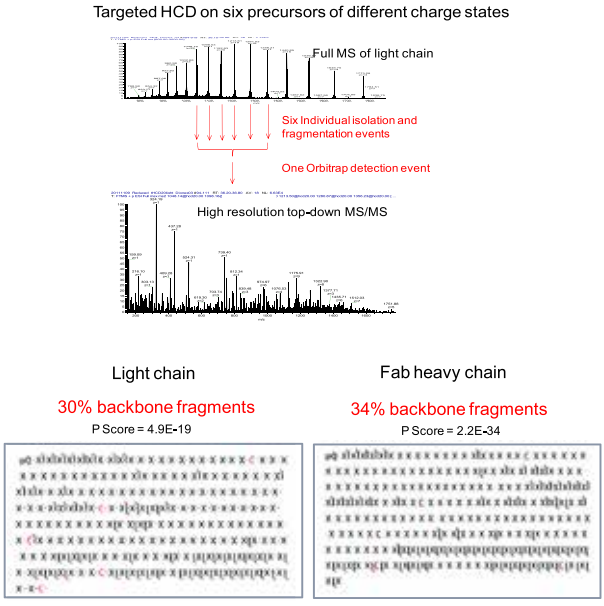

Figure 4. Top-down sequencing of light and Fab heavy chain using LC-MS/MS

Besides molecular mass, amino acid sequence can be obtained at intact protein level using a top-down LC-MS/MS approach. High resolution top-down HCD was performed using a multiplexing mode where multiple precursors, which were the same protein molecule carrying different number of charges, were isolated, fragmented separately and the resulting fragment ions were then detected all together in a single Orbitrap detection event (Figure 4 top). More than 30% of fragments from backbone cleavage were detected for both light chain and Fab heavy chain (Figure 4 bottom) with excellent P-score from ProSight PC software.

Top-down sequencing was also performed on a partially-reduced light chain which is 4.02 Da less in molecular mass than the fully reduced species. ProSight PC analysis of the HCD spectrum matched two disulfide linkages which is typical of this type of IgG molecule (Figure 5).

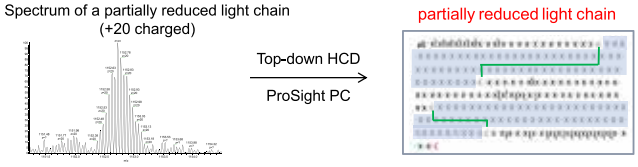

Figure 5. Top-down sequencing maps disulfide linkage on partially-reduced light chain

Conclusion

In this study, a workflow was developed that combines high resolution Orbitrap MS, fast chromatography, high throughput msx HCD and accurate data analysis to characterize intact mAb. The precise mass measurement and extensive, high confident amino acid sequence obtained from this workflow provides the following information for intact mAb and its substructure:

- Accurate measurement of intact molecular mass
- Reproducible quantification of glycoform relative abundances
- Confident amino acid sequence information and protein structural information

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Analysis Of Intact Macromolecular Assemblies On A Bench Top Orbitrap MS System

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Overview

Purpose: Measure proteins in native like state to study the biologically active conformations.

Methods: Protein samples were measured using a Thermo Scientific™ Exactive Plus EMR™ mass spectrometer with chip based static nanospray using an Advion™ TriVersa™ Nanomate™.

Results: It could be shown that for proteins of various molecular weight clear separation of isotope resp. isoform signals could be achieved due to full desolvation even under native conditions.

Introduction

Intact proteins are routinely measured using ESI-MS instrumentation under acidic, denaturing conditions, destroying large, non covalent protein assemblies and substrate bound complexes. Under native conditions, fully active protein assemblies can be studied, but these experiments are challenging due to the limited surface area of protein complexes for protonation at physiological pH.Ion signals are shifted to higher m/z values, which until recently, only TOF instruments were capable of detecting. TOF measurements have limited achievable resolution, making it difficult to resolve specific isoforms or substrate complexes. With an Exactive Plus EMR bench top Orbitrap instrument we were able to detect ion signals up to m/z 20,000 with high mass resolving power thus achieving remarkable signal distribution and precision for various large protein assemblies.

Methods

Sample Preparation

Carbonic Anhydrase, Herceptin and Pyruvate Kinase are commercially available and were purchased from Sigma-Aldrich, Germany. GroELsamples were provides by the laboratory of Prof. Dr. Albert Heck, Utrecht, The Nehterlands. All samples were desalted prior measurement using Bio-Rad™ Micro BioSpin™ columns, following the instructions of the BioSpincolumn manual. Desalting changed any storage buffer system to 5 µM ammonium acetate buffer, pH 6.8 ±0.2.

Sample introduction

Samples were introduced using an AdvionTriVersaNano-Matewith chip based nanospray ionization in positive mode, using an Advion nozzle chip with an internal spray nozzle diameter of 5 µm. According to the manufacturer's specifications this should result in a sample flow of approx. 100 nL/min.

Mass Spectrometry

All analyses were carried out on a Thermo Fisher Scientific Exactive Plus EMR mass spectrometer. Detection parameters were set according to the type of analyte measured.

Data Analy sis

Deconvolution of signals measured for determination of molecular masses of the analytes were carried out using ProteinDeconvolution 2.0 SP2 software.

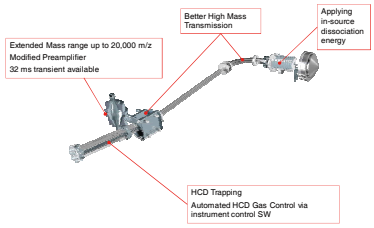

FIGURE 1. Improved ion path in the Exactive Plus EMR mass spectrometer

Results

Carbonic Anhydrase

Smaller proteins can easily measured by mass spectrometry, but under native conditions even these show quite different behavior. The signal of carbonic anhydrase for example, as member of this group of proteins, is reduced to two major charge states, making a classical deconvolution based on charge envelop pattern difficult. Due to the high resolution of the Orbitrap detection system, this 29 kDa protein still can be isotopically resolved (see fig. 2).

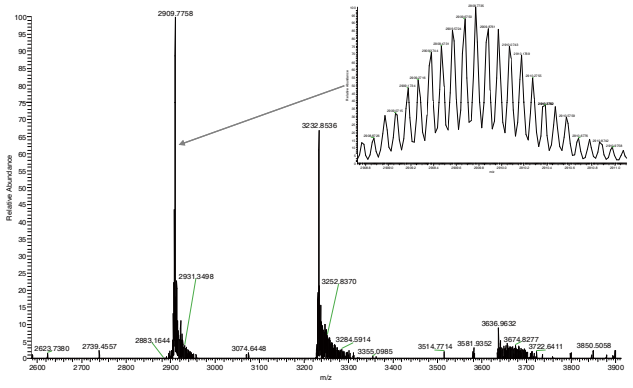

FIGURE 2. Spectrum of carbonic anhydrase in native state. Isotopic resolution is easily achieved.

Here the +9 charge state deconvolutesto a mass of 29069.6107 Da. The active protein zinc containing molecule has a theoretical mass of 29069.6034, relating to a mass accuracy of 0.25 ppm.

Herceptin

Herceptin is a therapeutic antibody in cancer treatment. The determination of the glycolysationstatus is important for characterization and quality control. For Herceptin, we could achieve a clear baseline separation and assignment of the major glycoformsknown (see fig. 3). In addition, present interfering adducts could be resolved clearly enough to separate them from the antibody signal, so a correct mass assignment could be achieved.

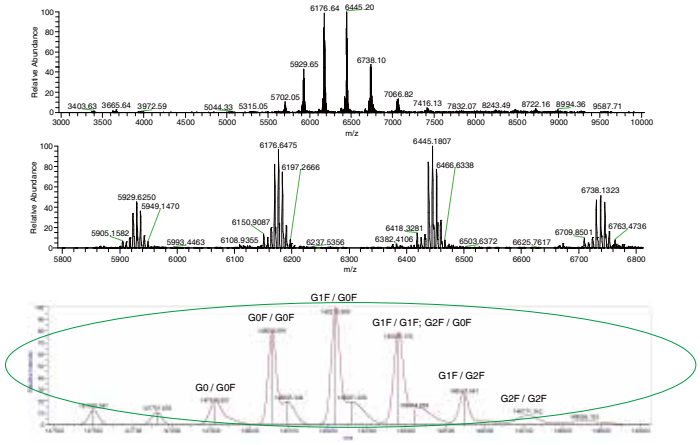

FIGURE 3. Experimental and deconvoluted spectrum of Herceptin, showing clear resolution of glycoforms and even resolving smaller adducts which would affect mass accuracy if not resolved

Pyruvate Kinase

In native state Pyruvate kinase is a tetrameric protein assembly of intermediate size. The full protein assembly appeared as a strong signal in the spectrum and due to full desolvation of the molecules in the mass spectrometer the full pattern of isoform combinations is visible (see fig. 4). Upon slight application for fragmentation energy the monomeric subunits with their isoform pattern are visible together with the full assembly. The mass difference of 324 amu is clearly visible in the deconvoluted spectra of the subunit as well as the multitude of isoform combinations for the tetrameric complex.

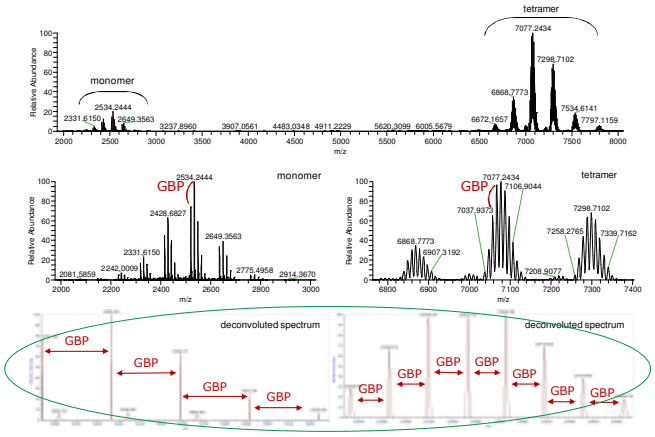

FIGURE 4. Experimental and deconvoluted spectra of Pyruvate Kinase showing the resolution of the isoforms with bound 2,5-anhydro-D-glucitol 1,6-bisphosphate (GBP)

GroEL

The chaperone protein GroEL from E. coli belongs to the group large proteins with a mass of roughly 800 kDa of the fulllyactive 14-mer complex. With proteins of this size, the resolving power of the MS instrumentation becomes a minor part for successful resolution of isoforms and conjugates, but desolvation capabilities are the limiting factor. With GroEL we could achieve full desolvation resulting in sharp baseline separation of the different charge states (see fig. 6).

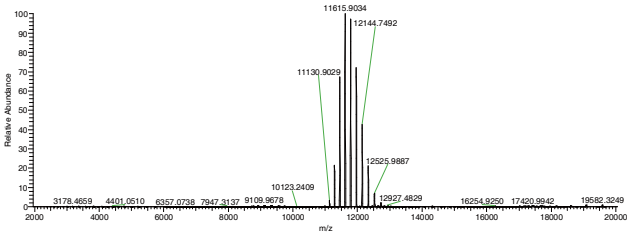

FIGURE 6. Spectrum of GroEL, showing the full assembly of the 14-mer

Application of elevated fragmentation energy lead to fragmentation of the assembly. The charge envelop of the first fragment, the 13-mer became the dominant signal reaching up to the upper mass range limint of m/z 20000, while the monomer signals were visible at the lower end of the spectrum at the same time (see fig. 7).

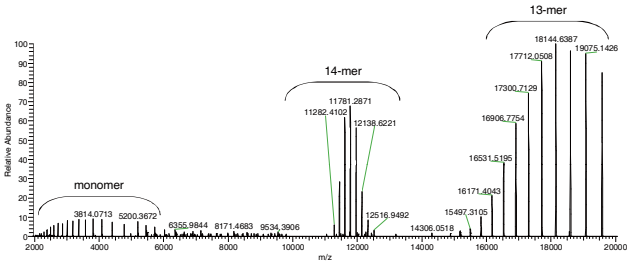

FIGURE 7. HCD spectrum of GroEL, showing the full assembly of the 14-mer together with the first fragmentation step (13-mer) and the according monomer signals.

Conclusion

With a Thermo Scientific Exactive Plus EMR mass spectrometer proteins easily can be studied in their native state, revealing the accurate mass of the fully active protein complex as well as clear separation of isoforms and conjugates. Large protein assemblies can be fragmented down to significant sub-assemblies and monomers for in-detail evaluation of quarternary structures.

For small proteins up to 35 kDa isotopic resolution can be achieved for determination of the monoisotopic mass. Larger proteins show clear separated signals for isoforms and conjugates. Acquisition time per compound lies in the rang of seconds.

Sample introduction with the Advion TriVersa NanoMate proved to be easy to handle, providing reproducible and stable spray conditions for best quality spectra acquired with minimum time consumption. It allows for automated data acquisition for maximum sample throughput.

Acknowledgements (if necessary)

We would like to thank Professor Albert Heck and his group from the University of Uthrecht, The Netherlands, for supplying samples of E. coli GroEL.

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Presented at HUPO 2013

A Complete Workflow Solution for Intact Monoclonal Antibody Characterization Using a New High-Performance Benchtop Quadrupole-Orbitrap LC-MS/MS

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Overview

Purpose: A LC/MS-based workflow solution was developed for robust, accurate and comprehensive intact monoclonal antibody (mAb) characterization.

Methods: Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometers were used for intact mass measurement and top-down sequencing. Full MS spectra of intact or reduced mAb were analyzed using Protein Deconvolution 1.0 that utilizes the ReSpect™ algorithm for molecular mass determination. The top-down msx HCD spectra were analyzed using Thermo Scientific ProSightPC software 2.0.

Results: A mass error of less than 10 ppm was routinely achieved for intact mAb mass measurement. Using an on-line high resolution top-down MSMS approach, over 40% of the fragmentation site was achieved for intact light chain that covers 100% sequence. Results from this study indicates that both precise mass measurement and extensive, high confident sequence information can be obtained for intact mAb using this workflow solution that combines high resolution MS, fast chromatography, high throughput msx HCD and accurate data analysis.

Introduction

Monoclonal antibodies (mAbs) are increasingly developed and utilized for the diagnostic and therapeutic treatment of diseases including cancer. Due to the heterogeneity of mAb products, thorough characterization is necessary for their reproducible as well as safe production. Among the analytical tools used for the analysis of therapeutic mAb, mass spectrometry has become more and more important in providing valuable information on various protein properties, such as intact mass, amino acid sequence, post-translational modification including glycosylation form distribution, minor impurities due to sample processing and handling and high order structure, etc. In this study, a high resolution LC-MS based workflow solution was developed for robust, accurate and comprehensive intact mAb characterization. The fast chromatography, the superior resolution and mass accuracy provided by the Q Exactive™ Orbitrap™ MS, and accurate data analysis of this workflow provides high-confident screening tool to accelerate biopharmaceutical product development cycles.

Methods

Samples: Four intact mAbs were used in this study. To reduce intact mAb, the sample was incubated for one hour at 60 C in 6 M guanidine-HCl containing 5 mM DDT.

HPLC: Thermo Scientific ProSwift RP-10R monolithic column (1 × 50mm) was used for desalting and separation of light and heavy chain. LC solvents are 0.1% formic acid in H2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80 ℃ during analysis. Flow rate was 60 µL/min. After injection of 5 µg mAb, a 15 min gradient was used to elute mAbs from the column

(0.0min, 20%B; 1.0min, 35%B; 3.0min, 55%B; 4.0min, 98%B; 7.0min, 98% B; 7.1min, 20%B; 15.0min, 20%B).

Mass Spectrometry: Q Exactive Orbitrap instruments (Figure 1) were used for this study. Intact and reduced mAbs were analyzed by ESI-MS for intact molecular mass. Top-down MSMS was performed using high energy collision dissociation with a unique spectrum multiplexing feature (msx HCD). In this data acquisition mode, fragment ions produced from several individual HCD events, each on a precursor of a different charge state of the reduced mAb, were detected together in the Orbitrap mass analyzer. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275℃ . S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500 or 140,000 for full MS and 140,000 for top-down MSMS. The AGC target was set at 1E6 for full scan and 2E5 for MSMS. Maximum IT was set at 250 ms.

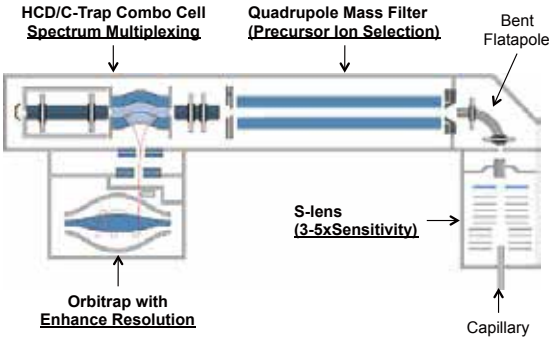

Figure 1. Schematics of Q Exactive Mass Spectromete

Data Processing: Full MS spectra of intact or reduced mAbs were analyzed using Protein Deconvolution 1.0 (Figure 2) that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. The averaged spectra were subsequently deconvoluted using an input m/z range of 2000 to 4000 m/z, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and minimum of at least 8 consecutive charge states from the input m/z spectrum to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses with the various combinations of commonly found glycoforms. The top-down msx HCD spectra were analyzed using ProSightPC™ software 2.0 under the single protein mode with a fragment ion tolerance of 5 ppm.

Key Features of The Q Exactive instrument:

- The incorporation of S-lens at the source dramatically enhanced its sensitivity.
- Quadrupole mass filter enables precursor selection for data-dependent MS2 and selected ion monitoring.
- Advanced signal processing increased resolution by two folds, which results in a maximum resolution of 140,000 and a maximum scan speed of 12Hz at a resolution of 17,500.
- Spectrum multiplexing and parallel ion injection/ orbitrap detection significantly improved duty cycle.

A screenshot of the software with the source and deconvoluted spectrum and a list of the components for that protein at the bottom of the page. In this application, the user has a choice from two algorithms, Xtract or ReSpect, depending on whether or not the target protein is isotopically resolved.

1) Select data file
2) Choose appropriate deconvolution parameters
3) Create an averaged spectrum from a chromatographic peak
4) Perform deconvolution
5) Print or save report

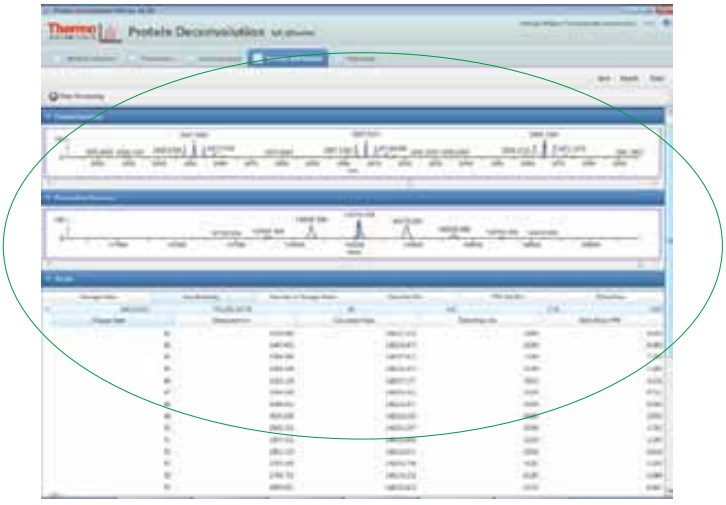

Figure 2. Protein Deconvolution 1.0

Results

Five micrograms of mAb were desalted and eluted from a ProSwift™ RP-10R monolithic column using a 15min gradient and analyzed using ESI-MS on the Q-Exactive. The was mAb eluted over one minute as shown in (A). The average spectrum over the elution time shows a nicely distributed complete charge envelope of the mAb (B). A zoom-in view of each charge state reveals five major glycosylation forms that are baseline separated (C).

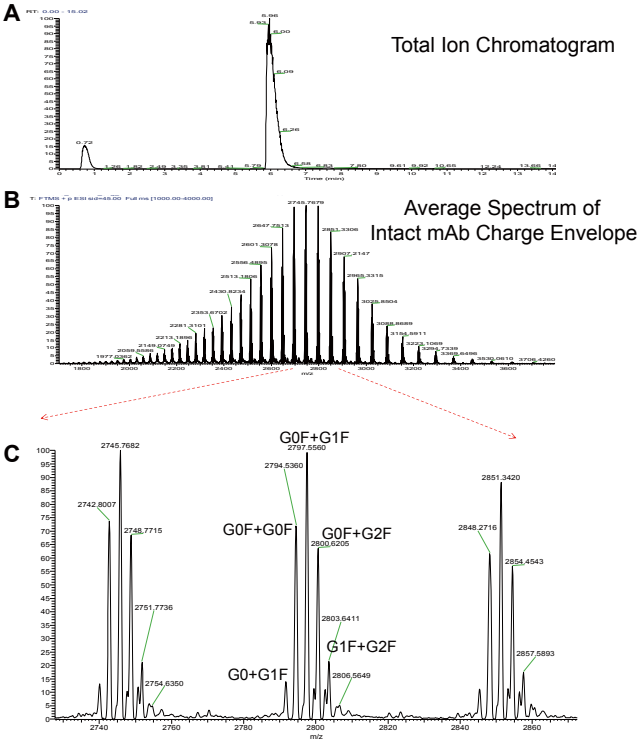

Figure 3. LC-MS Result of mAb

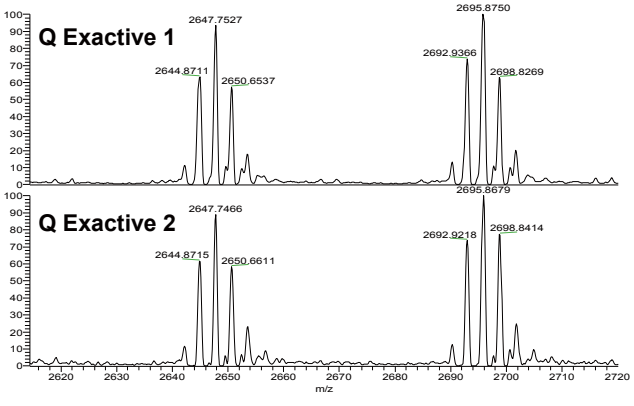

Figure 4. Consistency of instrument performance
Mass difference of major components is < 6 ppm between instruments

After each of the mAb datasets were analyzed using the Protein Deconvolution software, the masses were compared to the masses expected for the known amino acid sequence with the various combinations of glycoforms commonly found on mAbs. One such result is shown below in Figure 5.

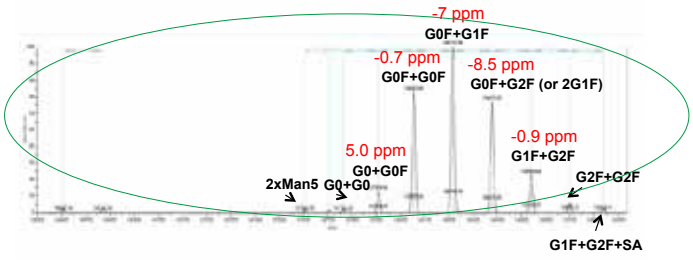

Figure 5. Deconvoluted spectrum for a mAb with known composition and mass errors

To measure the mass accuracy and reproducibility of mAb samples on the Q Exactive in conjunction with ReSpect, the mAb sample was analyzed several times using two different instruments over three different days. The results for ppm mass accuracy are shown in Table 1 and the results for relative abundance of the various glycoforms are shown in Table 2.

Table 1: ppm mass deviations from expected target masses for the 5 most abundant glycoforms

		ppm mass measurement errors				
RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	-10.5	0.7	-10.5	-13.8	-18.0
2	1	-3.2	-4.3	-6.9	3.2	N/A
3	1	-11.6	-1.1	-8.8	-11.2	-12.0
4	1	5.1	-5.0	-2.6	5.1	5.6
5	2	-14.3	3.0	-6.9	-5.4	-5.9
6	2	-8.6	-2.2	-12.2	-12.5	-12.9
7	2	-14.3	-6.6	-12.3	-14.8	-10.1

The average ppm error for all 34 measurements was 6.9 ppm with a standard deviation of 6.4 ppm. This indicates that the Q Exactive is a very powerful platform for confirmation of protein primary structure.

		Relative abundances				
RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	12.9	74.1	100.0	67.0	23.4
2	1	12.3	76.0	100.0	71.4	29.8
3	1	12.0	72.8	100.0	66.2	22.0
4	1	12.2	75.0	100.0	67.0	23.6
5	2	12.7	75.7	100.0	63.6	21.6
6	2	13.2	75.4	100.0	64.8	21.0
7	2	12.9	76.6	100.0	64.7	21.6

For the top 5 glycoforms, the relative intensity reproducibility is within a few percent.

To obtain amino acid sequence, on-line, top-down MS/MS was applied to the reduced mAb samples using msx HCD. Besides the improved throughput from spectrum multiplexing, the advanced signal processing provides improved resolution and higher Orbitrap scan speeds, which is critical for on-line protein top-down sequencing. As a result, high resolution, information rich spectra were generated on the one minute LC elution time for reduced mAb samples. For the light chain, over 40% sequence coverage was achieved, including the N-terminal variable region, with a mass error of less than 5 ppm for fragment ions. The result is shown below in Figure 6.

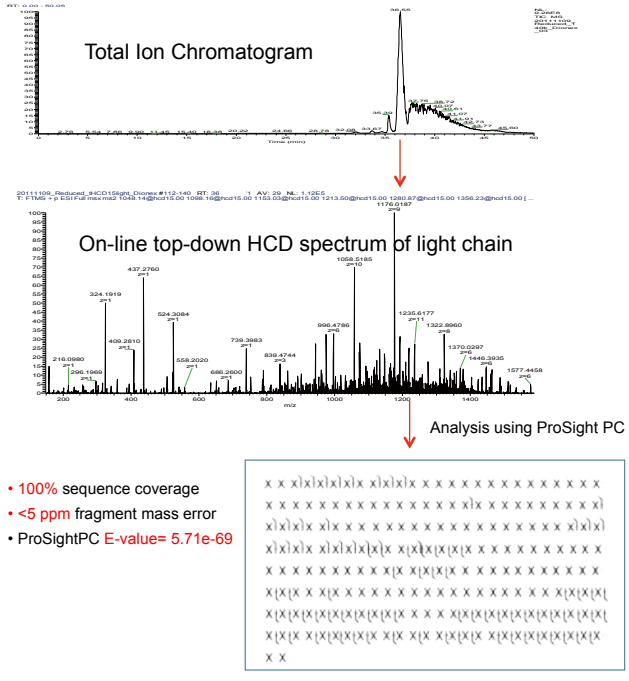

Figure 6. On-line top-down sequencing of light chain

Conclusion

- ProSwift RP-10R monolithic column provides robust and efficient separation of mAbs.
- Q Exactive MS produces accurate and reproducible mass analysis for intact mAb analysis.
- Q Exactive on-line top-down analysis generates extensive sequence information for reduced mAb, offering a fast way to confirm sequence identity.
- Protein deconvolution suite enables fast and accurate calculation of the intact mass of mAbs.
- ProSightPC software offers confident sequence assignment for high resolution top-down spectrum generated by Q Exactive instrument.
- Both precise mass measurement and extensive, high confident sequence information can be obtained for intact mAb using this workflow solution that combines high resolution MS, fast chromatography, high throughput msx HCD and accurate data analysis.

LC/MS Analysis of the Monoclonal Antibody Rituximab Using the Q Exactive Benchtop Orbitrap Mass Spectrometer

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Key Words

Monoclonal antibody, intact protein mass measurement, sequence confirmation, protein deconvolution, top-down sequencing

Goal

Analysis and characterization of a monoclonal antibody using an optimized LC/MS workflow based on monolithic columns coupled online with the Thermo Scientific™ Q Exactive™ benchtop Orbitrap™ mass spectrometer.

Introduction

Monoclonal antibodies (mAbs) are one of the fastest growing classes of pharmaceutical products. They play a major role in the treatment of a variety of conditions such as cancer, infectious diseases, allergies, inflammation, and auto-immune diseases. Because mAbs can exhibit significant heterogeneity, extensive analytical characterization is required to obtain approval for a new mAb as a therapeutic product. Mass spectrometry has become an essential tool in the characterization of mAbs, providing molecular weight determinations of intact proteins as well as separated light and heavy chains, elucidation of glycosylation and glycan structures, confirmation of correct amino acid sequences, and identification of impurities such as host cell proteins (HCP) inherent to the production process.

Rituximab, which is known under the trade names Rituxan® (Biogen Idec/Genentech) in the United States and MabThera® (Roche) in Europe, is a recombinantly produced, monoclonal chimeric antibody against the protein CD20. It was one of the first new generation drugs in cancer immune therapy. Rituximab was approved by the U.S. Food and Drug Administration in 1997 and by the European Commission in 1998 for cancer therapy of malignant lymphomas. The variable domain of the antibody targets the cell surface molecule CD20, that can be found in some non-Hodgkin lymphomas.

In this application note, the capabilities and performance of the Q Exactive benchtop Orbitrap mass spectrometer in analyzing the intact and reduced forms of rituximab are demonstrated as well as sequence confirmation analyses using a combined top-down and bottom-up approach. Furthermore, the sensitivity of two chromatographic

setups using monolithic columns coupled online to the mass spectrometer is evaluated. The data obtained demonstrate superior resolution and mass accuracy of the Q Exactive mass spectrometer and present it as a highconfidence screening tool for accelerated and accurate biopharmaceutical product development and characterization.

Experimental

Sample Preparation

The commercially available monoclonal antibody rituximab was used in all experiments. Rituximab is a sterile, clear, colorless, preservative-free, concentrated solution for intravenous infusion. It was supplied at a concentration of 10 mg/mL, formulated in 7.35 mg/mL sodium citrate buffer containing 0.7 mg/mL polysorbate 80, 9.0 mg/mL sodium chloride, and sterile water, and ready for injection. The pH was adjusted to 6.5 with sodium hydroxide or hydrochloric acid.

Prior to LC/MS analysis, rituximab was dialyzed due to polysorbate 80 in the sample. The dialysis was performed with a Thermo Scientific™ Slide-A-Lyzer™ dialysis cassette with a molecular weight cut off (MWCO) of 3.5 kDa. A 1 mL sample of rituximab was dialyzed for 48 h against 2 L of 20% aqueous acetonitrile (ACN) at 4 ℃ .

For analysis of the light and heavy chains of rituximab, disulfide bonds were reduced by incubation for 30 min at 60 ℃ with 5 mM tris(2 carboxyethyl)phosphine (TCEP).

For the bottom-up analysis of digested mAb, the sample was alkylated with 20 mM iodoacetamide (IAA) for 30 min at room temperature in the dark after the reduction step. The sample was purified with Thermo Scientific™ Pierce™ C18 tips dried in a Thermo Scientific™ SpeedVac™ concentrator and dissolved in 0.5 M triethylammonium bicarbonate buffer (TEAB). Sequencing grade modified trypsin (Promega) was added twice in a total ratio of 1:15 (w/w) at 0 h and 1.5 h and digestion was allowed to proceed for 2.5 h at 37℃ . The digest was stopped by addition of triflouroacetic acid (TFA) to approximately pH 3.

All samples were supplied in autosampler vials containing glass inserts (micro-inserts 0.1 ml, clear glass, VWR).

Liquid Chromatography

A monolithic 160 × 0.20 mm i.d. poly(styrene-divinylbenzene) copolymer (PS-DVB) capillary column, prepared according to a previously published protocol1, and a Thermo Scientific™ PepSwift™ monolithic 250 × 0.20 mm i.d PS-DVB capillary column were used. Protein separations were performed with a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano system that included a detector equipped with a 3 nL z-shaped capillary detection cell.

Separations were accomplished at 55 ℃ with a gradient of 20–60% acetonitrile (ACN) in 0.050% aqueous triflouroacetic acid (TFA) in 10 min at a flow rate of 1 µL/min. For the proteolytic digest with trypsin, the gradient was adapted to run at 0–50% B in 30 min. For the reduced antibody samples, a gradient from 35–45% B in 15 min was selected.

Protein separation in a higher scale was performed using a Thermo Scientific™ ProSwift™ RP-10R monolithic 50 mm × 1.0 mm i.d. column with an UltiMate 3000 RSLCnano system that included a 45 nL detection cell. The column was run with a flow rate of 60 µL/min and a column temperature set to 55 ℃ . The gradient used was 26–80% B in 20 min. For the reduced antibody, a gradient of 26–56% B in 20 min was chosen to separate the heavy and the light chain.

The recorded back pressure of the monolithic columns for the gradients described above was in the range of 190 to 260 bar for the PepSwift 250 mm × 0.2 mm i.d. column and 120 to 180 bar for the ProSwift RP-10R 50 mm x 1 mm i.d. column.

For all experiments, the solvents used were water with 0.05% TFA (A) and acetonitrile with 0.05% TFA (B). The LC gradients are described in Tables 1 and 2.

Table 1. LC gradients used for experiments with the PepSwift 250 mm × 0.2 mm i.d. column, at a flow rate of 1 µL/min

Time [min]	Intact mAb [%B]	Time [min]	Reduced mAb [%B]	Time [min]	mAb Digest [%B]
0.0	20	0.0	35	0.0	0
10.0	60	15.0	45	30.0	50
10.1	85	15.1	85	30.1	85
16.0	85	21.0	85	40.0	85
16.1	20	21.1	35	40.1	0
30.0	20	30.0	35	50.0	0

Table 2. LC gradient used for experiments with the ProSwift RP-10R 50 mm × 1 mm i.d. column, at a flow rate of 60 µL/min

Time [min]	Intact mAb [%B]	Time [min]	Reduced mAb [%B]
0.0	26	0.0	26
15.0	80	15.0	56
20.0	80	15.1	80
20.1	26	20.0	80
30.0	26	20.1	26
		30.0	26

Mass Spectrometry

The Q Exactive benchtop Orbitrap mass spectrometer was used for all experiments in this study. Experiments using the ProSwift RP-10R 50 mm × 1 mm i.d. column were performed using the Thermo Scientific™ IonMax™ source with the heated electrospray ionization (HESI) sprayer, applying 4 kV spray voltage and sheath gas and auxiliary gas flow rates of 15 and 5 units, respectively.

All other experiments were performed using the Thermo Scientific™ NanoFlex™ ion source equipped with 15 cm PicoTip® emitter (New Objective, Woburn, USA; 20 µm i.d., 360 µm o.d., 10 µm tip), running with a flow rate of 1 µL/min. A source voltage of 1.5 kV was applied.

Method details are provided in Table 3.

Table 3. Mass spectrometric parameters used for all experiments

	Intact Antibody	Reduced Antibody	Top Down AIF	5-plex MS/MS (Targeted MS^2)	Antibody Digest
Method type	Full MS	Full MS (2 segments)	Full MS-AIF	Targeted MS^2	Full MS-dd top 10 HCD
Total run time	30 min	0–15.8/15.8–30 min	25 min	25 min	40 min
Scan range *m/z*	1800–5000	800–3500/700–2500	300–2500	Fixed first mass 300	350–2000
Resolution (full MS/MS^2)	17,500/x	140,000/17,500	70,000	n.a./70,000	70,000/17,500
AGC Full MS	3.00×10^6	3.00×10^6	3.00×10^6	5.00×10^5	3.00×10^6 (MS)/1.00×10^5(MS^2)
Max inject time (Full MS/MS^2)	150 ms	150 ms/200 ms	150 ms	150 ms	100 ms/100 ms
Isolation window	n.a.	n.a.	n.a.	10 Th	2 Th
Microscans	10	5	5	5	1
Capillary temperature	275 ℃	275 ℃	275 ℃	275 ℃	275 ℃
S-lens RF level	80	80	50	50	50
SID [eV]	80	0/60	n.a.	LC 0/HC 20	n.a.
NCE [%]	n.a.	n.a.	10 to 30	10 to 30	25

Source CID

The source CID (SID) parameter is a DC offset (0–100 eV) that is added to the source DC offset. The source DC offset consists of three voltages: capillary DC, S-lens DC, and S-lens exit lens. The application of this DC offset by setting the source CID parameter results in collisions of the analytes inside the injection flatapole with residual gas molecules present in the source region of the instrument.

All-Ion Fragmentation

All-ion fragmentation (AIF) is a fragmentation type in which all ions generated in the source are guided through the ion optics of the mass spectrometer, accumulated in the C-trap, and sent together to the higher-energy collisional dissociation (HCD) cell for fragmentation. In this case, the quadrupole is not set to select a particular precursor but operated in RF-only pass-through mode. For the analysis of intact proteins, this is a useful method since different charge states often show different fragmentation behavior and it is not easy to predict which one works best.

Data Analysis

Full MS spectra were deconvoluted using Thermo Scientific™ Protein Deconvolution™ software version 2.0. From the intact antibody and the intact heavy chain, the spectra acquired at a resolution setting of 17,500

were deconvoluted using the ReSpect™ algorithm. High resolution spectra from the intact light chain acquired at a resolution of 140,000 and top-down spectra acquired at 70,000 resolution were deconvoluted using the Xtract algorithm. To identify glycoforms of the intact antibody and the intact heavy chain obtained after reduction, the masses were compared to the expected masses with the various combinations of commonly found glycoforms.

The top-down HCD and AIF spectra were deconvoluted using the Xtract algorithm in the Thermo Scientific™ Qual Browser™ utility. Fragment ion assignment was performed using Thermo Scientific™ ProSightPC™ software version 3.0 in single protein mode with a fragment ion tolerance of 5 ppm.

The dataset obtained from the proteolytic digest was processed with Thermo Scientific™ Proteome Discoverer™ software version 1.4, using the SEQUEST® algorithm. A three-protein-entry database was used consisting of the light chain, the heavy chain in two variants carrying either Ala or Val at position 219, and trypsin. Mass tolerances were set to 10 ppm for the precursor and 20 mmu for the fragment ions. Four variable modifications were considered: carbamidomethylation (Cys), oxidation (Met), deamidation (N, Q), Gln to pyro-Glu conversion, and N,N-dimethylation (Lys) (relevant for identification of trypsin autolysis products only).

Results and Discussion

Rituximab is an IgG1 class chimeric monoclonal antibody against the protein CD20, which consists of two light chains with 213 amino acids and two heavy chains with 451 amino acids each in length. The light and heavy chains are connected via 12 intrachain and 4 interchain disulfide linkages (Figure 1). The antibody is decorated with glycan structures attached to residue Asn^{301} of each of the two heavy chains. The composition and length of the attached glycans is quite diverse, resulting in a microheterogeneity of the molecule. The variety and relative abundance of the different glycostructures is essential for the efficiency of the antibody as a biological drug. The nomenclature of common glycans attached to antibodies are listed in Figure 2.

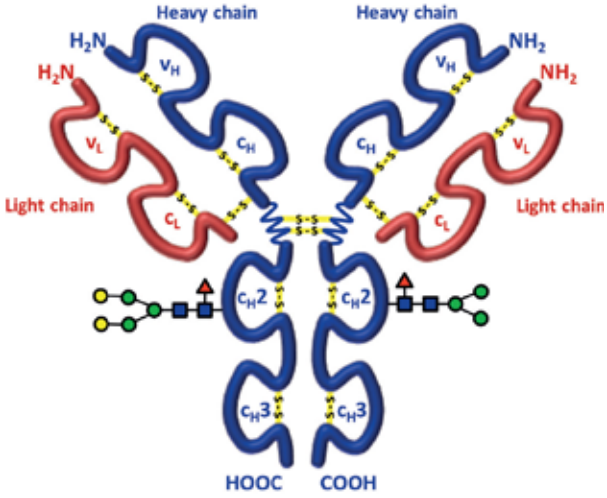


Figure 1. Schematic of molecular structure for the humanized IgG1 class monoclonal antibody rituximab

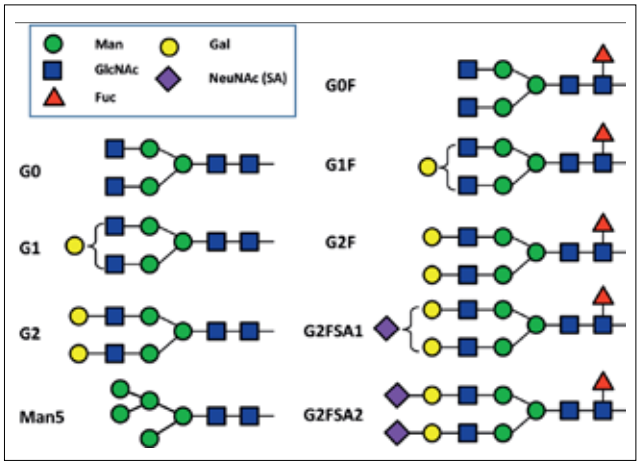


Figure 2. Nomenclature of carbohydrate structures commonly observed on antibodies

The full MS spectrum obtained from 20 ng rituximab applied to a 25 cm × 0.2 mm i.d. monolithic column is displayed in Figure 3. The mass spectrum, acquired over *m/z* 1800–5000 shows the typical charge distribution observed for large proteins. The most abundant charge state (z=+45) at *m/z* 3269, represented in the zoomed in insert, nicely pictures the four most abundant glycoforms of the intact antibody.

The intact mass of these four most abundant glycoforms and a series of less abundant glycoforms is obtained after the deconvolution of the full MS mass spectrum shown in Figure 4. The assignment of the peaks was based on the calculation of the proteins sequence, taking into account the various anticipated glycan structures shown in Figure 2.

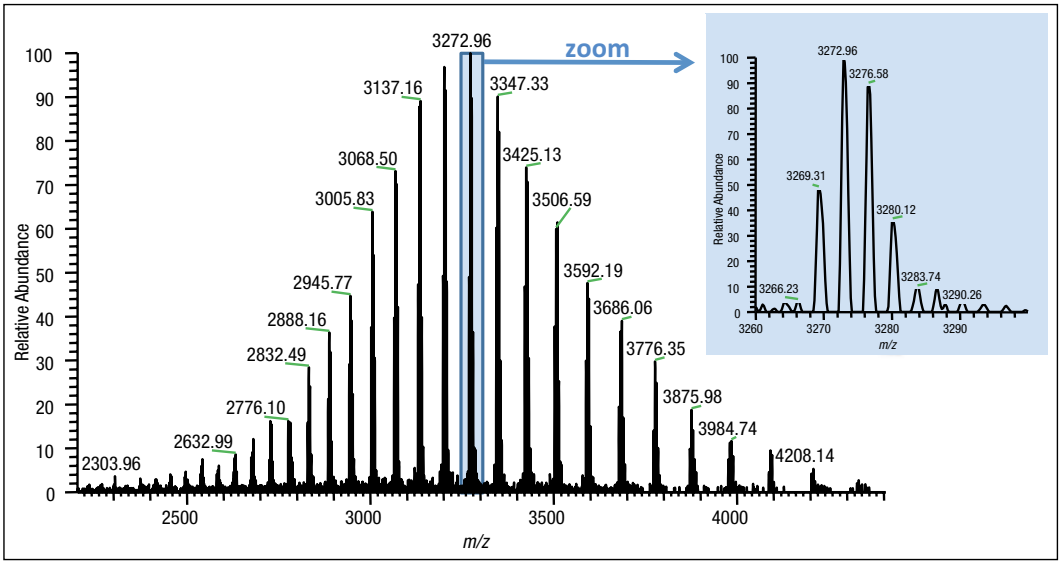
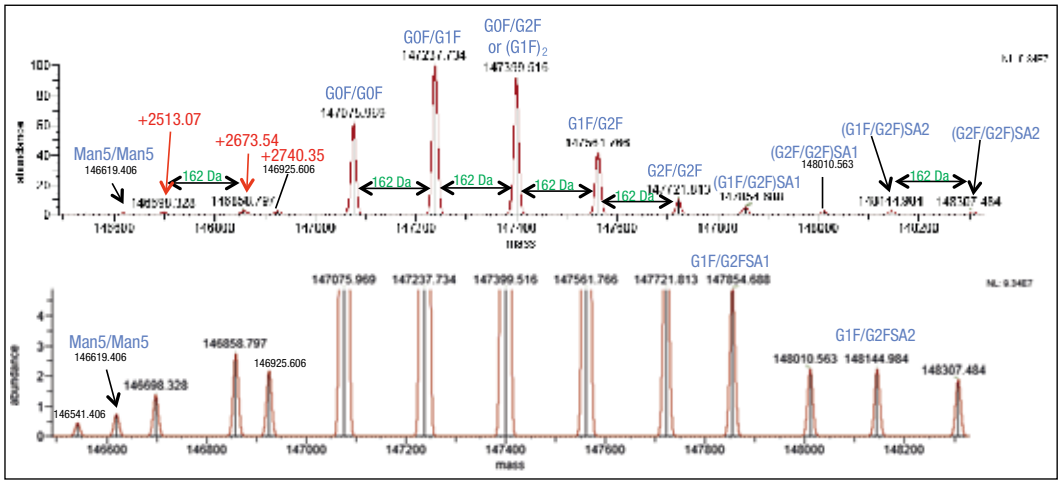


Figure 3. Single scan full MS spectrum (10 µscans) of rituximab, acquired from 10 ng sample loaded on a 250 × 0.2 mm i.d. column. The insert shows a zoomed in view of the most abundant charge state (z=+45). The observed peak pattern in the insert represents the different glycoforms of the molecule.



	G0F/G0F	G0F/G1F	G0F/G2F or $(G1F)_2$	G1F/G2F	G2F/G2F
Measured	147,075.969	147,237.734	147,399.516	147,561.766	147,721.813
Theoretical	147,074.985	147,237.126	147,399.267	147,561.408	147,723.549
Δ ppm	-6.7	-4.1	-1.7	-2.0	11.8

Figure 4. Deconvoluted mass spectrum of rituximab with annotated glycoforms (top) and comparison of theoretical and measured masses for the five most abundant glycoforms (table)

For the acquisition of the full MS spectrum of the intact antibody, the optimum setting of the source CID was evaluated (Figure 5). This setting was found to be crucial in obtaining a high quality spectrum. The application of 25–90% source CID is beneficial for most proteins. For this sample the optimum setting was 80% SID.

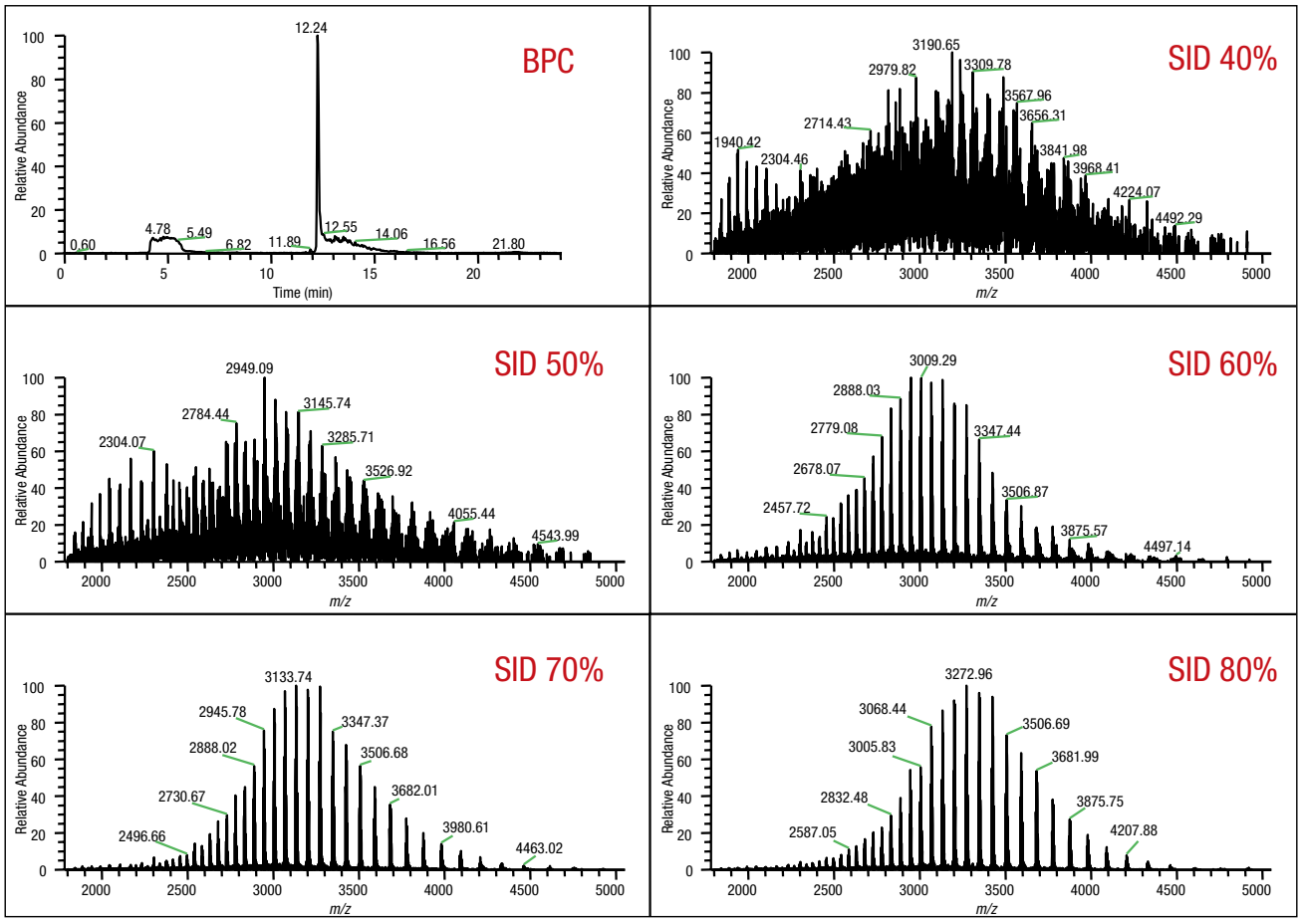


Figure 5. Full MS spectra acquired from 1 ng intact rituximab, applying increasing settings of source CID (SID)

The calculation of the masses for the light chain, unglycosylated heavy chain, and intact fully assembled antibody is presented in Table 4, showing the step-by-step calculation starting with the 213 respectively 451 amino acids of the light and heavy chain. Both protein sequences contain an N-terminal glutamine, which is anticipated to be modified to a pyro-glutamic acid, resulting in a deduction of mass of 17.0265 Da. Moreover, the C-terminal lysine present in the heavy chain is likely to be cleaved off, reducing the molecular weight by another 128.09497 Da.

For assembling the intact antibody, a total of 16 disulfide linkages is considered by abstracting 32 protons. The glycan structures on each of the two heavy chains will add between 1217.1 and 2352.1 Da in mass. It has to be considered that the two chains can carry different glycans, resulting in a mixed composition, e.g. G01/G2F. Chemical composition and masses of individual carbohydrates are listed in Table 5. The monoisotopic and average atomic masses of the elements used to calculate molecular weights in Tables 4 and 5 are listed in Table 6.

Table 4. Chemical composition and step-by-step calculation of monoisotopic and average mass for the light and heavy chain, including their modifications as well as the intact antibody rituximab with various glycoforms. Detected masses shown in Figures 4, 6, and 7 are presented in the blue cells.

Elemental compositions	C	H	N	O	S	MW (monoisotopic)	MW (average)
Light chain (LC) full sequence aa 1-213	1016	1577	273	328	6	23,042.34369	23,056.5
N-terminal pyro Glutamic acid	1016	1574	272	328	6	23,025.31714	23,039.4
N-terminal pyro Glutamic acid, 2 intrachain S-S bonds	1016	1570	272	328	6	23,021.28584	23,035.4
2 × LC (N-term. pyroGlu)	2032	3148	544	656	12	46,050.63428	46,078.9
2 × LC (N-term. pyroGlu, 2 intrachain S-S bonds each)	2032	3140	544	656	12	46,042.57168	46,070.8
Heavy chain (HC) full sequence aa 1-451	2197	3389	577	676	16	49,183.40813	49,214.0
N-terminal pyro Glutamic acid	2197	3386	576	676	16	49,166.38158	49,197.0
minus C-term. K (aa 1-450)	2191	3374	574	675	16	49,038.28661	49,068.8
minus 4 intrachain S-S bonds	2191	3366	574	675	16	49,030.22401	49,060.8
HC-G0F (pyro-Glu, - K, fully reduced)	2247	3466	578	714	16	50,482.82048	50,514.2
HC-G1F (pyro-Glu, - K, fully reduced)	2253	3476	578	719	16	50,644.87330	50,676.3
HC-G2F (pyro-Glu, - K, fully reduced)	2259	3486	578	724	16	50,806.92613	50,838.5
HC minus 4 intrachain S-S bonds + G0F	2247	3458	578	714	16	50,474.75788	50,506.1
2 × HC (pyroGlu, - K)	4382	6748	1148	1350	32	98,076.57323	98,137.7
2 × HC (pyroGlu, - K, 4 intrachain S-S bonds each)	4382	6732	1148	1350	32	98,060.44803	98,121.6
Man5 (HexNAc)2 (Hex)5	46	76	2	35	0	1216.42286	1217.1
G0 (HexNAc)4 (Hex)3	50	82	4	35	0	1298.47596	1299.2
G0F (HexNAc)4 (Hex)3 Fuc	56	92	4	39	0	1444.53387	1445.3
G1 (HexNAc)4 (Hex)4	56	92	4	40	0	1460.52878	1461.3
G1F (HexNAc)4 (Hex)4 Fuc	62	102	4	44	0	1606.58669	1607.5
G2 (HexNAc)4 (Hex)5	62	102	4	45	0	1622.58161	1623.5
G2F (HexNAc)4 (Hex)5 Fuc	68	112	4	49	0	1768.63951	1769.6
G1FSA (HexNAc)4 (Hex)4 Fuc SA	73	119	5	52	0	1897.68211	1898.7
G1FSA2 (HexNAc)4 (Hex)4 Fuc (SA)2	84	136	6	60	0	2188.77752	2190.0
G2FSA (HexNAc)4 (Hex)5 Fuc SA	79	129	5	57	0	2059.73493	2060.9
G2FSA2 (HexNAc)4 (Hex)5 Fuc (SA)2	90	146	6	65	0	2350.83035	2352.1
Man5/Man5 (HexNAc)4 (Hex)10	92	152	4	70	0	2432.84572	2434.2
G0F/G0F (HexNAc)8 (Hex)6 (Fuc)2	112	184	8	78	0	2889.06774	2890.7
G0F/G1F (HexNAc)8 (Hex)7 (Fuc)2	118	194	8	83	0	3051.12056	3052.8
G1F/G1F (HexNAc)8 (Hex)8 (Fuc)2	124	204	8	88	0	3213.17338	3215.0
G1F/G2F (HexNAc)8 (Hex)9 (Fuc)2	130	214	8	93	0	3375.22621	3377.1

Elemental compositions	C	H	N	O	S	MW (monoisotopic)	MW (average)
G2F/G2F (HexNAc)8 (Hex)10 (Fuc)2	136	224	8	98	0	3537.27903	3539.2
G1F/G2FSA (HexNAc)8 (Hex)9 (Fuc)2 SA	141	231	9	101	0	3666.32162	3668.3
G1F/G2FSA2 (HexNAc)8 (Hex)9 (Fuc)2 (SA)2	152	248	10	109	0	3957.41704	3959.6
G2F/G2FSA (HexNAc)8 (Hex)10 (Fuc)2 SA	147	241	9	106	0	3828.37445	3830.5
G2F/G2FSA2 (HexNAc)8 (Hex)10 (Fuc)2 (SA)2	158	258	10	114	0	4119.46986	4121.7
Sum 2 × HC +2 × LC (4 × pyroGlu, -2K)	6414	9896	1692	2006	44	144,127.20750	144,216.6
minus 32 S-S bond protons	6414	9864	1692	2006	44	144,094.95710	144,184.3
2HC + 2LC - 16 S-S bonds + (Man5)2	6506	10016	1696	2076	44	146,527.80282	146,618.5
2HC + 2LC - 16 S-S bonds + (G0F)2	6526	10048	1700	2084	44	146,984.02484	147,075.0
2HC + 2LC - 16 S-S bonds + G0F/G1F	6532	10058	1700	2089	44	147,146.07766	147,237.1
2HC + 2LC - 16 S-S bonds + G0F/G2F or (G1F)2	6538	10068	1700	2094	44	147,308.13049	147,399.3
2HC + 2LC - 16 S-S bonds + G1F/G2F	6544	10078	1700	2099	44	147,470.18331	147,561.4
2HC + 2LC - 16 S-S bonds + G2F/G2F	6550	10088	1700	2104	44	147,632.23613	147,723.5
2HC + 2LC - 16 S-S bonds + G1F/G2F SA	6555	10095	1701	2107	44	147,761.27872	147,852.7
2HC + 2LC - 16 S-S bonds + G1F/G2F (SA)2	6566	10112	1702	2115	44	148,052.37414	148,143.9
2HC + 2LC - 16 S-S bonds + G2F/G2F SA	6561	10105	1701	2112	44	147,923.33155	148,014.8
2HC + 2LC - 16 S-S bonds + G2F/G2F (SA)2	6572	10122	1702	2120	44	148,214.42696	148,306.1

Table 5. Chemical composition and masses of monosaccharides

	Sum Formula	Monoisotopic Mass	Average Mass	C	O	N	H
Sialic Acid	$C_{11}O_8NH_{17}$	291.09542	291.3	11	8	1	17
Galactose	$C_6O_5H_{10}$	162.05282	162.1	6	5	0	10
N-Acetylglucosamine	$C_8O_5NH_{13}$	203.07937	203.2	8	5	1	13
Mannose	$C_6O_5H_{10}$	162.05282	162.1	6	5	0	10
Fucose	$C_6O_4H_{10}$	146.05791	146.1	6	4	0	10

Table 6. Monoisotopic and average atomic masses of the elements used to calculate the molecular masses in Tables 4 and 5

	Monoisotopic Mass	Average Mass
C	12.0000000	12.01074
H	1.00782503	1.00794
N	14.0030740	14.00674
O	15.9949146	15.99940
S	31.9720707	32.06608

The initial calculation based on the sequence published in the DrugBank database[2] resulted in a mass that did not match the masses obtained in our experiments. Comparison of the DrugBank sequence with a previously published sequence[3] revealed a difference in one amino acid at position 219, located in the conserved region of the heavy chain, Ala versus Val. The sequence containing the Ala at position 219 did match well with the results obtained from intact mass measurements as well as with previously reported results.[4] To further verify this, a series of additional experiments was performed.

After reducing the antibody (without alkylation), the analysis of separated light and heavy chain was performed applying different resolution settings to account for whether or not isotopic resolution can be achieved based on molecular weight. Due to the smaller molecular weight of the light chain, it is possible to obtain an isotopically resolved spectrum, whereas for the heavy chain this is not possible since it is about twice as large as the light chain. To apply different resolution settings, the method was set up in two segments (140k resolution for the scans acquired from 0 to 15.8 min, and 17.5k resolution from 15.8 to 30 min) and the gradient was optimized to achieve wellseparated peaks of the light and heavy chain.

On both monolithic columns evaluated in this study (PepSwift 250 mm × 0.2 mm i.d. and ProSwift RP-10R 50 mm × 0.1 mm i.d.), the separation of the two peaks by more than 2 min was equally possible (Figure 6). The mass spectra obtained from the light chain and from the heavy chain (Figures 6b and 6c) were submitted for deconvolution. The isotopically resolved light chain spectrum was deconvoluted using the Xtract algorithm, resulting in a monoisotopic molecular weight of 23,025.3758 Da, which matches the calculated mass by 2.5 ppm. The heavy chain was deconvoluted using the ReSpect algorithm, resulting in three peaks, each of which represents one of the major glycoforms, G0F, G1F, and G2F (Figure 7).

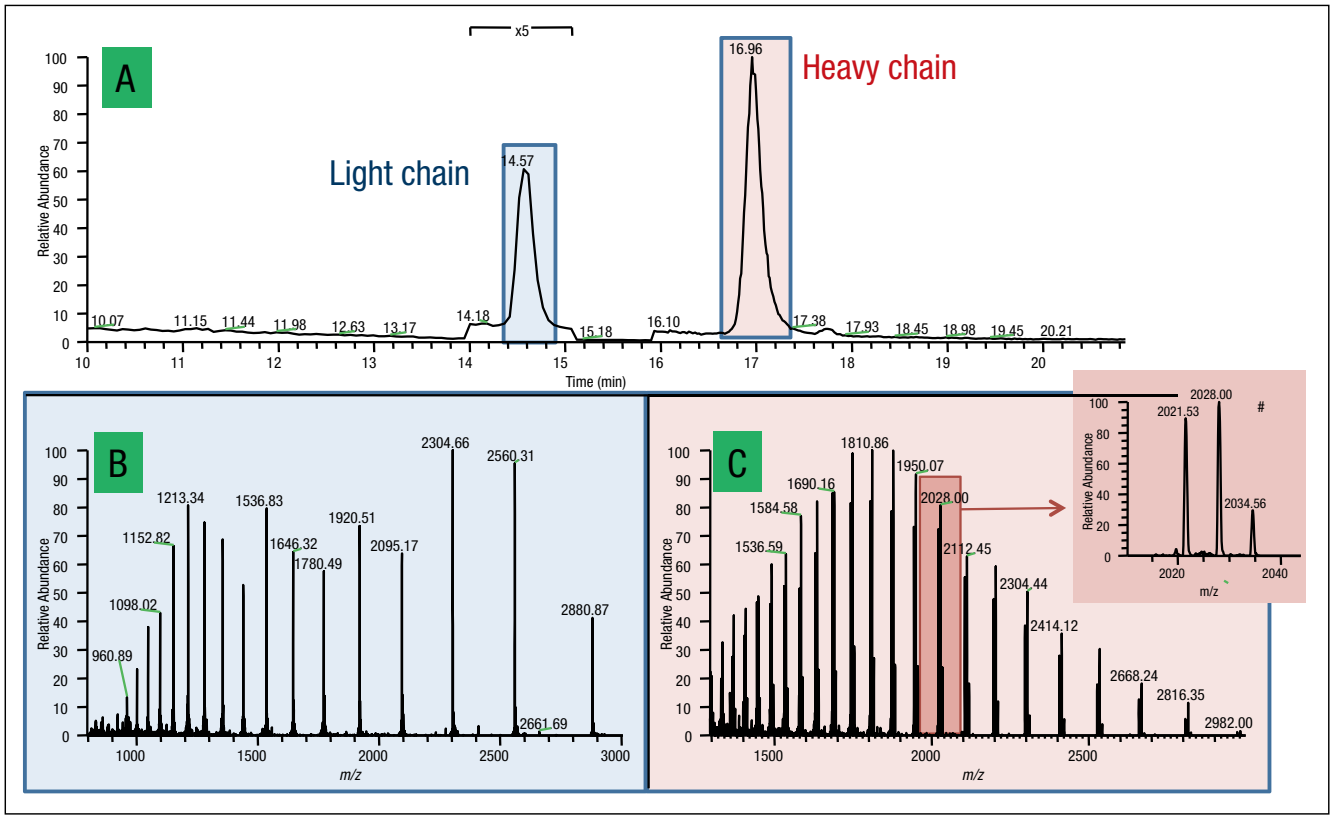


Figure 6. Chromatogram (A) and full MS spectra of light (B) and heavy chain (C) from reduced rituximab. The insert in panel C shows a zoomed in view of charge state z=+25, with the three peaks representing three different glycoforms.

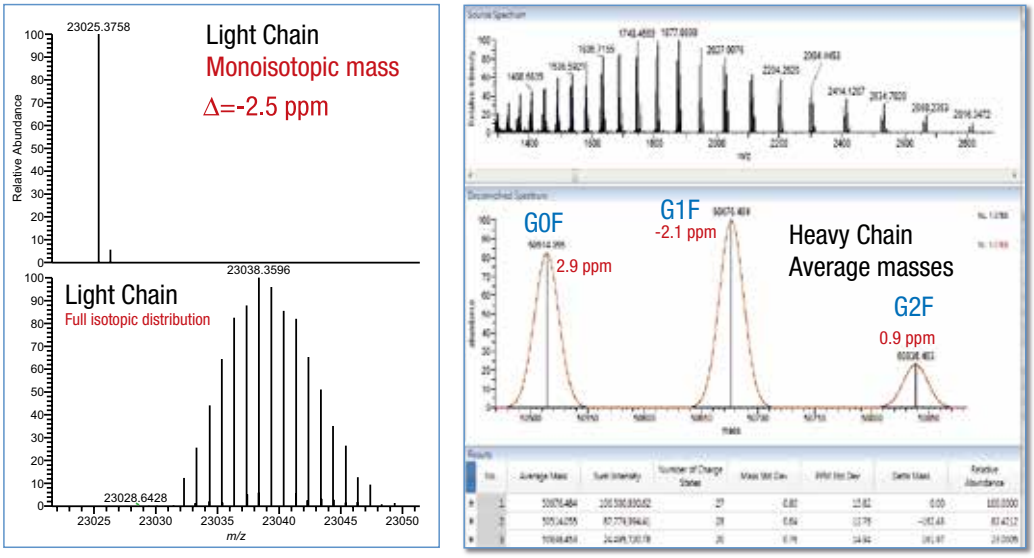


Figure 7. Deconvolution results of the light and heavy chain. The light chain, acquired at a resolution setting of 140,000 in full scan mode, was deconvoluted using the Xtract algorithm, obtaining an accurate monoisotopic mass as well as the full isotopic envelope (left). The heavy chain, detected at 17,500 resolution, was deconvoluted with the ReSpect algorithm providing average masses (right).

To assess the limit of detection of the instrument setup using the 250 × 0.2 mm monolithic PepSwift column, a series of LC/MS runs were acquired. Between 50 pg and 20 ng of the intact antibody was applied on column (Figure 8), starting with the lowest concentration. Two blanks were run before the sequence and between each sample to exclude carryover effects. With this setup, 500 pg was found to be the lowest amount that still achieved a good spectrum for deriving the most abundant glycoforms of the intact antibody. Here it is worth pointing out that for the lowest concentrations it was crucial to prepare the samples fresh without storing them for several hours in the autosampler prior to analysis.

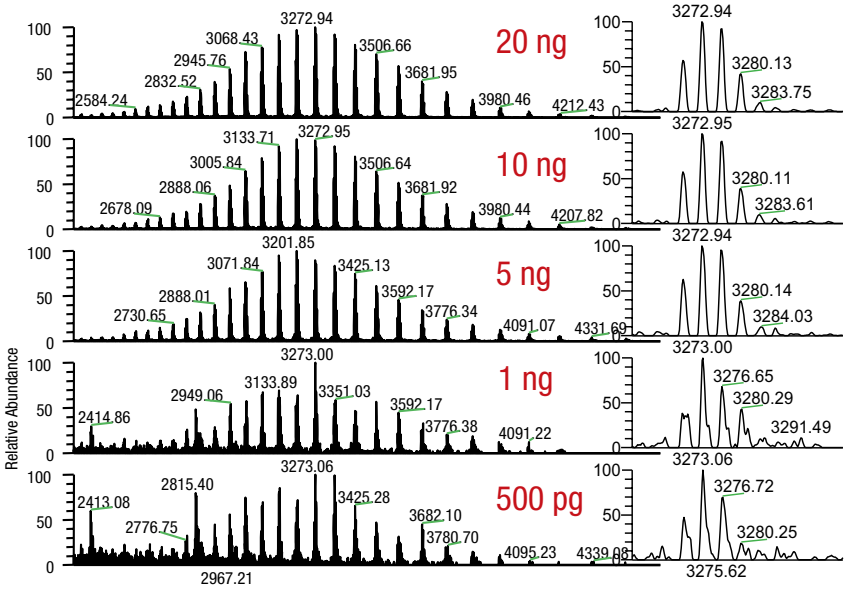


Figure 8. Full MS spectra from a dilution series of 20 ng to 500 pg of intact antibody, applied on a 250 mm × 0.2 mm i.d. monolithic PepSwift column

On the 50 mm × 1 mm i.d. monolithic ProSwift column, 30 ng and 150 ng of intact antibody were applied, both of which produced high quality spectra (Figure 9). Based on the 30 ng load it can be estimated that the lowest amount still yielding a sufficient spectrum quality to be between 5 and 10 ng.

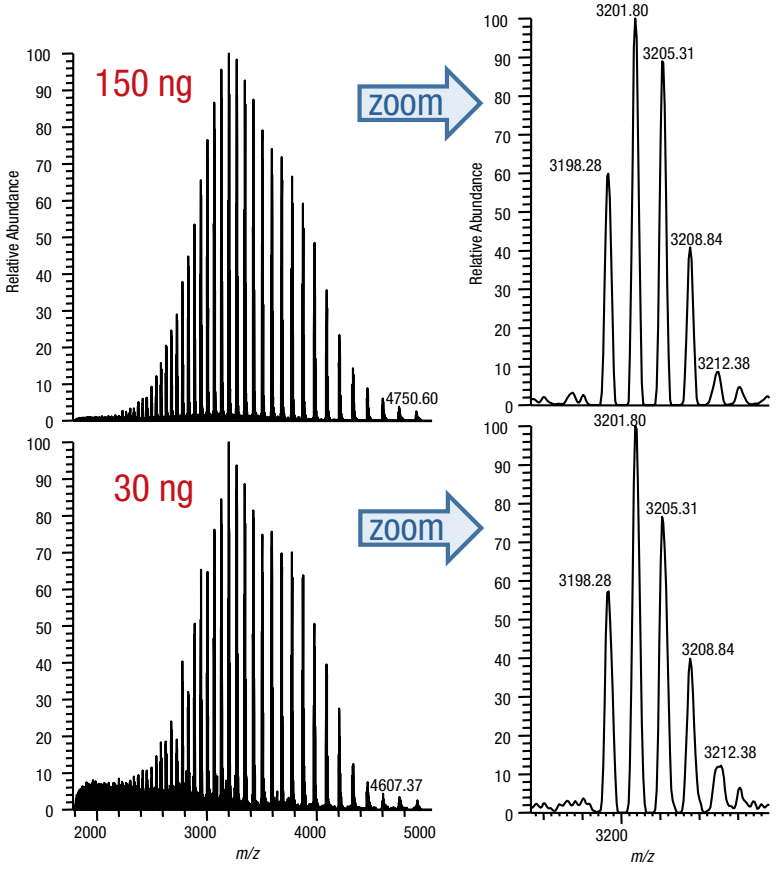


Figure 9. Full MS spectra (left) and zoomed in view of the highest abundant charge state (right) of 150 ng and 30 ng loads of intact rituximab applied on a 50 mm × 1 mm i.d. monolithic ProSwift column

In an attempt to further confirm the sequences of the light and heavy chains, two types of top-down experiments were performed: all-ion fragmentation (AIF) with fragmentation upon collision in the HCD cell and a multiplexed (5-plex), targeted MS^2 experiment on five selected charge states each of the light and heavy chains. All spectra were acquired at 70,000 resolution. For the targeted MS spectrum, a retention-time-dependent mass list was used, targeting first the earlier eluting light chain (RT 13.16 min: *m/z* 1536.96, 1646.6, 1773.3, 1920.7, 2095.4) and later the heavy chain (RT 16–20 min: *m/z* 1584.6, 1635.7, 1684.7, 1748.5, 1810.9). In this type of experiment, the first charge state listed on the inclusion list is selected and sent to the HCD cell for fragmentation. The product ions are stored in the HCD cell while the second charge state is isolated, sent to the HCD cell, fragmented, and stored in the cell until the fifth charge state has also been fragmented. All ions from the five individual isolation and fragmentation steps are sent together to the Orbitrap analyzer, resulting in one single fragment ion spectrum.

The fragment ion assignment for the light chain is displayed in Figure 10. There is good coverage of both the N- and C-terminal ends as well as some fragments in the center of the sequence, resulting in 28% coverage, respectively 15% of the theoretical fragments. For the heavy chain, fragmentation was less efficient with both methods and resulted in about 20 fragments, most of which represent the sequence termini.

To further confirm the sequences, a bottom-up approach was performed using a digest with trypsin following reduction and alkylation of the antibody. The chromatogram obtained from the digest is displayed in Figure 11. A database search against a four-entry database containing the light chain, both variants of the heavy chain, and trypsin revealed a sequence coverage of the light chain of 96% and for the heavy chain of 78.8% (Figure 12). The two short missing peptides from the light chain (LEIK and EAK) could be detected as intact masses only in the full MS spectra, whereas the peptide EAK was identified based on the accurate mass corresponding to the peptide containing a missed cleavage EAKVQWK. Taking into account the peptides identified based on MS/MS spectra and based on accurate masses of the small intact peptides, sequence coverage for the light chain is 100%.

Figure 10. Matched sequence coverage of the rituximab light chain based on fragment ions obtained from AIF experiments. Seventeen b- and 50 y-ions were assigned, corresponding to 15.7% of the theoretical number of fragments (67 of 426).

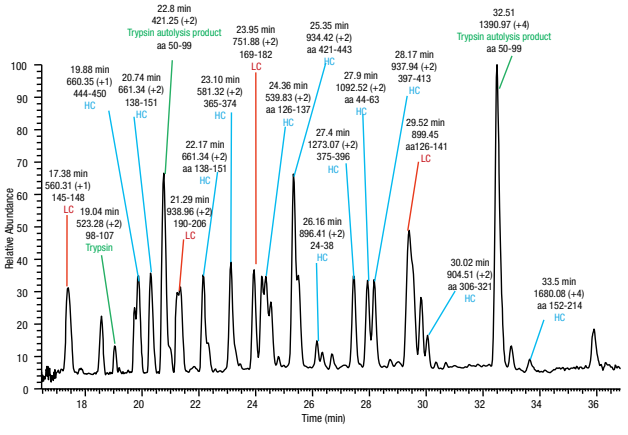


Figure 11. Base peak chromatogram of a digest using trypsin on the reduced and alkylated antibody rituximab

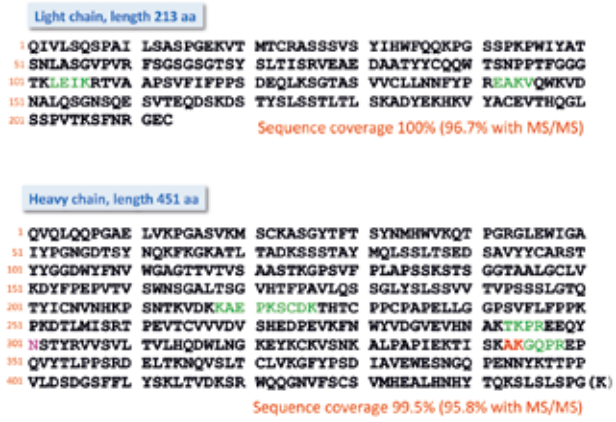


Figure 12. Amino sequence of light and heavy chains from rituximab. Amino acids shown in black letters represent the parts identified based on MS/MS spectra. Sequences confirmed based on MS full scan data as intact peptides only are shown in green. The two amino acids shown in red (AK) as part of the heavy chain could neither be covered on the MS nor on the MS/MS level. Resulting sequence coverage for the light chain is 100% (96% with MS/MS) and 99.5% (98.8% with MS/MS) for the heavy chain. Asparagin^{251} in the heavy chain represents the glycosylation site.

For the heavy chain, the peptide GQPR was also identified based on the accurate mass of the intact peptide. Lastly, the peptide containing the glycosylation site at position Asn^{301} was not identified in its unglycosylated form based on an MS/MS spectrum. A database search including the expected glycans as modifications was successful. In addition, the glycopeptides can easily be detected in the full scan spectra in different glycosylated forms and in different charge states, and the MS/MS spectra can easily be spotted due to the presence of a characteristic peak pattern. The G0Fcontaining peptide is shown as an example in Figure 13, representing the intact precursor and the typical fragmentation pattern obtained from glycopeptides using HCD-type fragmentation: the two hexonium ions at mass 204 (HexNAc) and 366 (Hex-HexNAc) as well as the fragment ions nicely showing the sequence ladder of released hexose (*m/z* 162), N-acetylhexosamine (203), and Fucose (146). Considering all peptides on the MS full scan level and based on MS/MS spectra via database searches, the sequence coverage of the heavy chain is 99.5%, leaving only two amino acids not covered (aa 343-344).

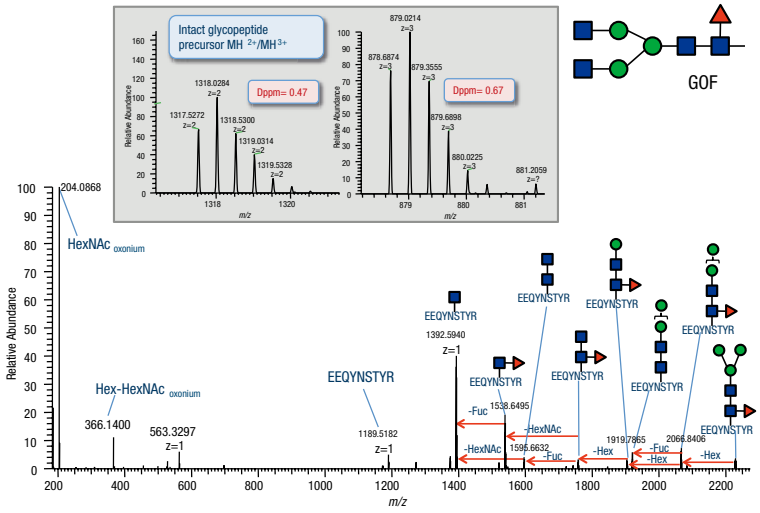


Figure 13. MS/MS spectrum of the glycopeptide aa 297-305 (EEQYN*STYR, *=G0F) obtained from the triply charged glycopeptide precursor. Inserts show the isotope patterns of doubly and triply charged intact precursors detected in the full scan spectrum.

Conclusion

In this study, a workflow is presented that combines fast chromatography, using two sizes of monolithic columns, and high resolution Orbitrap mass spectrometry of intact, as well as reduced, rituximab, sequence verification by AIF, and multiplexed HCD top-down fragmentation, supplemented by a bottom-up approach.

The data presented here also demonstrate the sensitivity of the applied LC-MS instrument setup, still obtaining a good quality MS spectrum from as low as 500 pg of the intact antibody loaded on column. Furthermore, for the analysis of the reduced mAb, a chromatographic separation of the light and heavy chains was achieved allowing for their detection at different resolution settings.

The data obtained from this workflow allow the determination of the molecular weight of the intact antibody, the confirmation/verification of the amino acid sequence of light and heavy chain, and the identification and evaluation of the relative abundance of various glycoforms of rituximab.

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References

1. Premstaller, A.; Oberacher, H. and Huber, C.G. High-Performance Liquid Chromatography- Electrospray Ionization Mass Spectrometry of Single- and Double Stranded Nucleic Acids Using Monolithic Capillary Columns. *Anal. Chem.* **2000**, *72*, 4386-4393.

2. http://www.drugbank.ca/drugs/DB00073

3. Nebija, D.; Kopelent-Frank, H.; Urban, E.; Noe, C. R. and Lachmann, B. Comparison of two-dimensional gel electrophoresis patterns and MALDI-TOF MS analysis of therapeutic recombinant monoclonal antibodies trastuzumab and rituximab. *Journal of Pharmaceutical and Biomedical Analysis*, **2011**, *56*, 684–91.

4. Kuribayashi, R.; Hashii, N.; Harazono, A. and Kawasaki, N. Rapid evaluation for heterogeneities in monoclonal antibodies by liquid chromatography/mass spectrometry with a column-switching system. *Journal of Pharmaceutical and Biomedical Analysis*, **2012**, *67-68*, 1–9.

单抗药物的分子质量、氨基酸序列以及糖基化位点鉴定

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1. 前言

单抗药物主要是由两条重链和两条轻链通过链内和链间二硫键以及非共价键组成，分子质量大约在 150kD 左右，每条重链和轻链在 N 端都包含一个可变区域，在 C 端都包含一个恒定区域，并且在每条重链上还存在一个 N 糖基化位点，该位点含有不同的糖链结构，如图 1 所示。目前，在生物制药领域中，单抗药物以其高效的治疗靶向性和特异性，越来越受到制药公司和医疗单位的青睐。为了保证单抗药物的有效性，以及单抗药物生产过程的实时监控，对于单抗药物的整体分子质量的测定、氨基酸序列的鉴定，糖基化位点的确认，二硫键的定位，糖链结构的解析等重要的分析技术，越来越受到制药公司的关注。随着生物质谱技术的不断发展和推广，基于生物质谱的单抗分析技术也开始全面、快速的开展起来。

目前，LTQ-Orbitrap Elite 组合式质谱仪结合了以前的双压线性离子阱质谱仪和新型高场 OrbitrapTM 质量分析器，提高了 MS 和 MSn 的性能和多功能性。该系统具有高于 240,000 的分辨率（FWHM）、更高的灵敏度、高扫描速度和更大的动态范围，同时结合了多种碎裂技术，为深度复杂样品分析，包括蛋白质组学、代谢组学、脂类组学等样品的鉴定，翻译后修饰和定量分析，提供了全面、高效和快速的质谱分析平台。

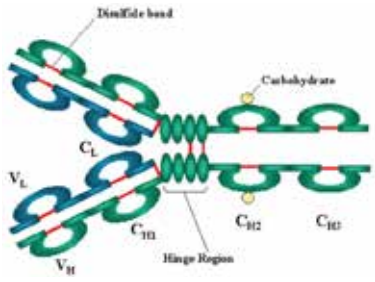

图 1. 单抗药物的简单结构示意图（V 表示可变区域，C 表示恒定区域，L 表示轻链，H 表示重链。）

2. 实验部分

2.1 分子质量测定

2.1.1 仪器和试剂

质谱仪器：LTQ-Orbitrap Elite（赛默飞世尔科技，美国）；
色谱仪器：Accela液相色谱系统（赛默飞世尔科技，美国）；
色谱柱：BioBasic 色谱柱（C8, 1 × 100 mm, 5 μm, 300 Å）
（赛默飞世尔科技，美国）；
试剂：二次去离子水，色谱级乙腈，色谱级甲酸。

2.1.2 仪器方法

色谱分析条件：具体见表 1；
质谱分析条件：具体见表 2；

2.1.3 数据分析方法

采用 Protein Deconvolution 2.0 软件对原始质谱图进行去卷积处理，得到完整的蛋白质分子质量信息。

表 1. 单抗分子质量测定的色谱分析条件

流动相	A：0.1% 甲酸水溶液；	B：0.1% 甲酸乙腈溶液
流速	300 μL/min	
柱温	75℃	
色谱梯度	时间 /min	B 相浓度 /%
	0	0
	0.5	8
	1.5	30
	11.5	50
	12	90
	14	90
	14.5	0
	16	0

表 2. 单抗分子质量测定的质谱分析条件

喷雾电压	4kV
毛细管加热温度	275℃
S-lens	60 %;
鞘气流速	25（arb）
辅助气流速	10（arb）
质量扫描范围	m/z 2000-4000
分辨率	15000（m/z 400）

2.2 氨基酸序列测定

2.2.1 仪器和试剂

质谱仪器：LTQ-Orbitrap Elite(赛默飞世尔科技,美国);

色谱仪器：Easy-nLC 1000 液相色谱系统（赛默飞世尔科技，美国）；

色谱柱：Nano 色谱柱（C18，75μm × 150mm，2μm, 100Å）（赛默飞世尔科技，美国）；

试剂：二次去离子水， 色谱级乙腈， 色谱级甲酸，PNGaseF 内切糖苷酶，二硫苏糖醇（DTT），碘乙酰胺（IAA），trypsin 内切蛋白酶。

2.2.2 仪器方法

色谱分析条件：具体见表 3；

质谱分析条件：具体见表 4；

2.2.3 数据分析方法

采用 Proteome Discoverer 1.3 软件对原始谱图进行数据库搜索，具体搜库参数为：包含单抗氨基酸序列的数据库；半胱氨酸 (C) 烷基化（+57.021Da）设置为固定修饰；甲硫氨酸 (M) 氧化 (+15.995Da) 和天冬酰胺 (N)、谷氨酰胺 (Q) 脱氨基化 (+0.984Da) 设置为可变修饰；trypsin 设置为酶；酶漏切位点为 2。设置肽段的置信水平为 99%，得到高置信的鉴定肽段和蛋白结果。

2.2.4 样品前处理方法

将单抗样品首先进行 PNGaseF 酶解，切除糖链后的蛋白质，再进行 DTT 还原和 IAA 烷基化，最后进行 trypsin 过夜酶解。

表 3. 氨基酸序列测定的色谱分析条件

流动相	A：0.1% 甲酸水溶液；	B：0.1% 甲酸乙腈溶液
流速	350 nL/min	
柱温	35℃	
色谱梯度	时间 /min	B 相浓度 /%
	0	3
	5	8
	55	20
	70	40
	78	90
	90	90

表 4. 氨基酸序列测定的质谱分析条件

喷雾电压	2.3 kV
毛细管加热温度	275℃
S-lens	50 %;
碰撞能量	27% 归一化能量
碎裂方式	HCD
质量扫描范围	m/z 300-1800
分辨率	一级 30,000（m/z 400），二级 15,000（m/z 400）

2.3 糖基化位点测定

2.3.1 仪器和试剂

质谱仪器：LTQ-Orbitrap Elite(赛默飞世尔科技,美国);

色谱仪器：Easy-nLC 1000 液相色谱系统（赛默飞世尔科技，美国）；

色谱柱：Nano 色谱柱（C18， 75μm × 150 mm，2μm,100Å）（赛默飞世尔科技，美国）；

试剂：二次去离子水，色谱级乙腈，色谱级甲酸，二硫苏糖醇（DTT），碘乙酰胺（IAA），trypsin 内切蛋白酶。

2.3.2 仪器方法

色谱分析条件：具体见表 3；

质谱分析条件：具体见表 4；

2.3.3 数据分析方法

采用 Byonic 1.1 软件对原始谱图进行数据库搜索，具体搜库参数为：包含单抗氨基酸序列和 N 糖基化结构的数据库；半胱氨酸 (C) 烷基化（+57.021Da）设置为固定修饰；甲硫氨酸 (M) 氧化（+15.995Da）和天冬酰胺 (N)、谷氨酰胺 (Q) 脱氨基化 (+0.984Da) 设置为可变修饰；trypsin 设置为酶；酶漏切位点为 2。最终通过人工确认，得到准确的糖基化修饰肽段的鉴定信息。

3. 结果与讨论

3.1 蛋白质分子质量测定

基于高分辨质谱 LTQ-Orbitrap Elite，为了保证采集谱图的质量和数量，我们设定高场 Orbitrap 检测器的采集分辨率为 15,000。

经过 LTQ-Orbitrap Elite 质谱采集的单抗原始质谱谱图如图 2-A 所示，我们可以观察到不同的电荷分布的质谱图，选取强度最高的 m/z 为 2744.0384 的一组质谱峰为例，放大之后的质谱峰如图 2-B 所示，可以看到质谱图的灵敏度和信噪比都很高。

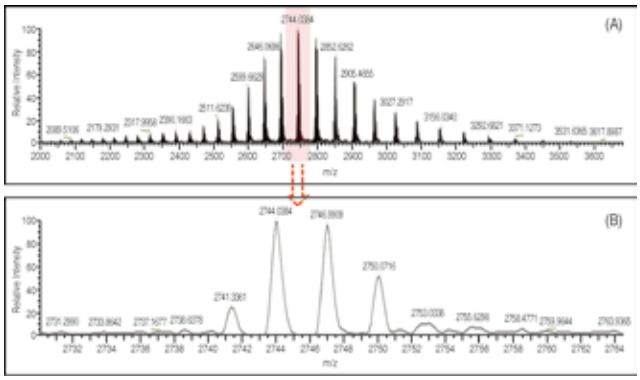

图 2. LTQ-Orbitrap Elite 质谱采集的单抗原始质谱图（A 为整体分布图，B 为 m/z 为 2744.0384 的放大图。）

经过 Protein Deconvolution 2.0 软件去卷积处理之后的单抗分子质量分布图（图 3），根据单抗的氨基酸序列理论分子质量进行计算，将观察到的质谱峰进行归属，可以初步推断该单抗药物存在多种形式的糖链结构，主要包括 G0、G0F、G1F 和 G2F，该结论与常见的单抗组成基本一致，同时根据质谱峰的峰强度信息，我们还可以计算得到不同糖型单抗的相对定量信息，如图 3 中蓝色数字所示。

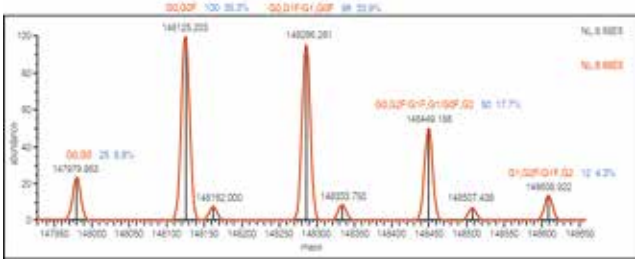

图 3. LTQ-Orbitrap Elite 质谱采集的单抗原始质谱图经过 Protein Deconvolution 2.0 软件去卷积处理之后的单抗分子质量分布图（G0、G0F、G1F 和 G2F 表示不同类型的糖型，蓝色数字表示质谱峰所对应的峰强度和相对定量信息。）

3.2 氨基酸序列测定

经过 LTQ-Orbitrap Elite 质谱数据采集和 Proteome Discoverer 1.3 软件的数据库搜索，以及肽段 99% 高置信水平卡值，在 trypsin 酶解实验中，单抗重链的蛋白序列覆盖度为 97 %，如图 4-A 所示，轻链的蛋白序列覆盖度为 98 %，如图 4-B 所示，由此证明 LTQ-Orbitrap Elite 质谱可以准确、有效地实现单抗氨基酸序列的成功鉴定。其中未鉴定到的氨基酸序列主要是由于酶切后肽段过短不适合质谱鉴定所造成的，为了实现 100% 的氨基酸序列覆盖，可以通过选择其它内切蛋白酶来实现。

单抗蛋白中一般都包含 N- 糖基化修饰，也就是说肽链的天冬酰胺 (Asn，N) 残基可以与糖链的还原端连接，形成不同糖链结构的糖基化蛋白，一般发生 N- 糖基化修饰的位点都是在保守序列 NXS 或 NXT 中（X 为除脯氨酸外的任意氨基酸）。在经过内切糖苷酶 PNGaseF 酶切后，天冬酰胺 (Asn，N) 残基会丢失糖链，产生 0.984Da 的质量增加。不过在样品处理过程中也可能会导致天冬酰胺、谷氨酰胺发生脱氨基化，同样会使天冬酰胺产生 0.984Da 的质量增加。最终的搜库结果显示一些天冬酰胺残基存在 0.984Da 的质量增加，这可能是由于样品发生了脱氨基化或者 N 糖链被酶解所致，进一步结合 N 糖基化位点的特定 NX(X ≠ P)S/T 结构域信息，我们初步推断该单抗中唯一可能发生糖基化的位点为：重链中氨基酸序列 EEQYNSTYR 中的 N 位点，为了得到准确的定性结果，我们进行了后续的糖基化位点鉴定实验。

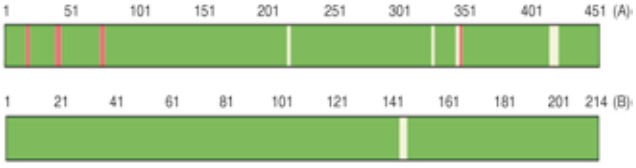

图 4. 单抗经过 trypsin 酶解和 LTQ-Orbitrap Elite 质谱实现的氨基酸序列覆盖度示意图（A 为重链氨基酸序列覆盖示意图，B 为轻链氨基酸序列覆盖示意图，其中绿色标记的氨基酸序列表示该段序列鉴定的可靠性非常高，达到 99% 以上，红色标记的氨基酸序列表示该段序列鉴定的可靠性为 95% 以下，无颜色标记的氨基酸序列表示该段序列没有被鉴定到，氨基酸序列上方的数字代表氨基酸的位置。）

3.3 糖基化位点确定

经过 LTQ-Orbitrap Elite 质谱数据采集和 Byonic 1.1 软件的数据库搜索，共鉴定到 6 个糖基化修饰的肽段，分别对应于 G0（  ）、G0F（  ）和 G1F（  ）（红三角表示岩藻糖，蓝方块表示 N- 乙酰氨基葡萄糖，绿圆表示甘露糖，黄圆表示半乳糖）等，每一条糖肽都经过人工确认，以其中的 EEQYN（G0F）STYR 糖肽为例，如图 5 所示，在二级质谱中，可以观察到 6 个糖诊断离子（见表 5）和 8 个糖肽碎片离子（见表 6），同时结合 N 糖基化修饰位点的特定结构域信息，从而确证该肽段中天冬酰胺(N)残基为N-糖基化修饰位点。

由于采用了高分辨、高精度的 LTQ-Orbitrap Elite 质谱，糖诊断离子的质量偏差都在 1ppm 以内，除了个别碎片离子外，基本所有的糖肽碎片离子的质量偏差也都

在 1ppm 以内，由此可以判断该糖肽碎片离子的归属准确、可靠。

表 5. EEQYN（G0F）STYR 糖肽经过 LTQ-Orbitrap Elite 质谱鉴定到的糖诊断离子列表

糖诊断离子	理论质核比 /M^+	实测质核比 /M^+
C6H7NO2	126.0549	126.0544
C7H7NO2	138.0549	138.0545
C6H9NO3	144.0655	144.0657
C8H9NO3	168.0655	168.0647
C8H11NO4	186.0760	186.0755
C8H13NO5	204.0866	204.0858

表 6. EEQYN（G0F）STYR 糖肽经过 LTQ-Orbitrap Elite 质谱鉴定到的糖肽碎片离子列表（红三角表示岩藻糖；蓝方块表示 N- 乙酰氨基葡萄糖；绿圆表示甘露糖）

糖肽碎片离子	理论质核比 /M^+	实测质核比 /M^+
y3	439.2302	439.2312
y4	526.2625	526.2614
C_4H_5NO	1272.5484	1272.5490
	1392.5859	1392.5878
	1538.6384	1538.5686
	1595.6598	1595.6610
	1757.7072	1757.7064
	1919.7545	1919.6420

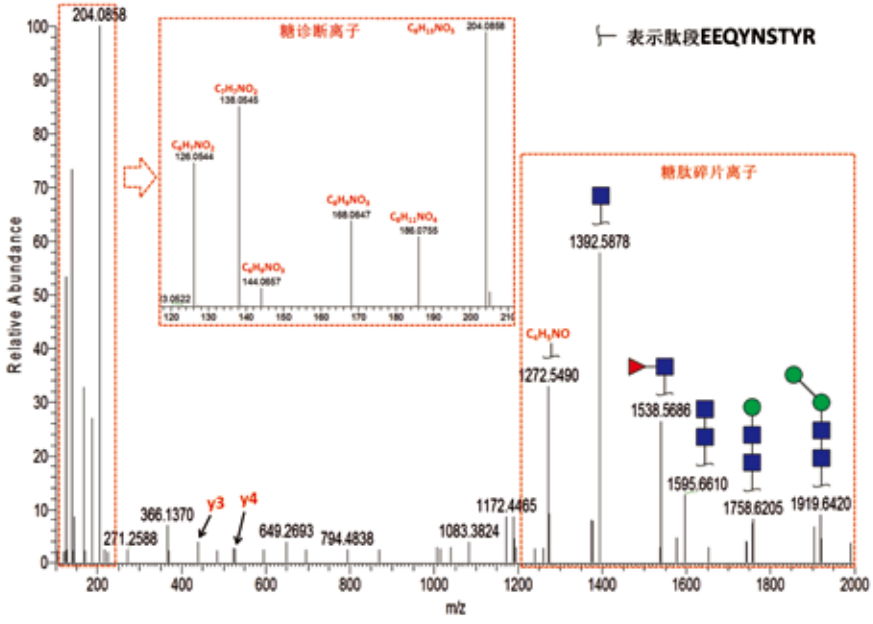

图 5. LTQ-Orbitrap Elite 质谱采集的 EEQYN（G0F）STYR 糖肽的二级质谱图（红三角表示岩藻糖；蓝方块表示 N- 乙酰氨基葡萄糖；绿圆表示甘露糖）

4. 结论

本文通过 LTQ-Orbitrap Elite 质谱建立了单抗药物的整体分子质量测定、氨基酸序列鉴定和糖基化位点确认的分析方法，为常规单抗药物研发分析和实时生产检测提供了高效、快速的分析平台。实验结果表明 LTQ-Orbitrap Elite 组合式质谱仪，凭借其超高的分辨率，超快的扫描速度，超高的质量精度、超低的灵敏度和超大的动态范围，极大地完善和推动了单抗药物的鉴定分析。

ADC 单抗药物的分子质量、氨基酸序列、糖基化位点以及 ADC 药物结合位点鉴定

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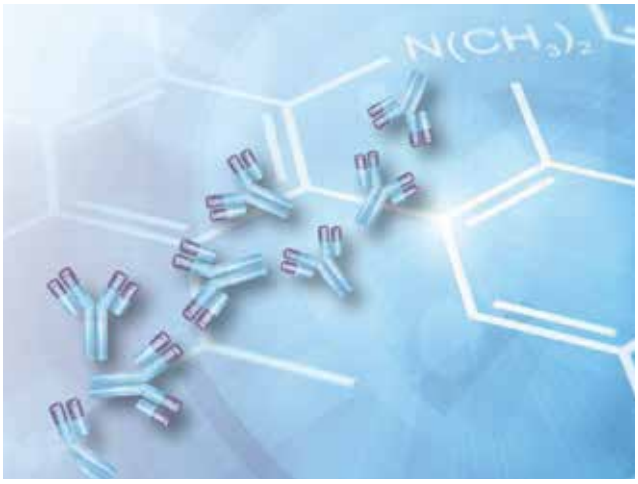
1. 前言

一直以来，单克隆抗体被认为是具有高度特异性的靶向药物，其对肿瘤细胞的靶向性非常高，被誉为治疗恶性肿瘤的“生物导弹”。ADC 技术，就是在抗体蛋白的天然氨基酸上非定点偶联具有抗肿瘤作用的化疗药物（或称小分子药物），以增加单克隆抗体的疗效、并降低小分子药物的毒性。相比于传统 ADC 技术，ADC 抗体药则是通过定点嵌入非天然氨基酸，实现在单克隆抗体上定点、定量接入抗肿瘤的小分子药物，以此获得单一的 ADC 纯品。对此，研究人士表示，这相当于在“生物导弹”上精确地装上了“核弹头”，使得治疗更加安全、有效、定向。

目前，在整个肿瘤药研究领域，ADC 抗体药物已得到了业内的普遍认可。不少跨国药企相继投身到 ADC 药物的研究开发中。为了保证 ADC 单抗药物的安全性和有效性，以及对单抗药物生产过程进行实时监控，对于 ADC 单抗药物的整体分子质量的测定、氨基酸序列的鉴定、糖基化位点的确认、ADC 药物结合位点的确认、二硫键的定位，糖链结构的解析等重要的 ADC 药物分析指标的测试和确认，越来越受到各大制药公司的关注。目前，随着生物质谱技术的不断发展和推广，基于生物质谱的单抗分析技术也开始全面、快速的建立起来。

目前，Q-Exactive 串联质谱仪结合了高选择性的四极杆质谱检测器和高分辨、高灵敏度的新型高场 OrbitrapTM 质量分析器，大大提高了离子的选择性、灵敏度和质量精度。该系统具有 140,000 的分辨率（FWHM）、更高的灵敏度、更快的扫描速度和更大的动态范围，同时结合了 HCD 碎裂技术，可以实现串级质谱碎裂和鉴定，为深度复杂样品分析，包括蛋白质组学、代谢组学、脂类组学等样品的鉴定，翻译后修饰和定量分析，提供了全面、高效和快速的质谱分析平台。同样，Q-Exactive 质谱可以有效地应用到 ADC 单抗药物的研发和生产监控中，基本的实验分析流程如下所示。



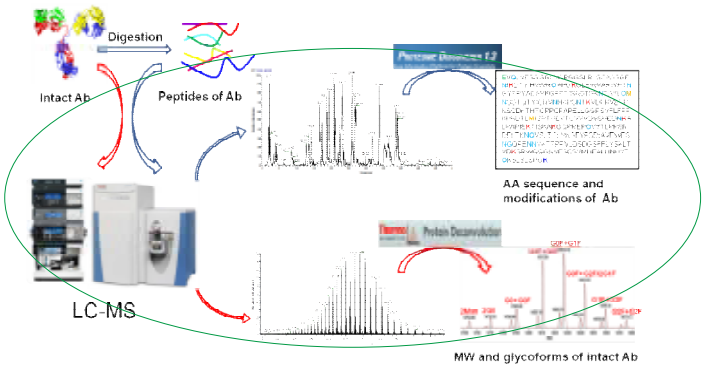


图 1. 单抗分析基本流程图

2. 实验部分

2.1 分子质量测定

2.1.1 仪器和试剂

质谱仪器：Q-Exactive（赛默飞世尔科技，美国）；

色谱仪器：Accela 液相色谱系统（赛默飞世尔科技，美国）；
色谱柱：Agilent Zorbax 300, SB-C8, 3 μm，2.1 × 50 mm；
试剂：二次去离子水，色谱级乙腈，色谱级甲酸。

2.1.2 仪器方法
色谱分析条件：具体见表 1；
质谱分析条件：具体见表 2；

2.1.3 数据分析方法
采用 Protein Deconvolution 2.0 软件对原始质谱图进行去卷积处理，得到完整的蛋白质分子质量信息。

表 1. 单抗分子质量测定的色谱分析条件

流动相	A：0.1% 甲酸水溶液；	B：0.1% 甲酸乙腈溶液
流速	500μL/min	
柱温	75℃	
色谱梯度	时间 /min	B 相浓度 /%
	0	5
	4	5
	12	90
	12.1	90
	16	90
	16.5	5
	20	5

表 2. 单抗分子质量测定的质谱分析条件

喷雾电压	4 kV
毛细管加热温度	275℃
S-lens	60 %;
鞘气流速	30（arb）
辅助气流速	5（arb）
质量扫描范围	m/z 2000-4000
分辨率	15000（m/z 400）

2.2 氨基酸序列、糖基化位点和 ADC 药物结合位点测定

2.2.1 仪器和试剂
质谱仪器：Q-Exactive（赛默飞世尔科技，美国）；
色谱仪器：Accela 液相色谱系统（赛默飞世尔科技，美国）；
色谱柱：Thermo, C18, 100Å ,1.9 μm, 2.1 × 100 mm
试剂：二次去离子水，色谱级乙腈，色谱级甲酸。

2.2.2 仪器方法
色谱分析条件：具体见表 3；
质谱分析条件：具体见表 4；

2.2.3 数据分析方法
采用 Proteome Discoverer 1.3 软件对原始谱图进行数据库搜索，具体搜库参数为：包含单抗氨基酸序列的数据库；半胱氨酸 (C) 烷基化（+57.021Da）设置为固定修饰；甲硫氨酸 (M) 氧化（+15.995Da）、天冬酰胺 (N) 和谷氨酰胺 (Q) 脱氨基化 (+0.984Da)、赖氨酸（K）ADC 结合（+956.3644Da）、天冬酰胺（N）G0F 糖基化 (+1444.5300Da) 设置为可变修饰；trypsin 设置为酶；酶漏切位点为 2。

表 3. 氨基酸序列测定的色谱分析条件

流动相	A：0.1% 甲酸水溶液；	B：0.1% 甲酸乙腈溶液
流速	200 μL/min	
柱温	35℃	
色谱梯度	时间 /min	B 相浓度 /%
	0	5
	4	5
	45	35
	50	90
	52	90
	52.5	5
	60	5

表 4. 氨基酸序列测定的质谱分析条件

喷雾电压	3.5 kV
毛细管加热温度	275℃
S-lens	60 %;
碰撞能量	27% 归一化能量
碎裂方式	HCD
质量扫描范围	m/z 300-1800
分辨率	一级 30,000（m/z 400），二级 15,000（m/z 400）

3. 结果与讨论

3.1 蛋白质分子质量测定

基于高分辨质谱 Q-Exactive，为了保证采集谱图的质量和数量，我们设定高场 Orbitrap 检测器的采集分辨率为 17,500。经过 Q-Exactive 质谱采集的 ADC 单抗药物的原始色谱质谱流出图和原始质谱图如图 2 和 3 所示，我们可以观察到 ADC 抗体药物在整个质量范围内表现出均匀的电荷分布，不同电荷的质谱峰之间可以实现基线分离，从而可以判断质谱的灵敏度、分辨率和信噪比都很高。同时选取强度较高的几组质谱峰进行放大，放大之后的几组质谱峰显示相似的分布模式。

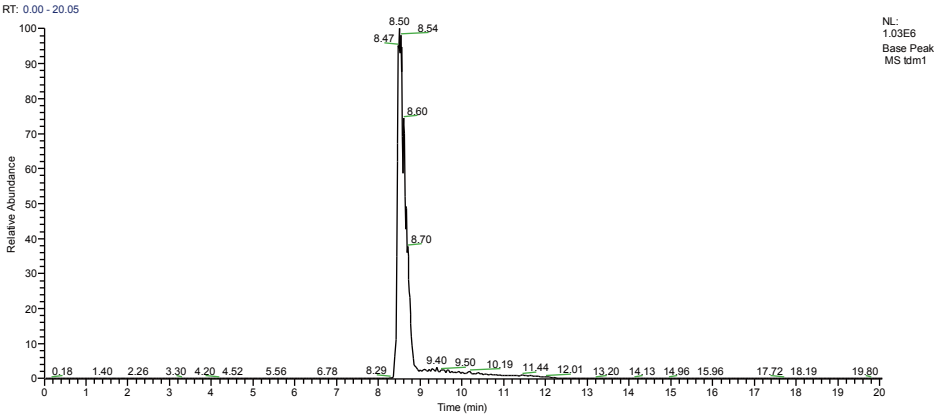


图 2. ADC 抗体药物色谱质谱流出图

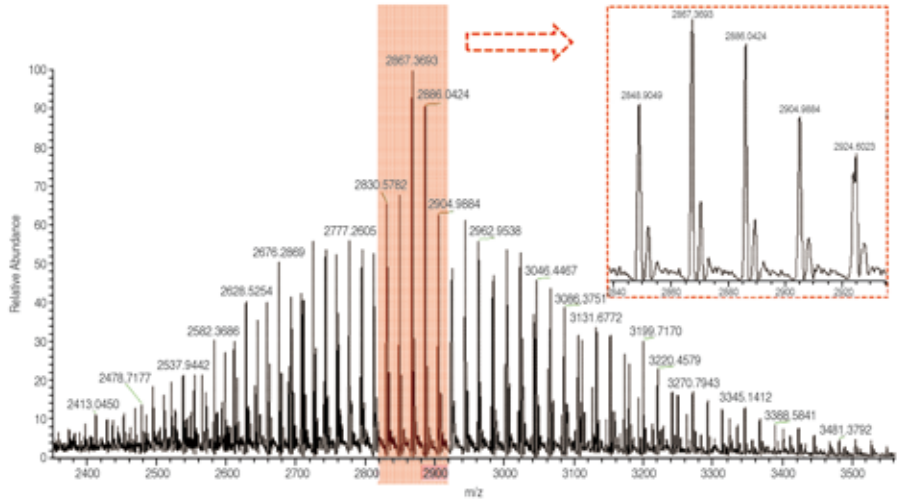


图 3. ADC 抗体药物原始质谱图（红色虚线框内为中间几组峰的放大图）

经过 Protein Deconvolution 2.0 软件去卷积处理之后的单抗分子质量分布图如下所示（图 4），根据单抗的氨基酸序列理论分子质量进行计算，将观察到的质谱峰进行归属，从图中我们观察到，该 ADC 单抗分子之间存在 956Da 左右的质量增加，由此推断该单抗分子结合了不同数目的分子质量在 956Da 左右的药物小分子，并且最多可以检测到 6 个药物小分子的结合。同时还观察到 160 Da 左右的质量增加，这主要是由于连接基团的存在引起的。从而初步推断该 ADC 单抗药物主要结合了 6 个 ADC 小分子药物，该结果与理论期待基本一致，同时根据质谱峰的峰强度信息，我们还可以计算得到该药物的 ADR 值，该数值对于 ADC 药物的有效性评估至关重要。

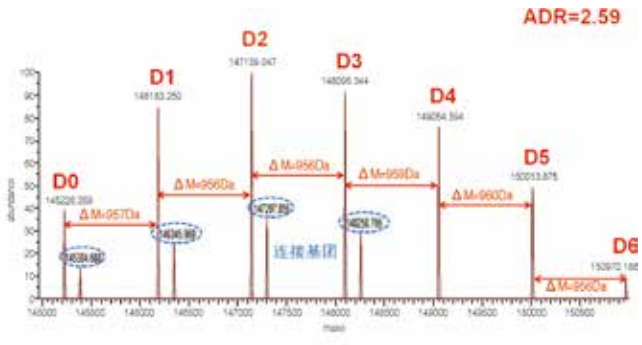


图 4. Q-Exactive 质谱采集的 ADC 单抗原始质谱图经过 Protein Deconvolution 2.0 软件去卷积处理之后的单抗分子质量分布图（D0-D6 表示不同 ADC 药物小分子结合的单抗变体，蓝色圆圈表示连接基团对应的质谱峰。）

3.2 氨基酸序列、糖基化位点和 ADC 药物结合位点鉴定

3.2.1 氨基酸序列覆盖

通过 trypsin 酶解后的肽段，在 Q-Exactive 质谱上采集到的色谱质谱流出图如图 5 所示，再经过 SEQUEST 数据库搜索后，ADC 单抗重链的蛋白序列覆盖度为 90.24 %，如图 6（A）所示，轻链的蛋白序列覆盖度为 100 %，如图 6（B）所示，其中未鉴定到的氨基酸序列主要是由于缺乏酶切位点和酶切后肽段较短造成的，为了实现 100% 的氨基酸序列覆盖，可以通过选择其它内切蛋白酶来实现。

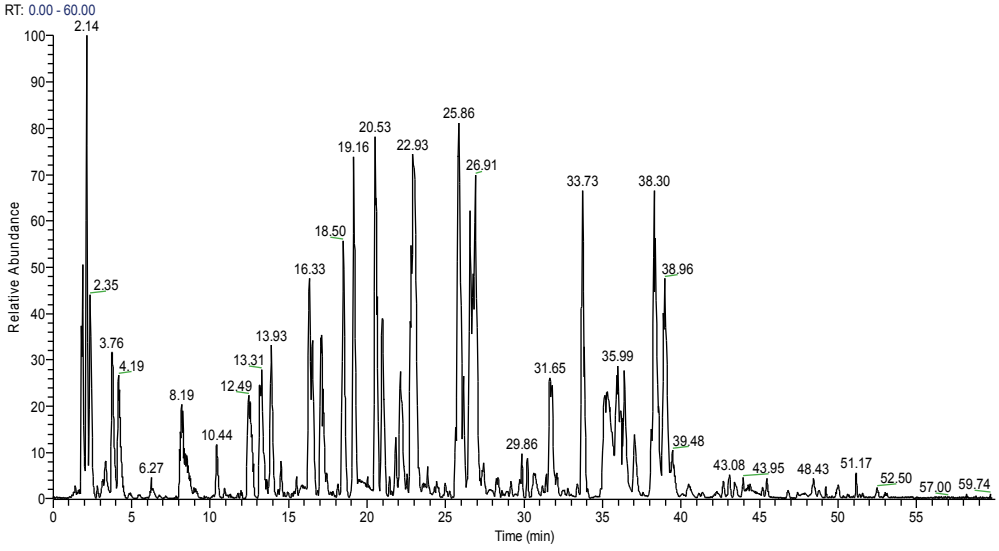


图 5. ADC 单抗药物的 Trypsin 酶解肽段的色谱质谱流出图

图 6. ADC 抗体药物经过 trypsin 酶解和 Q-Exactive 质谱实现的氨基酸序列覆盖度示意图。

（A 为重链氨基酸序列覆盖示意图，B 为轻链氨基酸序列覆盖示意图，其中绿色标记的氨基酸序列表示该段序列鉴定的可靠性非常高，达到 99% 以上，红色标记的氨基酸序列表示该段序列鉴定的可靠性为 95% 以下，无颜色标记的氨基酸序列表示该段序列没有被鉴定到，氨基酸序列上方的数字代表氨基酸的位置。）

3.2.2 糖基化位点确定

ADC 单抗蛋白与其他单抗蛋白相同，也都包含 N- 糖基化修饰，一般发生在保守序列 NXS 或 NXT 中（X 为除脯氨酸外的任意氨基酸）。在糖链完整的情况下，直接进行 trypsin 酶解，我们在搜库时进行 G0F 糖基化可变修饰设定，可以直接获得该 N 糖基化位点：重链氨基酸序列 EEQY N_{261}STYR 中的 N_{261} 位点，下图为该肽段的 G0F 串级质谱图，除了图中黄色和蓝色代表的该肽段的 b, y 碎片离子外，红色圆圈所示的为该糖肽的糖特征碎片离子（低 m/z 端）和糖肽碎片离子（高 m/z 端），从而可以准确确证该糖肽和糖基化位点的存在。

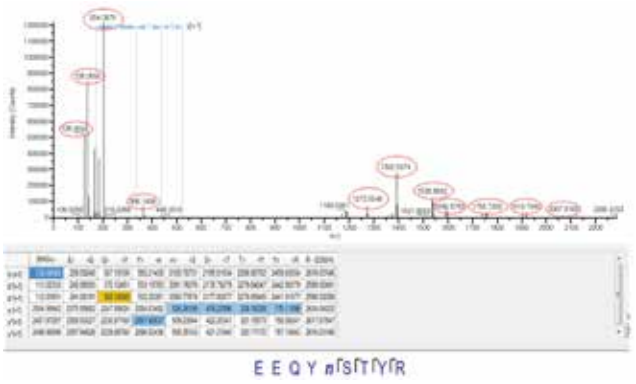


图 7. 重链氨基酸序列 EEQYN261STYR 的串级质谱图

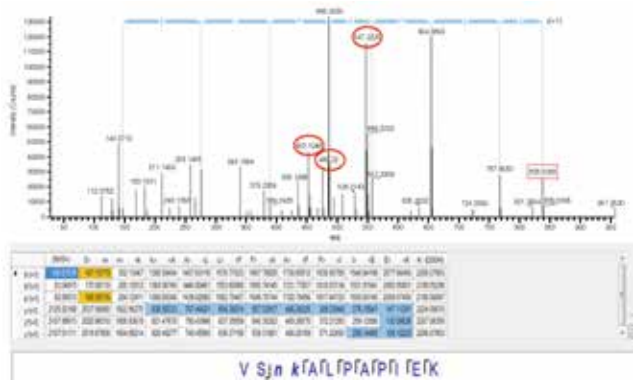


图 9. ADC 结合肽段 VSNKALPAPIEK 的串级质谱图

3.2.3 ADC 药物结合位点确定

根据 Proteome Discovery 软件搜库结果显示，我们发现在赖氨酸结合药物分子的肽段的串级质谱图中存在特征碎片离子 453.20、485.23 和 547.21，因此我们通过这些特征碎片对所有的可能发生 ADC 结合的肽段的串级质谱图进行人工确认，最终准确确认了轻链中 2 个赖氨酸位点和重链中 7 个赖氨酸位点发生了 ADC 小分子药物结合，具体位点如下图（图 8）所示，并且以其中一条肽段为例，给出了 ADC 结合肽段的串级质谱图（图 9），红色圈表示 ADC 特征碎片离子。同时我们也观察到 ADC 药物结合后的肽段的疏水性比较强，色谱流出比较靠后，这一现象完全符合理论推测。

>Light chain
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XKXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

>Heavy chain
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXKXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXKXXXXXXKX
XXXXXKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXKXXXXXXXXXXXXXXXXXXXXXXxx

图 8. ADC 药物小分子结合位点示意图

4. 结论

本文通过 Q-Exactive 质谱建立了 ADC 单抗药物的整体分子质量测定、氨基酸序列鉴定、糖基化位点和 ADC 药物结合位点确认的分析方法，为 ADC 单抗药物研发分析和实时生产检测提供了高效、快速的分析平台。实验结果表明 Q-Exactive 串联质谱仪，凭借其超高的分辨率，超快的扫描速度，超高的质量精度、超低的灵敏度和超大的动态范围，极大地完善和推动了 ADC 单抗药物的鉴定分析。

Automated Glycan Structural Isomer Differentiation Using SimGlycan Software

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Introduction

Currently, glycans are attracting attention from the scientific community as potential biomarkers or as post-translational modifications (PTMs) of therapeutic proteins.[1,2] For example, glycans on the surface of cells mediate interactions between cells and define cellular identities within complex tissues at all stages of animal life. In addition, specific glycan structures control the activities of the proteins to which they are attached, adding a post-transcriptional, post-translational layer of regulation onto protein function. Many glycans show disease-related expression level changes.[3,4] In some instances, the function of specific cell-surface signaling molecules requires the elucidation of an exact glycan structure at a precise site on an appropriate protein. However, the complex branching and isomeric nature of glycans pose significant analytical challenges to the identification of these structures.

Mass spectrometry (MS) has emerged as one of the most powerful tools for the structural elucidation of glycans.[5-7] This is due to its sensitivity of detection and its ability to analyze complex mixtures of glycans derived from a variety of organisms and cell lines. However, there are some drawbacks to using MS-based approaches. Mass spectrometers generate large volumes of data. Currently, processing of data from glycans is mostly done manually, which makes it tedious and time-consuming. In addition to the characterization of the sugar sequence, the analysis must elucidate branching, linkages between monosaccharide units, anomeric configuration, and the location of possible sulfate or phosphate groups.[5] The lack of reliable bioinformatics tools to simplify and expedite the elucidation of glycan structures is the biggest bottleneck in MS-based approaches.

The challenges of MS-based glycomics are further compounded by the need for multistage fragmentation (MS^n). This is a critical tool for glycan structure elucidation[8-11], as it allows for determination of structural heterogeneity and differentiation of isomers. However, it is complicated by the large number of spectra generated for a single structure. It is very common that one must acquire six or seven levels of fragmentation (MS^6 or MS^7) to differentiate potential glycan structural isomers.

SimGlycan™ software from PREMIER Biosoft provides support for glycan identification and structural elucidation.[12] It accepts raw data files from Thermo Scientific mass spectrometers and elucidates the associated glycan structure with high accuracy using database searching and scoring techniques. MS/MS data are searched against the SimGlycan software's database containing theoretical fragmentation of over 9500 glycans. Each proposed structure is assigned a score to reflect how closely it matches with the experimental data. Other relevant biological information for the proposed glycan structures, such as the glycan class, reaction, pathway, and enzyme, are also available via interactive links. To address the challenges of MS^n data interpretation, SimGlycan software version 2.92 has been introduced to provide comprehensive support for MS^n experiments performed on Thermo Scientific mass spectrometers.

In this note, we present for the first time an automated workflow for complete structural elucidation of glycans by combining MS^n fragmentation and data processing using SimGlycan software.

Goal

To demonstrate the use of SimGlycan software for automated structural elucidation of MS^n glycan spectra acquired on Thermo Scientific mass spectrometers.

Experimental Conditions

Sample Preparation

Chicken ovalbumin (1 mg, Sigma) was reduced, alkylated, and digested overnight with trypsin in 25 mM ammonium bicarbonate buffer (pH~8) at 37 ºC as described previously.[13] PNGase F solution (3 µL, Roche) was added to 200 µL of digested sample and the mixture was incubated for another 16 hours at 37 ºC.

Released glycans were separated from peptides using a Sep-Pak® C18 cartridge. The Sep-Pak C18 cartridge was conditioned by washing with acetonitrile, followed by water. PNGaseF-digested sample was loaded onto the cartridge. The released glycans were eluted with 1% ethanol while peptides remained bound to the cartridge.

Released ovalbumin oligosaccharides were first purified using porous graphite carbon column (PhyNexus) and then permethylated as described previously.[9]

Mass Spectrometry

All MS^n experiments were carried out on a Thermo Scientific Velos Pro linear ion trap mass spectrometer using direct infusion into a nanoelectrospray source. Details for the mass spectrometric settings and SimGlycan 2.92 search parameters are listed in Tables 1 and 2.

Table 1. Mass spectrometer settings

Source	nano-ESI	
Capillary Temperature	200 ℃	
S-lens RF Level	50%	
Source Voltage	1.3 kV	
Full MS Mass Range	150-2000 (*m/z*)	
Scan Rate	Enhanced	
Maximum Injection Time	Full MS	50 ms
	MS^n	50 ms
Isolation Width	3	
Collision Energy	30	
Activation Time	10 ms	
Predictive AGC Enabled	Yes	
No. Microscans for Full MS	5	
Target Value Full MS	3e4	
Target Value MS^n	3e4	

Table 2. SimGlycan 2.92 search parameters

Ion Mode	Positive
Adducts	Sodium
Precursor Ion *m/z* Error Tolerance	0.8 Da
Spectrum Peak *m/z* Error Tolerance	0.8 Da
Chemical Derivatization	Permethylated
Reducing Terminal	Reduced
Include Substituents while Searching Glycans	Yes
Class	Glycoprotein
SubClass	N-Glycan
Biological Source	Chicken
Pathway	Unknown
Search Structure	All
Glycan Type	All

Results and Discussion

Automated structural interpretation of MS^n glycan spectra were tested on glycans released from chicken ovalbumin (Figure 1). This was an ideal system to test the capability of SimGlycan software because the glycan content of ovalbumin has been characterized in depth.[14] In parallel, we manually interpreted the MS^n spectra and compared it with Glycome Technologies results presented at the 58th ASMS conference[15] which provided a perfect control.

Figure 2a shows the MS profile of permethylated glycans derived from ovalbumin and analyzed on a Velos Pro mass spectrometer. This was an ideal instrument for these experiments as the dual-pressure ion trap and S-Lens

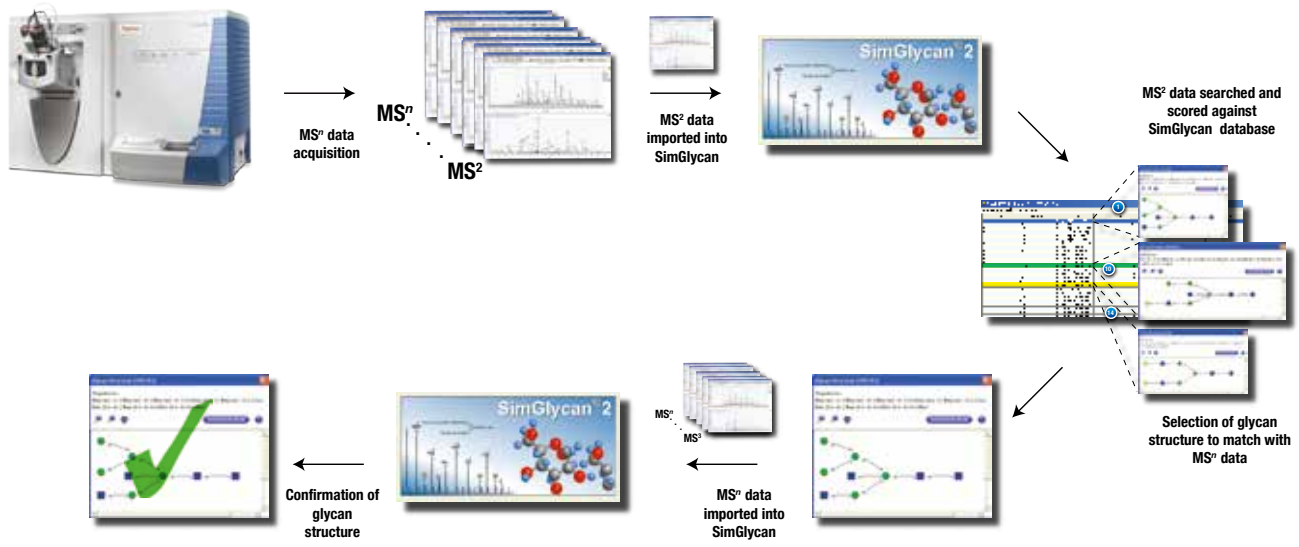


Figure 1. Workflow for automated structural interpretation of MS^n glycan spectra.

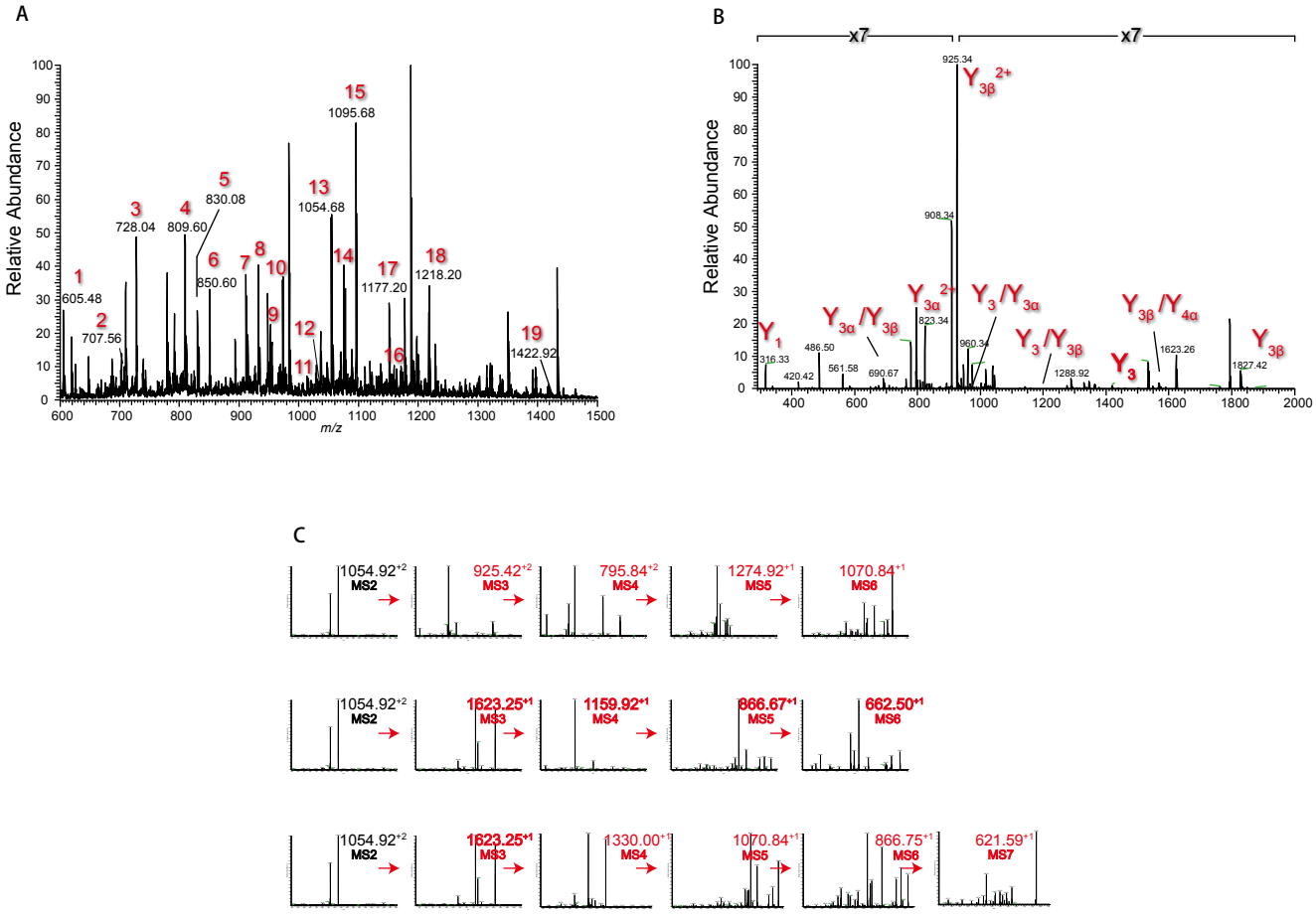


Figure 2. (a) Ion trap (IT) mass spectrum of permethylated ovalbumin released glycans (labeled peaks correspond to Table 3). (b) IT MS/MS spectrum of the peak at *m/z* 1054.68. (c) Set of sequential MS^n spectra acquired for peak at *m/z* 1054.68 (+2).

ion optics provide increased ion transmission along with better trapping and fragmentation efficiency, which are critical for performing MS^n experiments. Table 3 shows all glycans identified in this study. Figure 2b shows the MS^2 spectrum for a peak at *m/z* 1054.68 (+2) (#13 in Figure 2a), which was selected for software evaluation as it was interrogated before.[15] Though it is possible to identify the different monosaccharides that make up the overall glycan composition, it is very difficult from the MS^2 spectrum to determine linkages and branching. To fully characterize the glycan structure, sets of sequential MS^n spectra were acquired for this precursor (Figure 2c) and SimGlycan software was used for data interpretation. The Velos Pro mass spectrometer was operated in "Enhanced Scan" profile mode for all MS experiments. The enhanced scan mode provides for higher resolution and allows charge-stage determination of precursors and fragment ions.

The initial step in data analysis is to import the MS^2 spectrum into SimGlycan for automatic compositional identification. Based on the criteria selected, the SimGlycan software searches its database to match the MS^2 data. If we were to strictly rely on MS^2 data, then the MS/MS fragmentation pattern for *m/z* 1054.68 (+2) can be interpreted as a hybrid glycan with a bisecting GlcNAc from the top ranked glycan from SimGlycan database search results (Figure 3). Examination of the glycan list reported by SimGlycan software for the submitted MS^2 spectrum shows additional glycan compositions having the same mass but ranked much lower. These glycans, though reported to have much lower probability of matching the submitted MS^2 spectrum, could represent additional isomers that might be present, as not every major fragment in the spectra was assigned (Figure 2b).

To determine whether these glycans were additional isomers present in the sample, SimGlycan software was used to examine if any of the lower-ranked glycan structures matched the MS^n fragmentation pathway. From the list generated by the software, we selected specific structures to compare with the MS^n fragmentation pathway. Each successive level of fragmentation was then brought in to match with the specific precursor selected for fragmentation in the previous level of MS^n spectrum.

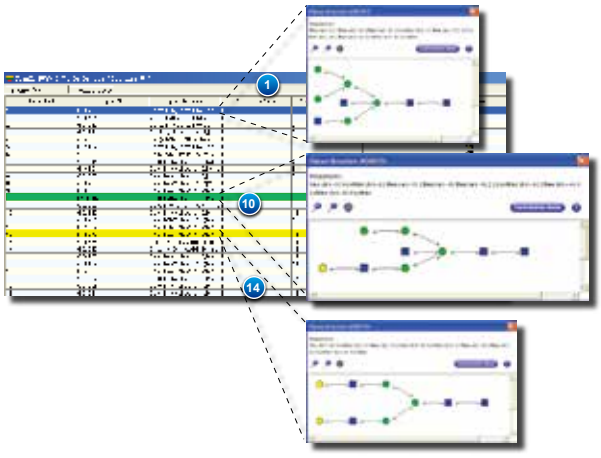


Figure 3. SimGlycan search results for ion trap MS/MS spectrum of precursor ion at *m/z* 1054.68 (+2). Symbolic representation of top-ranked and two lower-ranked glycans search results by SimGlycan software is shown.

From our list, we selected the asialyl digalactosyl biantennary glycan to confirm or deny as an isomer. It was ranked much lower based on MS^2 data, but had the same precursor mass as the top match (Figure 3, ranked 14 on the list). From the MS^2 spectrum (Figure 2b) of *m/z* 1054.68 (+2) we selected the fragment ion at *m/z* 1623.25 (+1) for further fragmentation. The detection of this ion indicated the loss of Gal-GlcNAc from the non-reducing end of the asialyl digalactosyl biantennary glycan. Figures 2c and 4a show the MS^3 spectra for this ion and the fragmentation match of this particular spectrum to the theoretical fragmentation of the selected structure. Of particular interest in the MS^3 spectrum is the fragment ion at *m/z* 1159.93 (+1), which corresponds to additional loss of Gal-GlcNAc structure. This is only possible from our selected asialyl digalactosyl biantennary glycan structure as additional loss is possible from the non-reducing end. Figure 4b shows the overall sequential fragmentation pathway for the proposed structure and how it is only compatible with the selected structure and the set of sequential MS^n spectra acquired in Figure 2c (1054.68 → 662.50). Figure 4c shows sequential (1054.68 → 1070.84 as in Figure 2c) fragmentation pathway for the hybrid glycan with the bisecting GlcNAc. This further confirms that this structure is also present in precursor at *m/z* 1054.68 (+2). An additional hybrid glycan is identified for this precursor in Figure 4d (1054.68 → 621.59 as in Figure 1c). As

illustrated in Figures 4b-d, SimGlycan software was able to resolve isobaric oligosaccharides and perform detailed characterization of selected structures.

Table 3 shows 2 other glycan structural isomers (labeled as 5 and 9) identified by SimGlycan software using the approach described above. As well as differentiating structural isomers, MS^n can be used to confidently elucidate correct glycan structure when insufficient fragmentation is generated at the MS^2 level. For example, the peak at *m/z* 1422.92(+2) represents a single glycan structure. However, the MS^2 spectrum does not provide enough information to clearly elucidate the correct structure. In fact, submission of MS^2 data to SimGlycan resulted in an incorrect structure being ranked number one due to the absence of key fragment ions. The correct structure of this precursor is shown in Table 3 (labeled as 19). Figure 5 highlights the MS^n sequential fragmentation pathway required for this glycan identification and the use of SimGlycan to interpret the MS^n spectra.

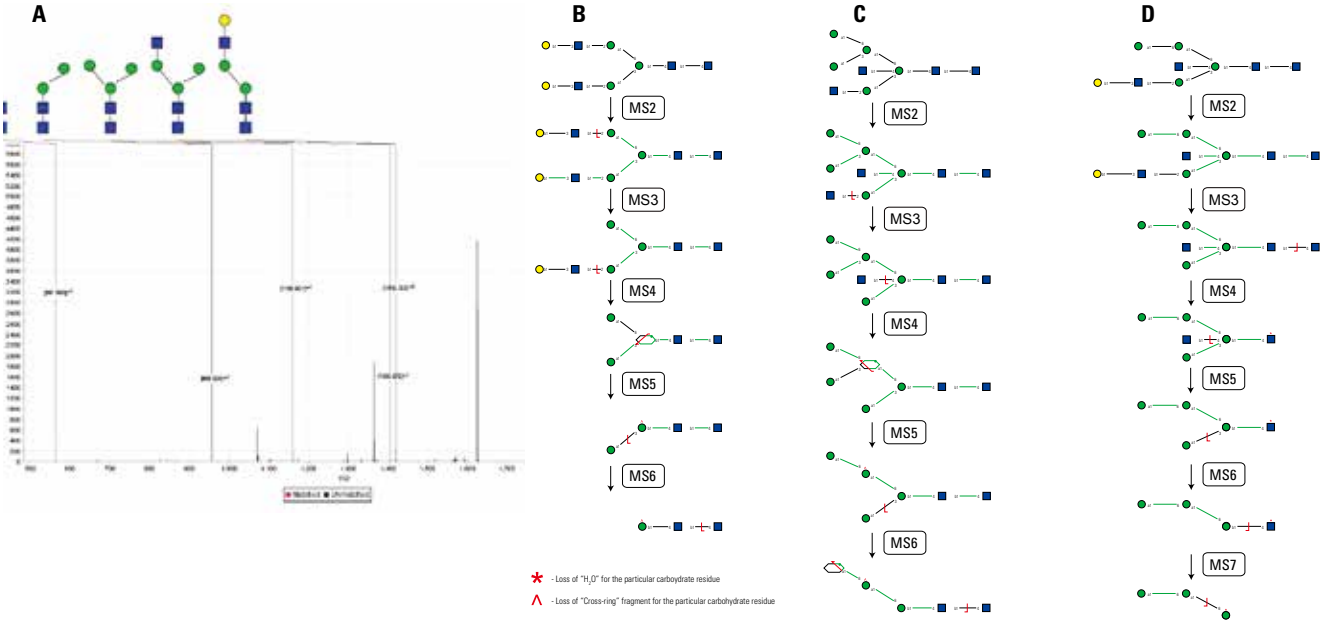
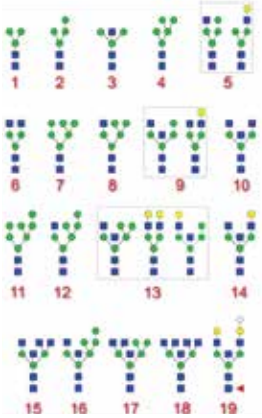


Figure 4. (a) Ion trap MS3 spectrum of precursor at *m/z* 1623.25 (+1). Symbolic representation of Y-type glycosidic fragments are shown. MS^n fragmentation pathway for (b) (Gal)2(Man)3(GlcNAc)4 (c) (Man)5(GlcNAc)4 and (d) (Gal)(Man)4(GlcNAc)4.

Table 3. Structures of chicken ovalbumin N-linked released glycans identified in this study (structures drawn using GlycoWorkbench[16])



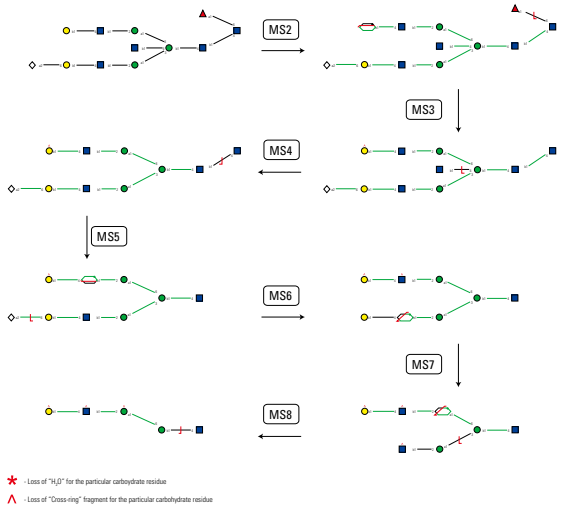


Figure 5. Ion trap MS^n fragmentation pathway for precursor at *m/z* 1422.92 (+2).

Conclusion

- The combination of permethylation, MS^n, and SimGlycan software enabled successful identification and differentiation of various structural isomers of chicken ovalbumin released glycans.
- The overall analysis time was reduced to matter of minutes thus enabling truly automated, high-throughput data analysis.
- SimGlycan software simplified data analysis by providing comprehensive support for performing MS^n experiments on Thermo Scientific ion trap and ion trap/Orbitrap hybrid mass spectrometers.

References

1. Shriver, Z.; Raguram, S.; Sasisekharan, R., Glycomics: a pathway to a class of new and improved therapeutics. *Nat Rev Drug Discov* **2004**, 3, (10), 863-73.
2. Kam, R. K. T.; Poon, T. C. W., The Potentials of Glycomics in Biomarker Discovery. *Clinical Proteomics* **2008**, 4, 67-79.
3. Kobata, A., Altered glycosylation of surface glycoproteins in tumor cells and its clinical application. *Pigment Cell Res* **1989**, 2, (4), 304-8.
4. Ono, M.; Hakomori, S., Glycosylation defining cancer cell motility and invasiveness. *Glycoconj J* **2004**, 20, (1), 71-8.
5. Bahr, U.; Pfenninger, A.; Karas, M.; Stahl, B., High-sensitivity analysis of neutral underivatized oligosaccharides by nanoelectrospray mass spectrometry. *Anal Chem* **1997**, 69, (22), 4530-5.
6. Viseux, N.; de Hoffmann, E.; Domon, B., Structural assignment of permethylated oligosaccharide subunits using sequential tandem mass spectrometry. *Anal Chem* **1998**, 70, (23), 4951-9.
7. Weiskopf, A. S.; Vouros, P.; Harvey, D. J., Electrospray ionization-ion trap mass spectrometry for structural analysis of complex N-linked glycoprotein oligosaccharides. *Anal Chem* **1998**, 70, (20), 4441-7.
8. Sheeley, D. M.; Reinhold, V. N., Structural characterization of carbohydrate sequence, linkage, and branching in a quadrupole Ion trap mass spectrometer: neutral oligosaccharides and N-linked glycans. *Anal Chem* **1998**, 70, (14), 3053-9.
9. Ciucanu, I.; Kerek, F., A Simple and Rapid Method for the Permethylation of Carbohydrates. *Carbohydrate Research* **1984**, 131, (2), 209-217.
10. Ciucanu, I.; Costello, C. E., Elimination of oxidative degradation during the per-O-methylation of carbohydrates. *J Am Chem Soc* **2003**, 125, (52), 16213-9.
11. Ashline, D. J.; Lapadula, A. J.; Liu, Y. H.; Lin, M.; Grace, M.; Pramanik, B.; Reinhold, V. N., Carbohydrate structural isomers analyzed by sequential mass spectrometry. *Anal Chem* **2007**, 79, (10), 3830-42.
12. Apte, A.; Meitei, N. S., Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan. *Methods Mol Biol* 600, 269-81.
13. Snovida, S. I.; Bodnar, E. D.; Viner, R.; Saba, J.; Perreault, H., A simple cellulose column procedure for selective enrichment of glycopeptides and characterization by nano LC coupled with electron-transfer and high-energy collisional-dissociation tandem mass spectrometry. *Carbohydr Res* 345, (6), 792-801.
14. Harvey, D. J.; Wing, D. R.; Kuster, B.; Wilson, I. B., Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins. *J Am Soc Mass Spectrom* **2000**, 11, (6), 564-71.
15. Ashline, D. J.; Fournier, J.; Cernisenco, C.; Second, T., Software-assisted peak annotation and isomer detection for oligosaccharide mass spectra: A case study. **ASMS 2010** poster, ThP13
16. Ceroni, A.; Dell, A.; Haslam, S. M., The GlycanBuilder: a fast, intuitive and flexible software tool for building and displaying glycan structures. *Source Code Biol Med* **2007**, 2, 3.

Integrated LC/MS Workflow for the Analysis of Labeled and Native N-Glycans from Proteins Using a Novel Mixed-Mode Column and a Q Exactive Mass Spectrometer

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Key Words

GlycanPac AXH-1, HILIC, WAX, glycomics, glycoproteins, glycopeptides, glycans, labeled *N*-glycans, Q Exactive, SimGlycan software

Goal

Develop a comprehensive method for the structural characterization of released glycans from proteins. The described integrated method covers sample preparation, separation, mass spectrometry data acquisition, and analysis.

Introduction

Glycans are widely distributed in biological systems in 'free state' as well as conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They play significant roles in many biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development.[1] Glycans can affect efficacy and safety of protein based drugs. For example, recombinant proteins and monoclonal antibodies (mAb) are often dependent on the structure and types of glycans attached to the proteins.[2] The structures of glycans are diverse, complex, and heterogeneous due to posttranslational modifications (PTMs) and physiological conditions. Minor changes in glycan structure can result in striking differences in biological functions and clinical applications. The structural characterization of glycans is essential in bio-therapeutics and bio-pharmaceutical projects.[3] In addition to the characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans. Hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterionicbased packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and compositionbased separation. A limitation of this approach is that identification of the glycan charge state is not possible due to the fact that glycans of different charge states are intermingled in the separation envelope.

The Thermo Scientific™ GlycanPac™ AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural analysis of glycans, either labeled or native, by LC-fluorescence or LC/MS methods.The GlycanPac AXH-1 column is based on innovative mixed-mode surface chemistry combining both weak anion-exchange (WAX) and HILIC retention mechanisms. The WAX functionality provides retention and selectivity for negatively charged glycans, while the HILIC mode facilitates the separation of glycans according to their charge, polarity, and size. As a result, the GlycanPac AXH-1 column provides unparalleled separation capabilities for glycans.

LC-MS/MS analysis of glycans requires the processing of large sets of data. The incorporation of SimGlycan® software (PREMIER Biosoft) alleviates this issue, thus enabling the development of a true high-throughput workflow.

This application note presents a step-by-step method for the release, labeling, separation, and structural elucidation of *N*-glycans from proteins by LC-MS/MS.

Experimental Conditions

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific™, AC610010040)
- LC/MS grade formic acid (Fisher Scientific, A117-50)
- Ammonium formate (Fisher Scientific, AC40115-2500)
- Thermo Scientific Premium 2 mL vial convenience kit, 60180-600
- PNGase F (New England BioLab, P0705L)
- Bovine fetuin (Sigma-Aldrich®, F2379)
- Thermo Scientific™ Hypercarb™ cartridge, 6 mL, 60106-403
- Trifluoracetic acid (Fisher Scientific, 28904)
- Sodium cyanoborohydride (Fisher Scientific, AC16855-0500)
- Anthranilamide (2AB) (Fisher Scientific, AC10490-5000)
- Glacial acetic acid (Fisher Scientific, AA36289AP)
- Dimethylsulfoxide (DMSO) (Fisher Scientific, D128500LC)
- Sodium hydroxide (NaOH) (Fisher Scientific, S318-100)
- Ammonium acetate (Fisher Scientific, A637-500)
- SEC column, 0.9 × 50 cm Sephadex® (GE Healthcare, G-10-120)
- GlykoClean™ G Cartridges, Prozyme, GC250
- 2-mercaptoethanol (Fisher Scientific, O3446I-100)

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 system, including pump: LPG-3400RS, thermal compartment: TCC-3000RS, pulled-loop well plate auto sampler: WPS-3000TRS, fluorescence detector with Dual-PMT: FLD3400RS, and 2 µ L micro flow cell: 6078.4330
- Q Exactive hybrid quadrupole-Orbitrap mass spectrometer
- Thermo Scientific™ SpeedVac™ Concentrator
- Thermo Scientific Lyophilizer (Labconco® FreeZone® -105 ºC 4.5 L benchtop freeze dry system) 16-080-207
- Thermo Scientific 24-Port SPE vacuum manifold, 60104-233

Buffer Preparation

- Ammonium formate (80 mM, pH 4.4): Dissolve 5.08 ± 0.05 g of ammonium formate (crystal) and 0.60 g of formic acid in 999.6 g of DI water. Sonicate the resulting solution for 5 min.
- 0.1 M sodium phosphate buffer, pH 7.25: Add 102.24 mg of Na_2HPO_4 and 38.14 mg of NaH_2PO_4 to 10 mL of DI water. Vortex to mix the solid completely. Verify that the pH of the solution is 7.25 ± 0.02.

Release of *N*-Glycans from Proteins

1. Dissolve 1 mg of the bovine fetuin protein in 500 µL of 0.1 M sodium phosphate buffer, pH 7.2 ± 0.05, in an Eppendorf tube.
2. Add 0.5 µL of 2-mercaptoethanol to this solution.
3. Finally, add 50 U (units) of PNGase F and incubate total solution at 37 ºC water bath for 18 h.
4. Cool to room temperature and purify the released glycans as described in the next section.

Purification of *N*-Glycans

Purify free glycans after digestion using a Hypercarb cartridge as follows:

1. Attach a single Hypercarb cartridge per reaction to a designated port in the SPE manifold.

2. Slowly, and with a consistent flow rate, pre-treat each cartridge with the following volumes of reagents in the order described: 15 mL of 1M NaOH, 15 mL of HPLC grade water, 15 mL of 30% acetic acid, 15 mL of HPLC grade water.
3. Prime the cartridge with 15 mL of 50% acetonitrile/0.1% trifluoroacetic acid (TFA), followed by 15 mL of 5% acetonitrile/0.1% TFA.
4. Load the entire sample volume into the cartridge and let it permeate into the resin by pulsing the vacuum on and off quickly.
5. Rinse the reaction tube with ~50 µL of HPLC grade water, transfer into the cartridge, and pulse the vacuum again.
6. Wash the cartridge with 15 mL of HPLC grade water, followed by 15 mL of 5% acetonitrile/0.1% TFA.
7. Elute the glycans with 4 × 2.5 mL of 50% acetonitrile/0.1% TFA into a labeled 15 mL conical tube.
8. Immediately freeze samples on dry ice and then lyophilize to dryness (16–24 h).
9. After lyophilization, dissolve the solid in 1 mL of water, dry the samples again in a 1.5 mL Eppendorf tube, and store at -20 ˚C.

2AB Labeling Reaction

Carry out the labeling reaction using a modified reported procedure.[4]

1. Prepare the 2AB labeling reagent (100 µL): Dissolve 2-aminobenzamide (4.6 mg) in 70 µL of DMSO.
2. Add 30 µL of glacial acetic acid (100%) to the mixture.
3. Transfer the complete solution to a black or lightprotected, screw-cap, 1.5 mL Eppendorf tubes containing 6.4 mg of sodium cyanoborohydride.
4. Incubate the solution at 60 ºC for 10 min to dissolve sodium cyanoborohydride completely. Occasionally vortex the solutions. When all the solids are completely dissolved, the 2AB labeling reagent is ready to use for the labeling reaction.
5. Add 20 µL of 2AB labeling reagent to 50 µg of free glycans and vortex to mix the solution. Then, incubate the mixture at 60 ºC for 3 h.

Clean Up of Labeled Glycans

1. After completion of the 2AB reaction, add 250 µL of acetonitrile to the vial at room temperature.
2. Purify the samples using a GlykoClean G cartridge; pre-equilibrate the column with the following solutions in the order they appear: wash with 3 mL of deionized water, 3 mL acetonitrile, 3 mL of 96% acetonitrile.
3. Add the labeled glycans to the pre-equilibrated column.
4. Wash with 96% acetonitrile.
5. Elute the glycans with 5 mL of DI water.
6. Lyophilize the solution to dryness.
7. Upon dryness, dissolve the sample in 500 µL of water.
8. Further purify the labeled glycans using a sizeexclusion chromatography (SEC) Sephadex® column to get highly pure labeled oligosaccharides.
9. Inject the samples onto an SEC column connected to a UV detector. Equilibrate the column with 10 mM ammonium acetate at a flow rate of 0.35 mL/min until a steady baseline of 205 nm is achieved.
10. Run the column with 10 mM ammonium acetate for 90 min and collect glycan containing fractions using UV detection at 205 nm.
11. Dry the combined fractions by lyophilization, re-suspend with 1 mL of DI water. Quantify the glycans[5] and then store the remaining sample at -20 ºC for future use.
12. Ready for use as 2AB labeled *N*-glycan from fetuin.

Sample Preparation for Injection

1. Mix 25 µL of purified labeled glycans at 0.2 nmol/µL in DI water with 75 µL of acetonitrile.
2. Transfer the total solution to the auto sampler vial for analysis.

Note: Store the standard at -20 ºC.

Separation Conditions

Column	GlycanPac AXH-1, 2.1 × 150 mm, 1.9 µm
Mobile phase	A: acetonitrile + water (80:20, v/v)
	B: ammonium formate (80 mM, pH 4.4)
Flow rate (µL/min)	400
Column temperature (ºC)	30
Sample volume (injected) (µL)	1
Mobile phase gradient	Refer to Table 1

Table 1. Mobile phase gradient

Time (min)	% A	%B	Flow (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

MS Conditions

MS instrument	Q Exactive hybrid quadrupole-Orbitrap MS
Source	HESI-II probe
Ionization mode	Negative ion

Full MS

MS scan range (*m/z*)	380–2000
Resolution	70,000
Microscans	1
AGC target	1×10^6
Max IT (ms)	60

dd-MS2

dd-MS2 resolution	17,500
Microscans	3
MS/MS AGC target	2×10^5
MS/MS max IT (ms)	250–1000
Isolation window (m/z)	2
NCE	35
Stepped NCE	8%
Dynamic exclusion (s)	90

Source Conditions

Source position	C
Sheath gas flow rate (arb units)	20
Auxillary gas flow rate (arb units)	5
Sweep gas flow rate	0
Spray voltage (kV)	3.30
Capillary temperature (ºC)	275
S-lens RF level	50
Heater temperature (ºC)	300

Data Processing and Software

Chromatographic software	Thermo Scientific™ Chromquest™ v 5.0 Chromatography Data System
MS data acquisition	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
MS/MS data analysis	SimGlycan software v 4.5

SimGlycan Search Parmeters

Ion mode	Negative
Adduct	H
Chemical derivatization	Underivatized
Match fragment ion for charge state	< Precursor *m/z* charge state
Precursor ion *m/z*	10 ppm
Fragment ion	0.05 Da
Modification	2AB
Class	Glycoprotein
Sub class	*N*-glycan (Intact Core)
Biological source	Bovine Fetuin
Pathway	Unknown
Search structure	All
Glycan type	All
% of evident glycosidic linkages	2
Fragmentation pattern	Specify Expected Fragments in the Spectra
Glycosidic	B: Yes; C: Yes; Y:Yes; Z:Yes
Cross-ring	A:Yes; X:Yes
Glycosidic/Glycosidic	Z/Z: Yes; Y/Y: Yes; B/Y or Y/B: Yes; C/Z or Z/C: No; Z/Y or Y/Z: No; B/Z or Z/B: No; C/Y or Y/C: Yes
Cross-ring/Glycosidic	A/Y or Y/A: Yes; A/Z or Z/A:Yes; X/Y or Y/X: Yes; X/Z or Z/X: No; X/B or B/X: Yes; X/C or C/X: Yes

Results and Discussion

The protocol outlined in this application note yields detailed information on the set of glycans present in proteins including mAbs. The protocol describes a fully integrated workflow that combines novel column technology (GlycanPacAXH-1 column), mass spectrometry (Q Exactive mass spectrometer), and a bioinformatics tool (SimGlycan software). This fully integrated workflow is demonstrated for *N*-glycans released from bovine fetuin glycoprotein, but can be used for released *N*-glycans from any glycoprotein.

The GlycanPac AXH-1 column described in this application note can be used for qualitative and quantitative characterization of neutral and charged glycans present on proteins. The elution of glycans is based on charge: the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated to penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. Separation of glycans based on charge, size, and polarity–combined with MS–provides complete structural and quantitative information.

2AB labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer (Figure 1). Data-dependant MS/MS spectra were acquired on all precursor ions ($z \geq 2$), and SimGlycan software was used for structural elucidation. A representative example of the analysis is shown in Figure 2. The Q Exactive mass spectrometer was selected for these experiments because of its 140,000 FWHM resolution at *m/z* 200, high scan speeds at all resolution settings, and sensitivity. All of these contribute to the detection of minor glycan species and generation of highquality MS/MS spectra even for low-abundance glycans.

Additionally, the Q Exactive mass spectrometer has the ability to generate higher-energy collisional dissociation (HCD) with high-resolution, accurate-mass (HR/AM) fragment ions. This allows for differentiation of near-mass fragment ions, which were observed to be useful for correctly assigning branching and linkage. The variation of collision energy can provide different fragment ions within the mass spectrometer. To maximize both glycosidic and cross-ring fragments, normalized stepped collision energy (NSCE) was incorporated. This provided optimum conditions for generation of a maximum number of both cross-ring and glysodic cleavages in a single spectrum, thereby increasing confidence in the identification (Figure 2). The detailed structural information obtained from the MS/MS data shown in Table 2 further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity.

The use of LC-MS/MS for glycan analysis increases the complexity of data analysis due to the large number of MS/MS spectra generated. SimGlycan software was incorporated to simplify data analysis.[6,7] SimGlycan software predicts the structure of a glycan from the MSn data. It accepts the raw MS^n files, matches them with its own database of theoretical fragmentation of over 22,000 glycans, and generates a list of potential glycan structures. Each proposed structure is assigned a score to reflect how closely it matches with the experimental data.

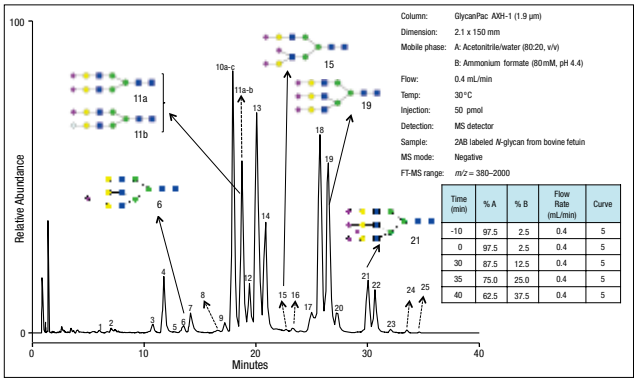


Figure 1. LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by GlycanPac AXH-1 column with MS detection

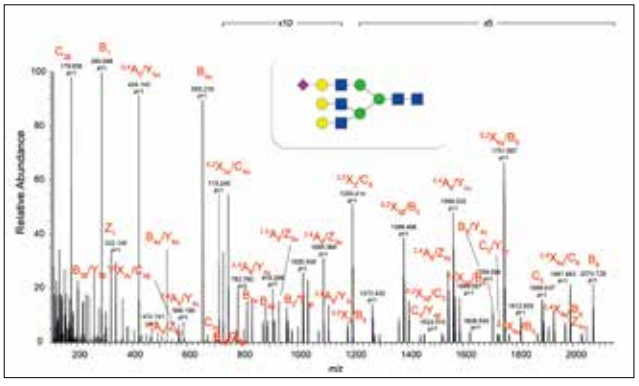
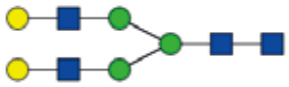
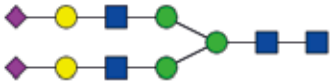
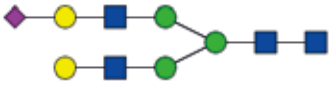
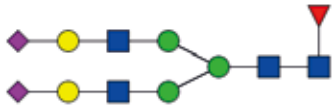
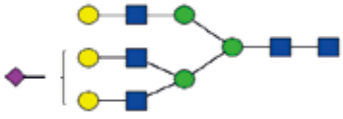
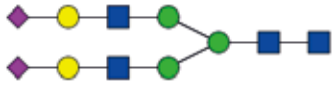
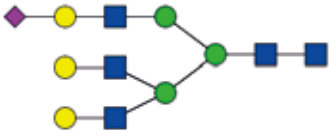


Figure 2. HCD MS/MS spectrum of a 2AB-labeled monosialylated triantennary *N*-glycan from bovine fetuin

Table 2. Structural identification of glycans present in each peak by the separation of 2AB labeled *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure (2AB labeling is not shown)	Peak (Figure 1)	Compound structure (2AB labeling is not shown)
1		8	
2		9	
3		10a	
4		10b	
5		10c	
6		11a	
7		11b	

N-aetyl Glucosamine (**GlcNAc**)

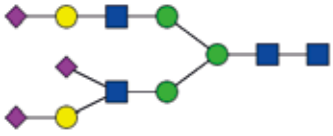
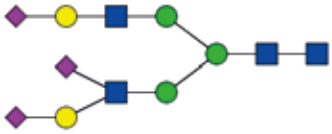
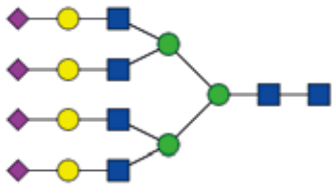
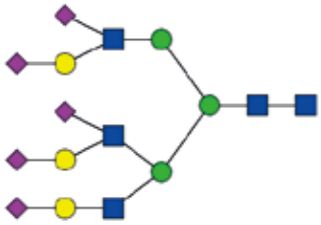
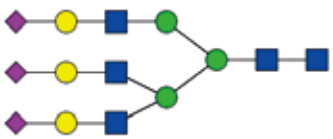
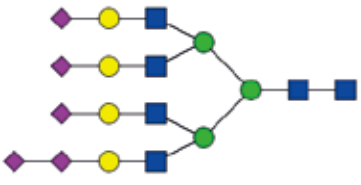
Mannose (**Man**)

Galactose (**Gal**)

N-Aetyl Neuraminic Acid (**Neu5Ac**)

N-Glycolyl Neuraminic Acid (**Neu5Gc**)

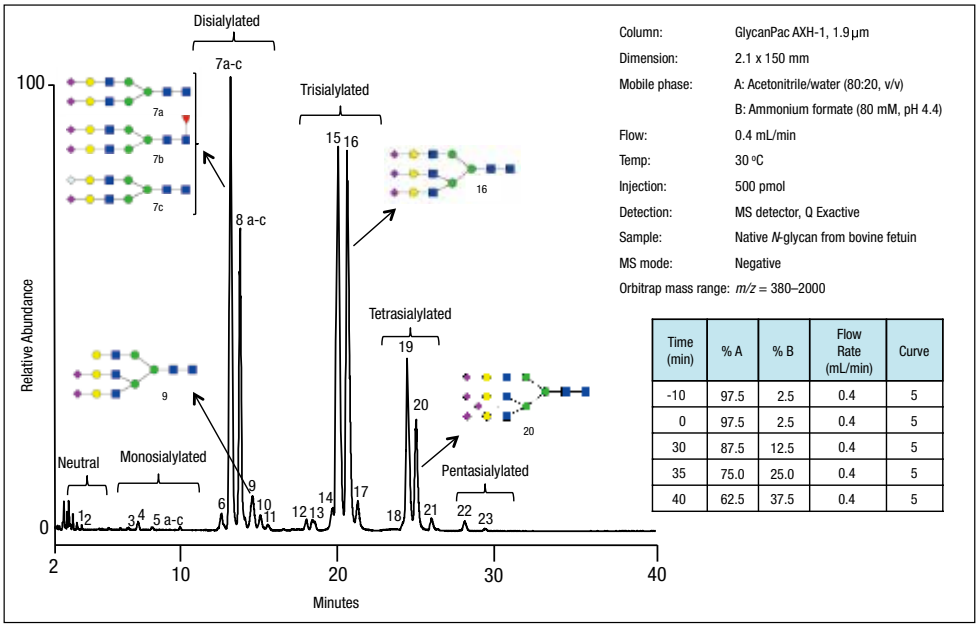
L-Fucose (***L*-Fuc**)

Peak (Figure 1)	Compound structure (2AB labeling is not shown)	Peak (Figure 1)	Compound structure (2AB labeling is not shown)
12		19	
13		20	
14		21	
15		22	
16		23	
17		24	
18		25	

LC-MS Analysis of Native *N*-Glycans Released from Proteins

The GlycanPac AXH-1 column is also suitable for analysis of native glycans. Analyzing unlabeled glycans not only eliminates the extra reaction step and cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Figure 3 shows the LC/MS analysis of native *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column. Detailed information is in Table 3. A representative MS/MS spectrum for a trisialylated triantennary glycan is shown in Figure 4.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 3. LC/MS analysis of native *N*-glycan from bovine fetuin

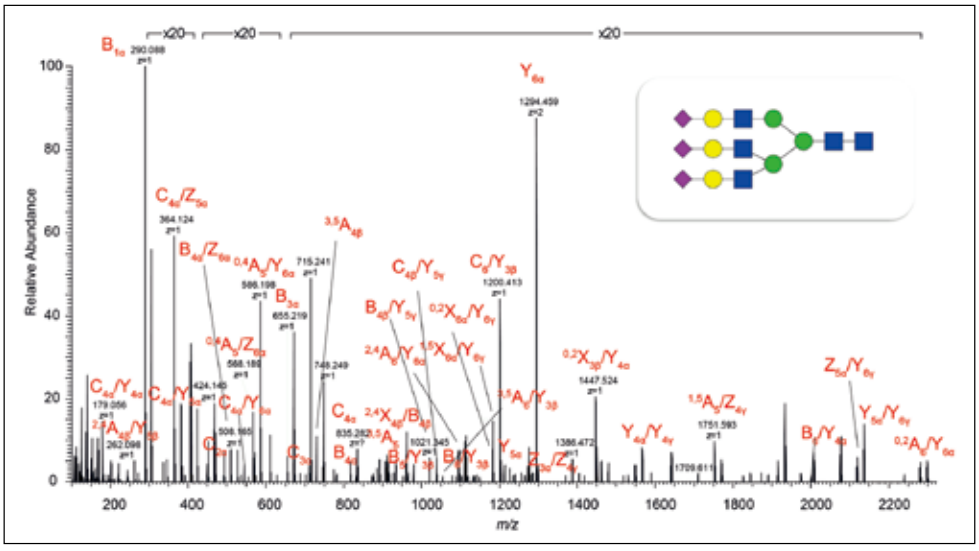
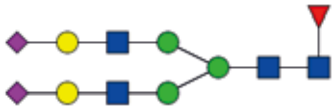
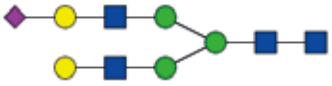
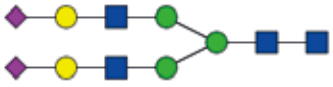
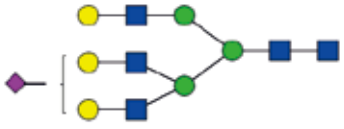
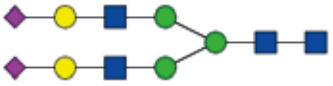
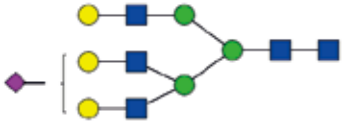
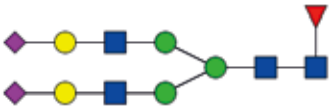
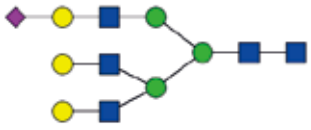
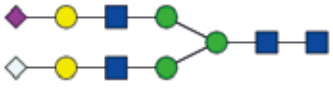


Figure 4. MS/MS spectra for a native trisialylated triantennary *N*-glycan released from bovine fetuin

Table 3. Structural identification of glycans present in each peak by the separation of native *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure	Peak (Figure 1)	Compound structure
1		6	
2		7a	
3		7b	
4		7c	
5a		8a	
5b		8b	
5c		8c	

N-aetyl Glucosamine (**GlcNAc**)

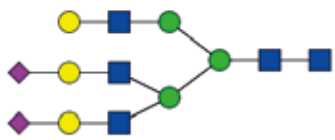
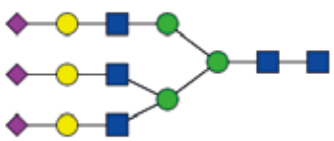
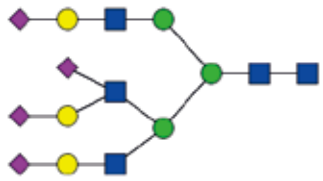
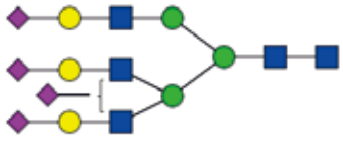
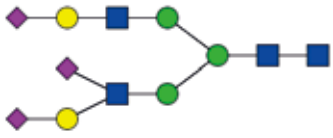
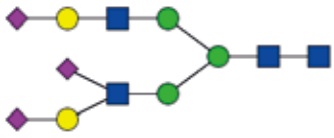
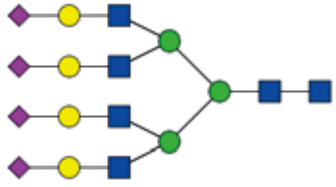
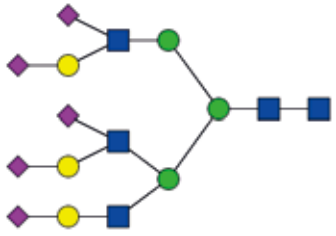
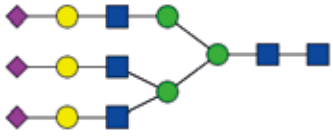
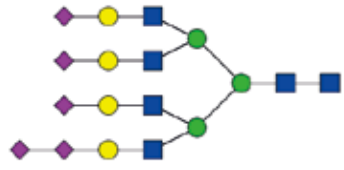
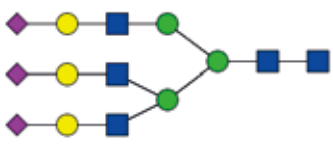
Mannose (**Man**)

Galactose (**Gal**)

N-Aetyl Neuraminic Acid (**Neu5Ac**)

N-Glycolyl Neuraminic Acid (**Neu5Gc**)

L-Fucose (***L*-Fuc**)

Peak (Figure 1)	Compound structure	Peak (Figure 1)	Compound structure
9		17	
10		18	
11		19	
12		20	
13		21	
14		22	
15		23	
16			

Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially for glycans containing multiple sialic acids (Figure 3). However, labeled glycans require smaller amounts (10 times) of samples for MS analysis as compared to native glycans. Thus, the GlycanPac AXH-1 column is useful for the analysis of biologically relevant glycans including glycans from antibodies, either labeled or native, by LC-fluorescence or LC-MS methods. If the amount of the sample is not extremely limited, analysis of unlabeled glycans using the GlycanPac AXH-1 is highly feasible.

Conclusion

- A fully integrated workflow for structural characterization of native and fluorescently labeled *N*-glycans released from proteins was demonstrated successfully.
- Novel GlycanPac AXH-1 column demonstrated excellent separation of released *N*-glycans especially forsilalylated species. It allowed for their sensitive detection by the Q Exactive mass spectrometer and identification by SimGlycan software.
- This LC-MS integrated technology is also useful for the separation and structural characterization of reduced *O*-linked glycans from proteins, mucins, and the analysis of charged and neutral glycosylaminoglycans and glycolipids.

References

1. Varki, A. Biological Roles of Oligosaccharides: All the Theories Are Correct. *Glycobiology* **1993**, 3, 97–130.
2. Bertozzi, C.R.; Freeze, H.H.; Varki, A.; Esko, J.D. Glycans in Biotechnology and the Pharmaceutical Industry, Essentials of Glycobiology, Second Edition; Cold Spring Harbor Laboratory Press: New York, 2009; Chapter 51.
3. Guidance for Industry, Scientific Considerations in Demonstrating Biosimilarity to a Reference Product, Draft Guidance; U.S. Department of Health and Human Services Food and Drug Administration, February 2012 [Online] www.fda.gov/downloads/Drugs/Guidance Compliance Regulatory Information/Guidances/UCM291128.pdf (accessed Jan. 18, 2013).
4. Rohrer, J.S. Monosaccharide analysis of glycoproteins by high-performance anion-exchange chromatography with pulsed amperometric detection (2012) in: Bhattacharyya, L. and Rohrer, J.S. (Eds) Applications of Ion Chromatography in the Analysis of Pharmaceutical and Biological Products, John Wiley and Sons Inc., Hoboken, New Jersey. 2012, pp 339–350.
5. Bigge, J.C.; Patel, T.P.; Bruce, J.A.; Goulding, P.N.; Charles, S.M.; Parekh, R.B. Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid, *Anal. Biochem*. **1995**, *230*, 229–238.
6. Apte, A; Meitei, N.S. Bioinformatics in Glycomics: Glycan Characterization with Mass Spectrometric Data Using SimGlycan. *Methods Mol. Biol*. **2010**, *600*, 269–81.
7. Saba, J.; Apte, A.; Meitei, N.S.; Viner, R., Thermo Scientific Application Note 516: Automated Glycan Structural Isomer Differentiation Using SimGlycan Software.

Characterization of Protein Glycosylation Using ESI Chip™ Static Nanospray IonTrap MS^n Mass Spectrometry

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Introduction

The accurate identification of glycopeptides is challenging because of their complex structures, labile nature, and microheterogeneity. A variety of mass spectrometry based techniques for determining the structure of glycopeptides have been used, including Time of Flight (TOF), triple quadrupole, and ion trap, coupled with a variety of ionization techniques including fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI). This application report describes the use of a static nanospray ionization technique in combination with a novel linear ion trap (Thermo Scientific LTQ) for identification and characterization of glycosylation in complex mixtures.

Goal

Determine in a single experiment the complete structure of glycosylation group(s) attached to the protein, the amino acid sequence of the glycosylated peptide(s), and the exact site(s) of the glycosylation linkage using MS^n techniques.

Experimental Conditions

Reduction, alkylation, and proteolytic digestion

One milligram of ribonuclease B (Sigma, St. Louis, MO) was reconstituted in 100 µL of solution containing 20 mM Tris-HCl pH 7.8, 6 M guanidine-HCl and 10 mM DTT. The mixture was incubated for 30 minutes at 50℃ , and 50 µL of 0.2 M iodoacetamide and 50 µL of 0.2 M ammonium bicarbonate pH 7.8 were added. The mixture was incubated at room temperature in the dark for two hours. The alkylated solution was dialyzed against 20 mM ammonium bicarbonate pH 7.8 overnight at 4 ℃ using a Slide-A-Lyzer® MINI Dialysis Unit. The dialyzed protein was digested by adding a 1:60 ratio (enzyme:sample) of a proteolytic enzyme (Promega, Madison, WI) at a concentration of 0.52 mg/mL in 50 mM ammonium bicarbonate pH 7.8 and incubated overnight at 37℃ .

Sample Analysis

Samples were introduced into the LTQ using the NanoMate™ 100 mounted in front of the LTQ and 5 µL samples (at 1 pmol/µL in 50% methanol; 0.1% formic acid) were infused at a flow rate of 100 nL/min.

MS Spectrometry

Mass Spectrometer: LTQ run in tune mode
Ionization Mode: Nano-electrospray
Ion Polarity: Positive
Spray voltage: 1.55 kV
Spray pressure: 0.2 psi
Capillary temperature: 150℃
Normalized Collision Energy™: 20–25% for MS^n experiments
Maximum scan time: 50 ms
Number of micro scans summed for each scan: 2–3

Results and Discussion

The proteolytic digest of bovine pancreatic ribonuclease B (1 pmol/µL) was analyzed using

chip-based static nanospray mass spectrometry as described in the experimental section. Tandem mass spectrometry was performed on all of the major peaks, and additional fragmentation (MS^n) was recorded manually. The full-scan mass spectrum (Figure 1) shows the complex peptide mass fingerprint of ribonuclease B. Several peptides were identified using fragmentation information from MS/MS spectra using the Thermo Scientific BioWorks™ 3.1 software package. The software correlates theoretical MS/MS data from a database with actual observed spectra for identification of proteins (Eng J.K, McCormack A.L, and Yates J.R.I. *J. Am. Soc. Mass Spectrom*. 1994). The identified peptides and the protein coverage are shown in Table 1. Nine out of fourteen possible proteolytic peptides were detected, resulting in over 87% coverage. The software did not identify several major peaks, although the fragmentation pattern of those peaks appeared to be of high quality. The unidentified peaks show a typical pattern for high mannose-type glycopeptides with mass shifts of 81 Da for doubly charged ions. Collision-induced fragmentation of the unidentified peaks (Figures 2 a-e) confirms the presence of high mannose-type glycosylation by generating doubly charged fragment ions that differ by 81 Da. Additionally, the presence of N-acetyl-D-glucosamine can be easily detected in the tandem mass spectra based on the marker ion at 204 Da. Assuming complete proteolytic digestion, the only possible site for N-linked glycosolation in ribonuclease B is the peptide NLTK. The molecular weight information of the unidentified peaks, together with the molecular weight of the amino acid sequence and the fragmentation information, confirms the presence of five different high mannose type glycopeptides in ribonuclease B. As shown in Figures 3 a-c, the MS^n capability of the LTQ was used to characterize the nature and extent of the glycosylation. Sequential MS/MS experiments allow the sequential linkage of the oligosaccharide units to be determined. The large ion populations trapped by the LTQ facilitate acquisition of high quality tandem mass spectra of up to MS^5 and enables complete characterization of the carbohydrate structure.

Since glycopeptides tend to fragment first within the saccharide portion of the glycopeptide, it is often difficult to determine the peptide sequence and the site of oligosaccharide attachment. The need for high sensitivity MS^n is clearly shown in Figure 4, where an MS^4 experiment enables the peptide sequence to be determined.

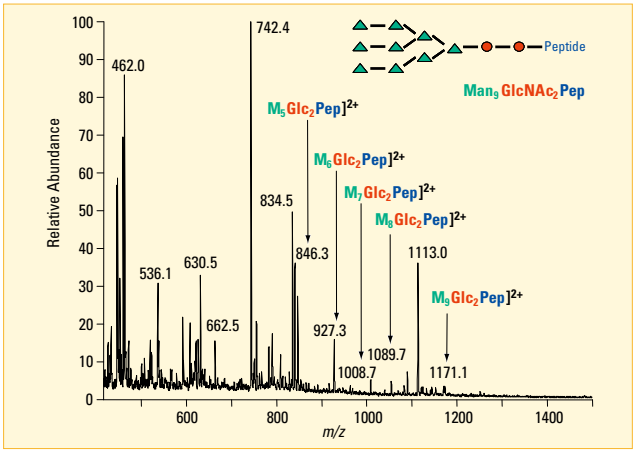


Figure 1. Full scan mass spectrum of the proteolytic digest of pancreatic ribonuclease B at a concentration of 1 pmol/mL as described in the experimental section

Table 1. Amino acid sequence and the predicted peptides from a proteolytic enzyme digestion of bovine ribonuclease B. Identified peptides are shown in bold; N-glycosylation site is shown in red

ETAAAK FER **QHMDSSTSAASSSNYCNQMMK** SR **NLTK**

DR CK **PVNTFVHESLADVQAVCSQK NVACK**

NGQTNCYQSYSTMSITDCR ETGSSK YPNCAYK TTQANK

HIIVACEGNPYVPVHFDASV

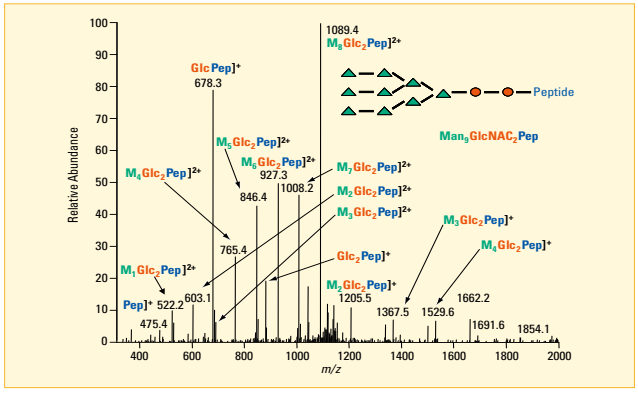


Figure 2a. Tandem mass spectra derived by collisioninduced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 1171.1

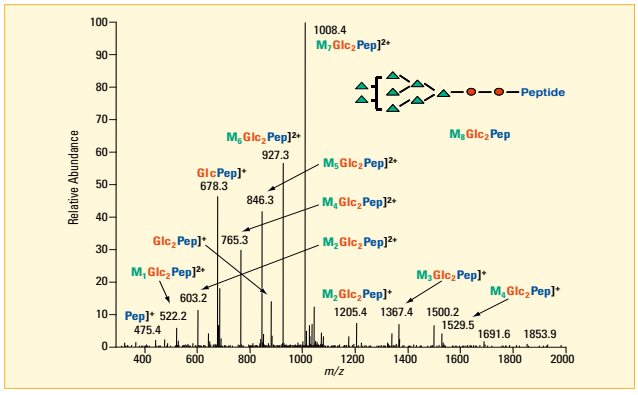


Figure 2b. Tandem mass spectra derived by collisioninduced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 1090.8

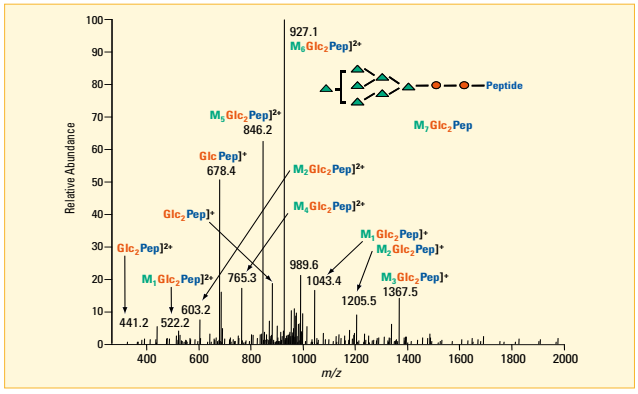


Figure 2c. Tandem mass spectra derived by collisioninduced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 1008.4

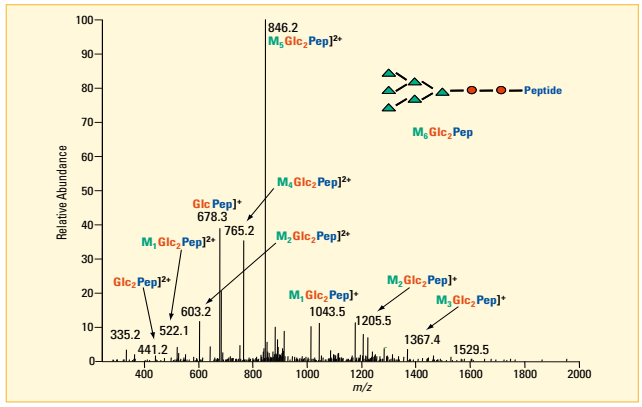


Figure 2d. Tandem mass spectra derived by collisioninduced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 927.3

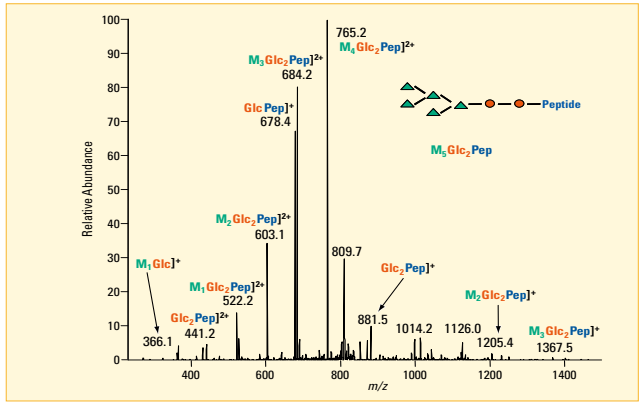


Figure 2e. Tandem mass spectra derived by collisioninduced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 846.0

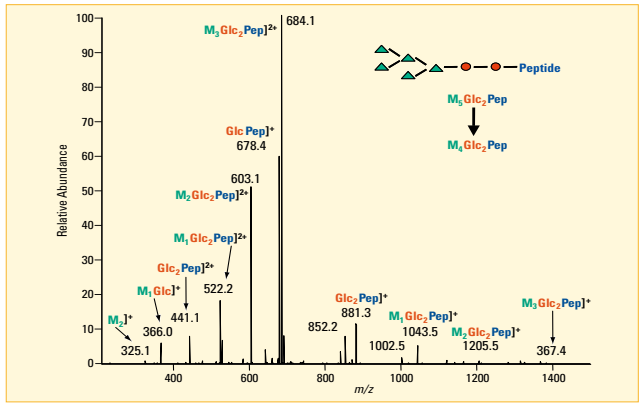


Figure 3a. MS^{3-5} mass spectra derived by collisioninduced fragmentation of $(M+2H)^{2+}$, m/z =864,0 → 765.0, for the stepwise removal of terminal mannose residues

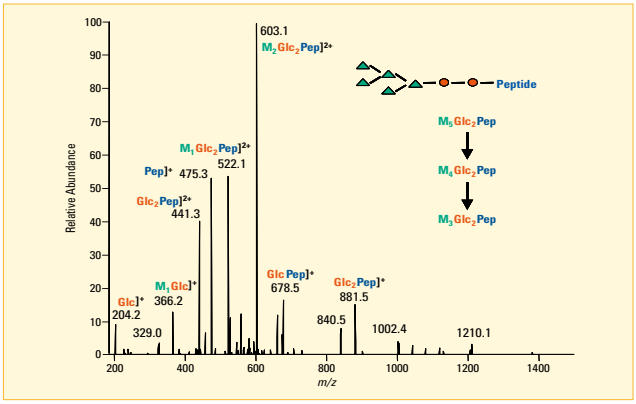


Figure 3b. MS^{3-5} mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, *m/z* = 864,0 → 765.0 → 684.0, for the stepwise removal of terminal mannose residues

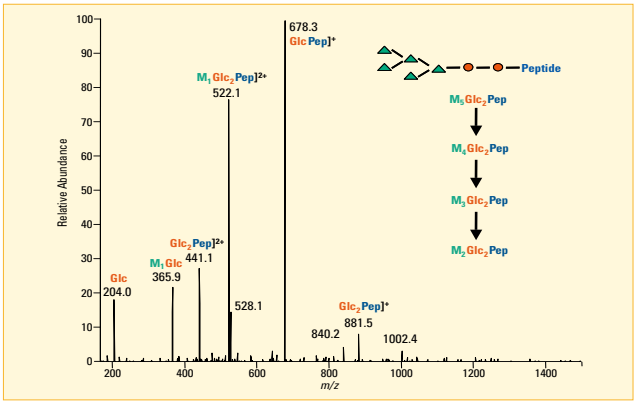


Figure 3c. MS^{3-5} mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, *m/z* = 864,0 → 765.0 → 684.0 → 603.0 for the stepwise removal of terminal mannose residues

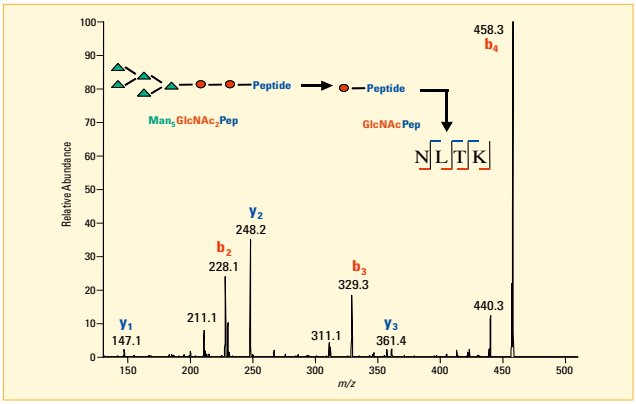


Figure 4. MS^4 mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, *m/z* = 864,0 → 765.0 → 475 for the identification of the amino acid sequence of the ribonuclease glycopeptide

Conclusion

The LTQ ion trap mass spectrometer has the sensitivity and scan functionality for the analysis of glycoproteins. Using the unique MS/MS and MS^n functions, combined with BioWorks protein identification software, the glycosylated peptide was identified and the sugar structure was determined. The MS^4 spectra provided the additional information of the site of sugar attachment at the peptide backbone. The Advion NanoMate allows full automation of the nanospray process, resulting in excellent reproducibility while minimizing sample consumption and eliminating cross-contamination.

新一代组合型质谱 LTQ-Orbitrap Elite 用于复杂糖蛋白完整糖肽结构解析

张伟 赛默飞世尔科技
谭青乔 中信国健药业

1. 前言

糖基化修饰是生命活动中最广泛、最重要的蛋白质翻译后修饰之一，不仅影响着蛋白质的空间构象、生物活性、运输和定位，而且在分子识别、细胞通信、信号转导等特定生物过程中发挥着至关重要的作用。糖蛋白根据其糖链结构及糖基化位点主要有 N- 糖蛋白与 O- 糖蛋白两大类。据推断，有超过 50% 的蛋白质都发生了糖基化修饰，但由于糖基化的高度复杂性，绝大多数糖蛋白尚未被发现，现有数据库中只有约 10% 的蛋白质被注释为糖蛋白 [1]。首先，糖链的组成与结构非常复杂，糖链为非模板合成且呈二维结构，糖苷键连接位置和构象的不同都会形成差异的精细结构。据报道，仅仅由 6 个不同单糖组成的寡糖链，其结构就可能达到惊人的 10^{12} 种 [2]。其次，当组成与结构各异的糖链连接在蛋白质上形成糖蛋白时，又构成微观不均一性，即糖基化位点上糖链结构多样化的问题，一个蛋白可能存在多个糖基化位点，而每个位点上又可能存在多种结构的糖链 [3]。

随着以单抗为代表的糖蛋白药物的开发，质谱已广泛应用于糖基化解析，通常先利用酶切或化学手段将糖链从糖蛋白释放，再分别进行糖基化位点的鉴定和糖链结构的解析 [4]。然而针对完整糖肽的解析，目前尚无成熟技术。本文利用新一代组合型质谱 LTQ-Orbitrap Elite 具有的 HCD/ETD “双碎裂模式”，解析一种复杂糖蛋白的完整糖肽，利用最新 Byonic 与 SimGlycan 软件解析，成功发现 2 个 N- 糖基化位点，16 个 O- 糖基化位点，83 种位点特异性糖链组成与结构信息，在未脱糖状态下使蛋白序列覆盖度高达 92.1%。

2. 实验部分

2.1 样品信息

样品为某种重组表达的高度糖基化蛋白，前处理使用二硫苏糖醇（DTT）、碘乙酸（IAA）对二硫键还原烷基化，胰蛋白酶（Trypsin）酶解蛋白，最终浓度为 1.5 μg/μL。

2.2 液相色谱方法

色谱柱：纳流速 C_{18} (2 μm, 100 Å, 75 μm × 50 cm)，常规流速 HILIC-NH_2 (3 μm, 100 Å, 2.0 mm × 15 cm)
色谱仪：C18 柱使用纳流速液相 Easy-nLC 1000，HILIC-NH_2 柱使用常规流速液相 Accela 600
上样量：2 μL
流动相：A: 0.1% 甲酸水溶液，B: 0.1% 甲酸乙腈溶液
梯度：C_{18}: 3%-90% B, 120 min, 250 nL/min; HILIC-NH2: 95%-5% B,120 min, 200 μL/min

2.3 质谱方法

质谱仪：LTQ-Orbitrap Elite 组合型高分辨质谱仪
离子源：
Easy-nLC 1000 使用 EASY-Spray 纳流电喷雾离子源：喷雾电压 2.3 kV，传输毛细管温度：275℃，S-Lens：60%；
Accela 600 使用 HESI-II 加热电喷雾离子源：喷雾电压 3.5 kV，
鞘气：30，辅助气：10，传输毛细管温度：275℃，SLens：60%
扫描方式：数据依赖性扫描：一级全扫描（分辨率 60,000），Top15 二级全扫描（分辨率 15,000）；扫描范围：母离子 m/z 400-3500，子离子 m/z 100-3500；动态排除：时间 30s，排除范围 m/z -0.5~+1.5
碎裂模式：高能碰撞诱导解离 HCD（NCE 35%），电子转移解离 ETD（100 ms for 2+）

2.4 数据分析

数据使用 Byonic 软件搜库鉴定糖肽 / 非糖肽，具体参数

为：酶：trypsin (KR)；专一性：全酶切；漏切位点：2；母离子质量精度：15 ppm；子离子质量精度：20 mD；固定修饰：C+58.005；可变修饰：M+15.995；糖基化修饰：N, O；肽段卡值标准：Score>150，Delta Mod>5，Log Probability<-1；糖基化位点卡值标准：Score>190，Delta Mod>10，Log Probability<-2。SEQUEST 搜库参数相同，卡值标准为 5% FDR。糖肽糖链部分结构使用 SimGlycan 软件解析。

3. 结果与讨论

3.1 分析策略

实验整体策略见图 1。本实验采用 nano-C_{18} 与氨基 HILIC 两种分离方式分别对样本进行色谱分离。C_{18} 是常用的蛋白 / 肽段分离色谱柱，而纳流速的 C_{18} 柱使被分析物的单位浓度显著提高，灵敏度达到最佳，能够有效分析低丰度 / 低离子化效率的肽段。氨基 HILIC 属亲水色谱，根据寡糖 / 聚糖组成和结构进行分离，是糖链结构解析、糖组学研究的有效工具。

同时，由于目标蛋白的糖基化高度复杂，本实验同时采用 HCD 与 ETD 两种碎裂模式进行质谱采集。HCD 倾向于碎裂低电荷 / 低分子量的肽段，存在糖基化修饰时，优先碎裂单糖之间的糖苷键，同时高能碎裂能够获得更充分的碎片信息。ETD 倾向于碎裂高电荷 / 高分子量的肽段，存在糖基化修饰时，优先碎裂肽段骨架的酰胺键。因此，HCD 与 ETD 具有良好的互补性，两者结合可以有效应用于完整糖肽的解析。

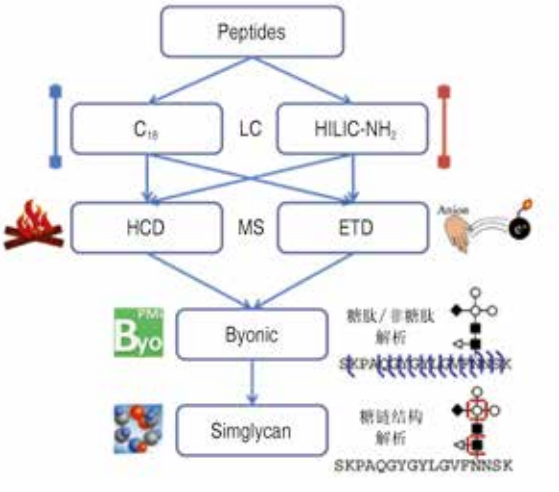


图 1. 糖肽解析流程图

糖肽谱图同时含有肽骨架与糖链的碎片信息，因此质谱数据的解析也是分析难点。Byonic 是专业的糖肽解析软件，采用创新的“Preview”算法和丰富的糖链组成数据库，实现对肽段、糖基化位点的鉴定，并根据精确质量数给出糖链组成。SimGlycan 是专业的糖链结构解析软件，含有 9650 种糖链的理论碎片信息，是目前最大的商品化糖数据库。本实验先使用 Byonic 对数据进行序列鉴定，获得糖基化位点与糖链的单糖组成信息，再使用 SimGlycan 对数据进行糖链结构解析，最终获得糖肽序列、糖基化位点与位点特异的糖链结构。

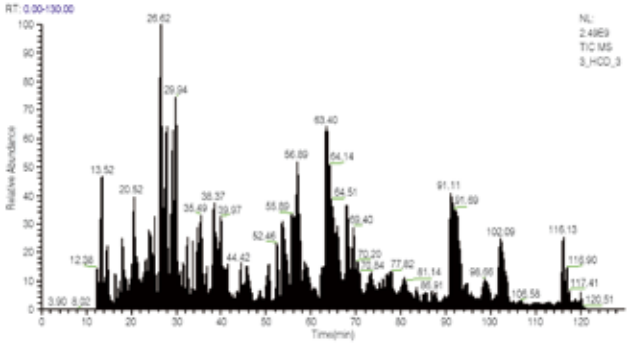


图 2. 样本 LC-MS/MS 总离子流图（TIC）

3.2 序列鉴定

图 2 展示了样本经 nano-C_{18}+HCD 流程分析获得的 TIC 图，色谱峰分布均匀，达到理想分离效果，质谱响应达 9 次方。所有质谱数据经 Byonic 进行序列鉴定，蛋白序列覆盖度达 92.1%，而使用传统算法 SEQUEST 进行搜库，序列覆盖度只有 76.6%（图 3）。

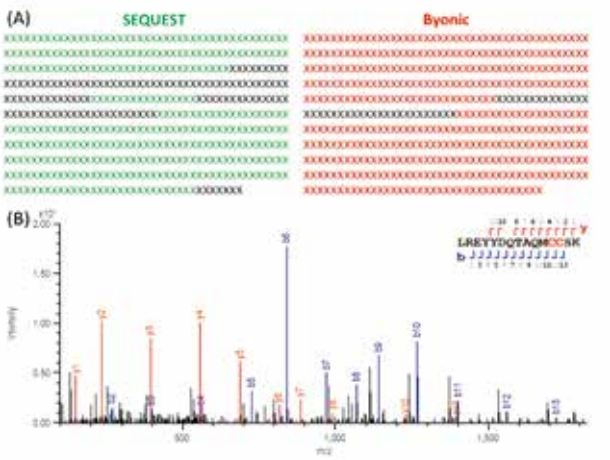


图 3. (A) SEQUEST/Byonic 序列鉴定覆盖度对比；(B) Byonic 对肽段二级谱图匹配

SEQUEST 算法无法鉴定完整糖肽，只能根据未发生糖基化的肽段分析序列，一些完全发生糖基化的肽段难以解析。相反，Byonic 鉴定时根据其糖链数据库，将糖基化作为可变修饰，因而能够直接解析糖肽。因此，Byonic 解析糖蛋白序列覆盖度显著提高。

3.3 糖肽解析

在上述 Byonic 鉴定结果中，共包含 28 条糖肽，对应 18 个糖基化位点，其中，N- 糖基化位点 2 个，O- 糖基化位点 16 个；同时，共获得位点特异的糖链组成 83 种，对应到每个位点最多 17 种，最少 1 种，平均每个位点 5.2 种。HCD 与 ETD 两种碎裂模式显示出优异的互补性，使解析结果明显增多（图 4）。

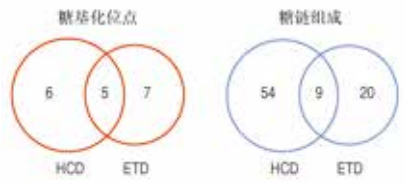


图 4. HCD/ETD 碎裂获得的糖肽解析结果比较

通过比较 TKPREEQYNSTYR 这条 N- 糖肽（N317）的质谱谱图（图 5）可以看出，ETD 与 HCD 都成功鉴定到这条糖肽，而 ETD 所获得的碎片信息（c/z）更加丰富，位点同时被 c、z 离子覆盖。相反，HCD 所获得的碎片信息（b/y）明显减少，位点没有被碎片离子覆盖。但值得注意的是，HCD 谱图的高分子量端出现显著的簇峰，峰之间相差 162 Da（Δm/z 81, 2+）或 203 Da（Δm/z 101.5, 2+），表明糖肽的糖链部分发生碎裂。

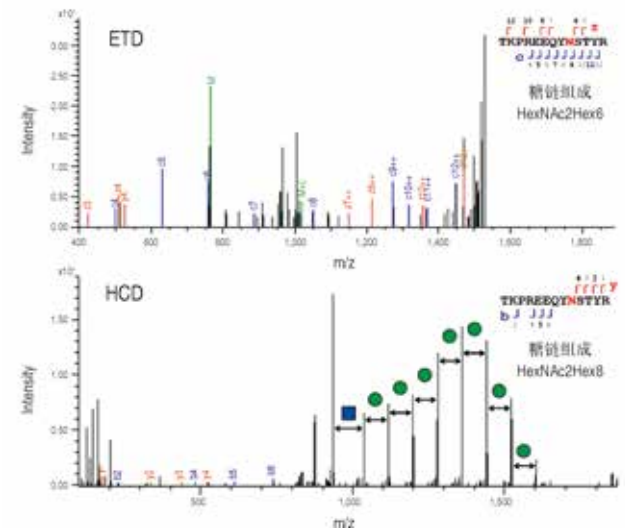


图 5. 糖肽 TKPREEQYNSTYR 的 HCD/ETD 碎裂谱图解析

ETD 倾向于碎裂较强的肽骨架氨基酸之间的酰胺键，保留修饰基团，因此该糖肽在 ETD 中完整保留了糖链，获得较为充分的肽骨架碎片（c/z 系列），能够更加有效鉴定肽段。HCD 倾向于碎裂较弱的翻译后修饰基团，造成该糖肽的糖链在 HCD 模式下发生显著碎裂，呈现一系列相隔一个单糖的碎片峰，而肽骨架的碎裂被显著抑制，b/y 系列离子不充分且信号较弱；同时，带有完整糖链的碎片难以产生，因此无法得到包含位点 N 的 b/y 碎片信息。

对于糖链较小的 O- 糖肽，HCD 对糖链的影响没有 N- 糖肽那么大，而且 ETD 碎裂的信号响应（10^3）明显要弱于 HCD（10^4），因此综合而言，两者鉴定到的糖肽和位点数量相当，展现了优异的互补性。

3.4 位点特异性糖链结构解析

Byonic 不考虑糖链碎片信息，只根据肽段碎片的精确分子量搜库得到糖链的组成信息，本实验进一步使用 SimGlycan 分析糖链具体结构。SimGlycan 根据糖链碎片信息解析糖链结构，因此在解析具体结构的同时，也从另一方面对 Byonic 的鉴定结果进行了验证，使最终结果更加可信。

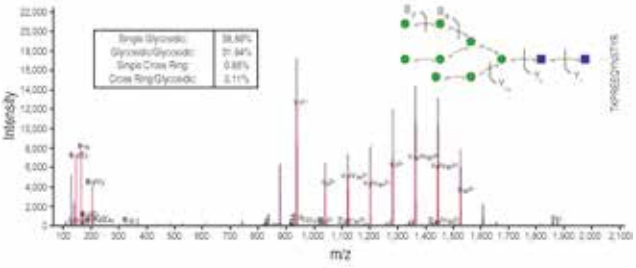
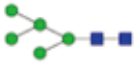
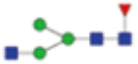
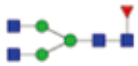
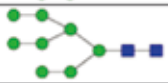


图 6. 糖肽 TKPREEQYNSTYR 的 HCD 碎裂谱图糖链解析

本实验对所有鉴定到 N317 糖基化位点谱图进行 SimGlycan 解析，最终 75.6% 的谱图得到验证，相应 93.8% 的糖链获得结构信息。如表 1 所示，N317 位点共有 15 种糖链结构，主要为高甘露糖型与双链复杂型 N- 糖链，还包含了核心岩藻糖、唾液酸等重要结构。图 6 进一步展示了图 5 中 HCD 谱图的糖链结构解析结果及部分碎片匹配图，充分的糖链碎片离子信息表明 Hex2HexNAc8 的结构为三分支的高甘露糖型。

表 1. N317 位点的糖链结构

组成 NHFAG	结构	组成 NHFAG	结构
21000		34000	
23000		44000	
25000		33100	
26000		43100	
27000		34100	
28000		44100	
33000		45110	
43000			N:HexNAc; H:Hex; F:Fuc; A:NeuAc; G:NeuGc

另外，糖链存在诸多同分异构体，特别是组成一致，连接方式不同的精细结构差异，仅靠二级碎片难以区分。当然，LTQ-Orbitrap Elite 所具有的多级解析能力（MS^n），同样能够针对糖链实现有效的精细结构解析。

4. 结论

本文使用新一代组合型质谱 LTQ-Orbitrap Elite，利用技术领先的 HCD/ETD“双碎裂模式”，结合先进的糖基化解析软件 Byonic 与 Simglycan，成功解析了一种高度糖基化蛋白的完整糖肽。实验共获得 2 个 N- 糖基化位点，16 个 O- 糖基化位点，83 种位点特异性糖链组成与结构，在未脱糖状态下蛋白序列覆盖度高达 92.1%，最大化地实现了复杂糖蛋白的高效解析。技术领先的 LTQ-Orbitrap Elite 是糖组学、蛋白质组学、生物制药领域复杂大分子解析的强大工具。

参考文献：

[1] Apweiler, R.; Hermjakob, H.; Sharon, N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database [J]. Biochim. Biophys. Acta 1999, 1473 (1): 4-8.

[2] Morelle, W.; Canis, K.; Chirat, F.; Faid, V.; Michalski, J. C. The use of mass spectrometry for the proteomic analysis of glycosylation [J]. Proteomics 2006, 6 (14): 3993-4015.

[3] 代景泉，蔡耘，钱小红；蛋白质糖基化分析方法及其在蛋白质组学中的应用 [J]. 生物技术通讯 2005, 16 (3): 287-292.

[4] Geyer, H.; Geyer, R. Strategies for analysis of glycoprotein glycosylation [J]. Biochim. Biophys. Acta 2006, 1764 (12): 1853-1869.

An Ultra High Resolution Glycan Column for Isomeric Separation and the Structural Identification of Labeled *N*-Glycans from Proteins Including Antibodies

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Overview

Purpose: Separation and identification of various complex *N*-linked glycans from proteins using a novel high resolution mixed-mode column and an Orbitrap Fusion Tribrid mass spectrometer.

Methods: Fluorescently labeled glycans from various proteins were separated and analyzed on a Thermo Scientific™ GlycanPac™ AXR-1 column coupled to a Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ mass spectrometer. Data analysis was performed using SimGlycan® software.

Results: The GlycanPac AXR-1 column coupled to Orbitrap Fusion mass spectrometer enabled resolution of twice as many peaks and identification of four times as many structures for 2AB-labeled *N*-linked glycans from bovine fetuin compared to other commercially available column technologies. Furthermore, this column allows direct injection of aqueous samples.

Introduction

Glycans are involved in a wide range of biological and physiological processes including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development. The functions of glycans are often dependent on the structure and types of glycans attached to the proteins. N-linked glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are affected by glycosylation. Understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, the glycan content of glycoprotein products, as well as thorough characterization of biosimilars have become increasingly important. The structures of glycans are highly diverse, complex and heterogeneous due to posttranslational modifications. Thus, it is challenging to comprehensively characterize glycan profiles and determine their structures [1].

Various modes of HPLC separation have been developed for the analysis of glycans [2-4]. Glycans are highly hydrophilic polar substances, and therefore one common separation mode utilizes amide hydrophilic interaction liquid chromatography (HILIC columns), which separates glycans based on hydrogen bonding, resulting in a size and composition-based separation. Amide HILIC columns are particularly useful for the separation of 2AA-labeled N-linked glycans released from antibodies, such as mAbs, where the majority of the glycans are neutral. However, amide HILIC amide columns do not provide a good separation when glycans are highly charged (charge ≥ 2) such as sialylated *N*-linked glycans. Here glycans of different charge states are intermingled in the separation envelope.

Recently, we have developed a novel mixed-mode column (GlycanPac AXH-1) with both weak anion-exchange (WAX) and HILIC properties [5], which separate N-linked glycans based on charge, size, and polarity. The GlycanPac AXH-1 column provides unique charge-based separation and broader applicability in term of qualitative and quantitative structural analysis of 2AB and 2AA labeled as well as native N-linked glycans from proteins by both fluorescence and mass spectrometry (MS) detection [6, 7]. Here we describe the new GlycanPac AXR-1 mixed-mode column which provides an even higher resolution separation based on isomeric structure along with separation based on charge, size, and polarity.

The GlycanPac AXR-1 column is based on novel mixed-mode column chemistry, combining both WAX and reversed-phase (RP) retention mechanisms for optimal selectivity and high resolving power. The WAX functionality provides retention and selectivity for negatively charged glycans, while the reversed-phase mode facilitates the separation of glycans of the same charge according to their isomeric structure, polarity, and size. As a result, the GlycanPac AXR-1 column provides exceptional resolution with more than 4 times the glycan structures identified compared to existing commercial columns, including amide HILIC columns and the GlycanPac AXH-1 column, for 2AB-labeled *N*-linked glycans released from bovine fetuin. The GlycanPac AXR-1 column is designed for HPLC and UHPLC methods using either fluorescence or MS detection, and uses volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile, presenting the eluting glycans ready for introduction into MS instruments.

Methods

Sample Preparation

Glycans were released from glycoproteins with PNGase F enzyme (New England BioLabs). The released glycans were labeled with 2-aminobenzamide (2AB) and 2-amino benzoic acid (2AA) with slight modification from the reported procedure of Bigge et. al.,[8] Prior to analysis, samples were dissolved in 100 µL D.I. water in a 250 µL auto sampler vial.

Liquid Chromatography

All glycans were separated on a GlycanPac AXR-1 column (1.9 µm, 2.1 × 150 mm) by a Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC instrument with either a fluorescence or MS detector.

Mass Spectrometry

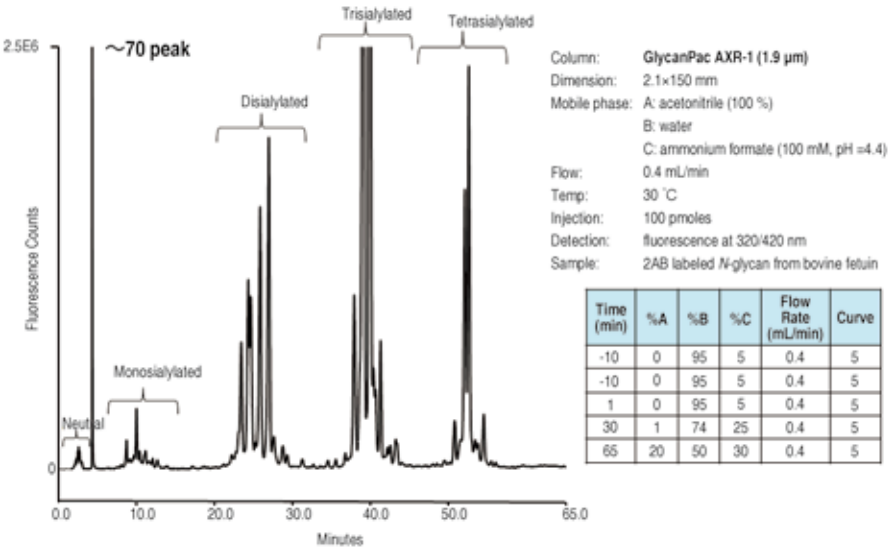
MS analysis was performed using an Orbitrap Fusion Tribrid mass spectrometer in negative ion mode. LC-MS^2 and LC-MS^3 experiments were conducted for structural elucidation.

Data Analysis

SimGlycan® 4.5 software (PREMIER Biosoft) was used for MS/MS data analysis.

Results

The GlycanPac AXR-1 column is designed for high-resolution separation of neutral and charged glycans (native and labeled) present in glycoproteins, glycolipids and glycopolymers. However, it should be noted that for neutral glycans, such as those released from antibodies, it is advantageous to use 2AA-labeling technique to enhance retention as well as selectivity on the GlycanPac AXR-1 column. Figure 1 shows the separation of neutral and acidic 2AB-labeled N-linked glycan from bovine fetuin using a GlycanPac AXR-1 (1.9 µm, 2.1 × 150 mm) column. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, close to the void, followed by monosialylated, disialylated, trisialylated, tetrasialylated,and finally pentasialylated species. Peaks in each cluster represent the glycans of the same charge separated by ion exchange interaction. Within each cluster, glycans containing the same charge are further separated according to their isomeric structures, sizes, and polarity by reversed-phase interaction. The GlycanPac AXR-1 (1.9 µm) column provides ≥ 70 resolved peaks with ≥ 1% intensity for 2AB-labeled N-linked glycans from bovine fetuin. *N*-linked glycan structures present in each peak were identified using LC-MS^2 and LC-MS^3 data.



Time (min)	%A	%B	%C	Flow Rate (mL/min)	Curve
-10	0	95	5	0.4	5
-10	0	95	5	0.4	5
1	0	95	5	0.4	5
30	1	74	25	0.4	5
65	20	50	30	0.4	5

FIGURE 1. Separation of 2AB-labeled *N*-linked glycans from bovine fetuin by charge, size, polarity and isomeric structure using GlycanPac AXR-1 (1. 9µm) column by ternary gradient condition.

LC-MS/MS Analysis of 2AB-Labeled *N*-linked Glycans from Bovine Fetuin using GlycanPac AXR-1 Column

The coupling of the GlycanPac AXR-1 column to MS is particularly attractive because MS enables in-depth analysis of complex glycans due to its ability to provide structural information. 2AB-labeled N-linked glycans from bovine fetuin were separated on the GlycanPac AXR-1 column and analyzed on an Orbitrap Fusion mass spectrometer.

The LC-MS profile of the GlycanPac AXR-1 column showed the highest number of resolved peaks (≥ 70) for bovine fetuin glycans ever achieved (Figure 2), more than doubling the number the existing commercially available stationary phases can resolve. The commercially available HILIC amide column (1.7 µm, Figure 2) was only able to resolve 26 peaks. Most commercial stationary phases are poor for separating glycan structural isomers. A single LC peak using these columns can have many structural isomers. So in most instances mixed MS^2 spectrum are generated that contain fragment ions from multiple glycans making it extremely difficult to assign correct structures. The GlycanPac AXR-1 column can resolve structural isomers (Figure 4). The ability to resolve structural isomers introduces complexity to analysis. Namely, far more MS/MS spectra need to be triggered in a single LC-MS^2 analysis. Additionally, wider dynamic range and sensitivity are needed from MS to detect and generate good quality MS^2 spectra not only for the most abundant glycans but the low abundant species as well (Figure 5). Orbitrap Fusion with it'-s wide dynamic range and ultrahigh mass resolution of makes it the ideal platform for looking deeper into the glycome and confidently identifying low-abundance glycans. Overall, 135 unique glycan structures were identified using a combination of GlycanPac AXR-1 column and Orbitrap Fusion

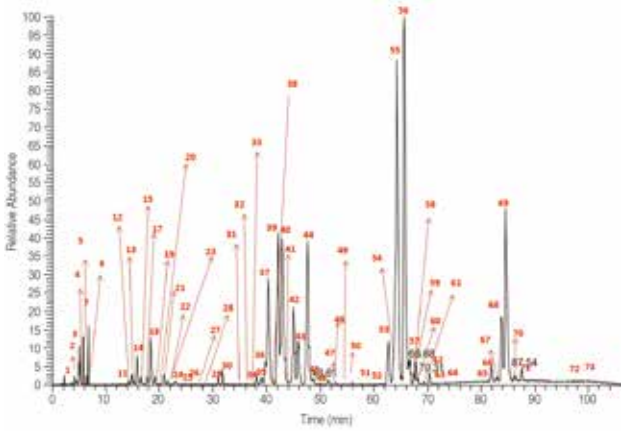


FIGURE 2. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by GlycanPac AXR-1 column with MS detection.

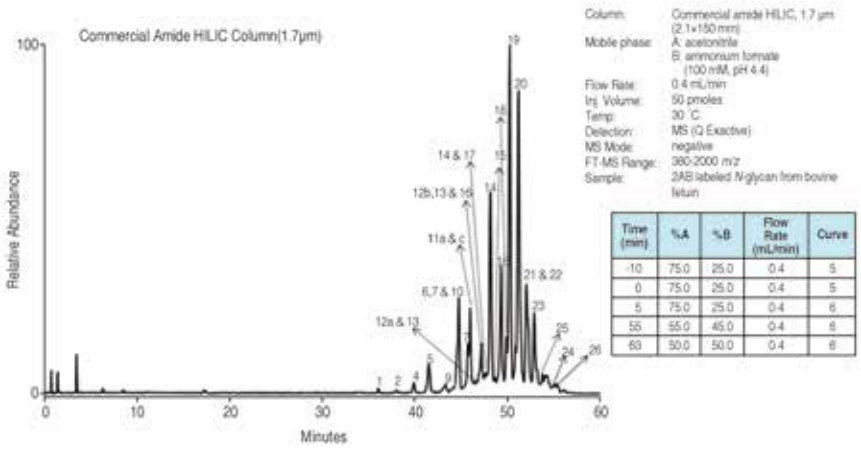


FIGURE 3. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by a commercial amide HILIC column (1.7 µm) with MS detection.

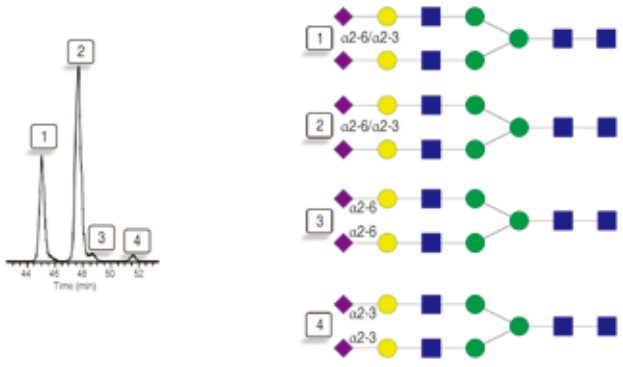


FIGURE 4. Separation of disialylated biantennary glycans from bovine fetuin based on α2-3 and α2-6 sialic acid linkage by the GlycanPac AXR-1 column.

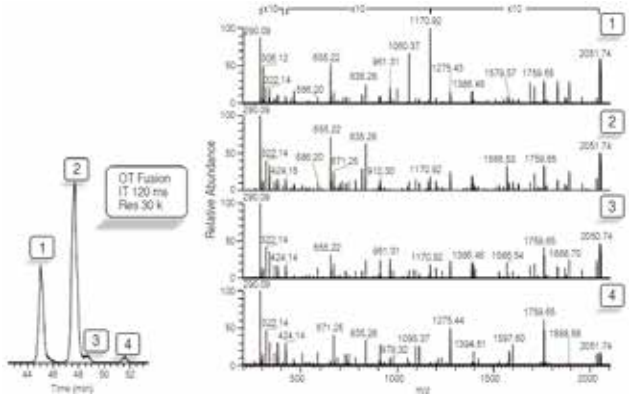
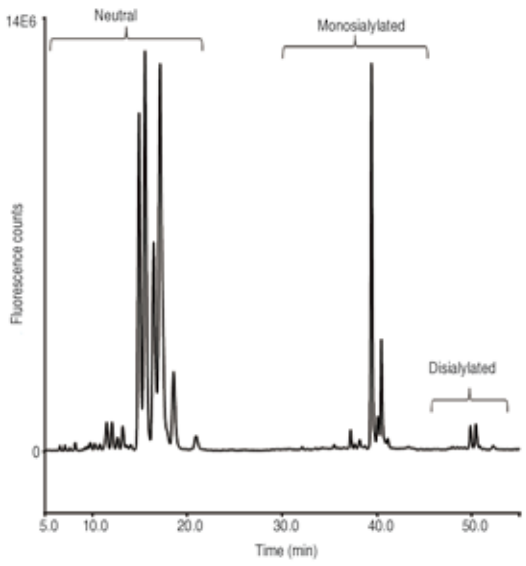


FIGURE 5. LC-MS/MS (HCD) spectra of 2AB-labeled *N*-linked glycans from bovine fetuin.

Analysis of 2AA-Labeled Antibody Glycans

Unlike 2AB, 2AA labeling introduces a formal negative charge to each glycan. This promotes greater binding to the GlycanPac AXR-1 column, thus improving retention of both neutral and negatively charged glycans. Antibodies are the most common proteins developed for therapeutics, and are under development for the treatment of numerous diseases. However, antibody glycosylation is a major source of heterogeneity with respect to both structure and therapeutic function. Glycosylation variants are primary factors in batch-to-batch antibody variation, altering product stability in vivo, and significantly influencing Fc effector functions in vivo. Both the U.S. FDA and European regulations require understanding of glycan profiles in these proteins because of their profound influence on safety and efficacy of biopharmaceuticals. Figure 6 shows the separation of neutral and acidic 2AA-labeled N-Linked glycans from a human IgG using a GlycanPac AXR-1 (1.9µm, 2.1 × 150 mm) column. As with the fetuin sample in the previous figures, the IgG-derived glycan elution profile consists of clusters of peaks in which the neutral glycans elute first, followed by monosialylated and disialylated forms. Analytes in each cluster represent the glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their isomerism and size by reversed-phase interactions. As shown in Figure 6, 2AA-labeled neutral glycans elute between 5 and 22 min, 2AA-labeled monosialylated glycans elute between 30 and 45 min and 2AAlabeled disialylated glycans elute between 45 and 55 min. More than 40 peaks are identified from the separation of 2AA-labeled *N*-glycans from this human IgG.



Column: GlycanPac AXR-1 (1.9 µm)
Dimensions: 2.1×150 mm
Mobile phase: A: Acetonitrile
B: water
C: 50 mM Ammonium Formate, pH 4.4)
Flow: 0.3 mL/min
Temp: 30 °C
Injection: 100 pmoles
Detection: Fluorescence at 320/420 nm
Sample: 2AA labeled *N*-glycan from human IgG

Time (min)	%A	%B	%C	Flow Rate (mL/min)	Curve
-10	0	92	8	0.3	5
0	0	92	8	0.3	5
1	0	92	8	0.3	5
25	0	92	8	0.3	5
26	0	50	50	0.3	5
60	10	40	50	0.3	5

FIGURE 6. Separation of 2AA-labeled *N*-linked glycans from human IgG by charge, isomers, and size using a GlycanPac AXR-1 (1.9 µm) column

Conclusion

- The GlycanPac AXR-1 column separates glycans based on charge, isomeric structure, size, and polarity, providing twice the number of resolved peaks and more than 4 times the glycan structures identified compared to existing commercial amide HILIC columns for 2AB-labeled *N*-linked glycans released from bovine fetuin.
- Faster Orbitrap enables higher scan rates at higher resolution. This translates to increased sensitivity and better quality MS/MS data for both abundant and low abundance glycans. This also enables LC-MS^3 workflows, providing additional stage of information for glycans structural elucidation

References

1. Bertozzi, C. R. *et al*., Glycans in Biotechnology and the Pharmaceutical Industry, Essentials of Glycobiology. 2nd edition. Chapter 51 Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.
2. Chester, T.L., *Anal. Chem*. **2013**, 85 (2), 579-589.
3. Ruhaak, L.R. et al., *Anal. Bioanal. Chem.* **2010**, 397, 3457-3481.
4. Chen, X. et al., *Anal. Biochem.* **2007**, 370, 147-161.
5. Thermo Scientific GlycanPac AXR-1 Product Specification: http://www.dionex.com/en-us/webdocs/114170-PS-GlycanPac-AXH1-Column-PS20695_E.pdf
6. Application Note 20786: Structural Analysis of Labeled *N*-Glycans from Proteins by LC-MS/MS Separated Using a Novel Mixed-Mode Stationary Phase.
7. Application Note 20827: Structural Analysis of Native *N*-Glycans Released from Proteins Using a Novel Mixed-Mode Column and a Hybrid Quadrupole-Orbitrap Mass Spectrometer.
8. Bigge, J. C. et al., *Anal. Biochem.* **1995**, 230, 229-238.

A Fully Automated Workflow for Glycopeptide Analysis

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Overview

Purpose: Development of an automated workflow for comprehensive site-specific glycan/glycopeptide analysis in human serum.

Methods: Proteins from human serum were enriched at the glycoprotein and glycopeptide level using different strategies. Enriched glycopeptides were labeled with isobaric mass tags and analyzed on a hybrid ion trap-Oribtrap™ mass spectrometer using combination of higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) fragmentation.

Results: The described automated workflow significantly improved the rate of success of ETD analysis and simplified the overall glycoproteomics workflow.

Introduction

Glycosylation is a post-translational modification (PTM) that plays crucial roles in biochemical processes. Structural characterization of glycoproteins and glycopeptides is analytically challenging. Successful application of mass spectrometry (MS) in glycoproteomics depends on adopting a workflows that addresses specific questions relating to a particular sample type. Targeted enrichment of glyco-peptides is one such procedure. In principle, it reduces the complexity of the overall sample matrix, facilitating more sensitive and accurate analysis of the glycopeptides. Others have explored the separate use of TiO_2 or graphite as a means to selectively enrich glycopeptides for MS analysis. Here we report on the selective enrichment and characterization of glycopeptides based on combining TiO_2 and graphite. This approach was compared to common used zwitterionic Hydrophilic Interaction Liquid Chromato-graphy (ZIC-HILIC)-based strategies. Glycopeptide analysis was performed with a novel acquisition strategy termed high-energy collisional dissociation-accurate mass product ion-dependent electron transfer dissociation (HCD-PD-ETD) (Figure 1). The advantage of this approach is that it streamlined data analysis, improved dynamic range and duty cycle. Additionally, we utilized a novel bioinformatics tool to automate the analysis of the data from the combined fragmentation techniques (Figure 2).

Methods

Sample Preparation

Glycoproteins from human serum were isolated using a Thermo Scientific Glycoprotein Isolation Kit Con A per manufacturer's suggestion. Isolated glycoproteins were reduced, alkylated and subjected to enzymatic digestion. Samples were split into multiple fractions and enriched on ZIC®-HILIC (EMD Chemicals Inc.), Thermo Scientific Pierce TiO_2 Phosphopeptide Enrichment Kit and/or graphite, or SAX respectively according to the manufacturer's protocol. Upon enrichment, the samples were split into two fractions from each enrichment strategy. For each enrichment strategy, one fraction was labeled with Thermo Fisher Pierce Tandem Mass Tags (TMT^0-126™) according to the manufacturer's protocol, while the other fraction was subjected to PNGase F digestion in the presence of H_2O^{18}.

LC/MS

A Thermo Scientific EASY-nLC nano-HPLC system

and Michrom Magic™ C18 spray tip 15 cm x 75 µm i.d. column (Michrom BioResources) were used. Gradient elution was performed from 5-45% ACN in 0.1% formic acid over 60 min at a flow rate of 300 nL/min. The samples were analyzed with a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer with ETD. The following MS and MS/MS settings were used: FT: MS^n AGCTarget = 5e4; MS/MS = 1 µscans, 200 ms max ion time; MS = 400-2000 *m/z*, 60000 resolution at *m/z* 400, MS Target = 1e6; MS/MS = Top 10 Data-Dependent™ acquisition HCD Product Dependent acquisition ion trap ETD (Figure 2), Dynamic Exclusion = repeat count 1, Duration 30 sec, Exclusion duration 90 sec; HCD Parameters: Collision Energy = 35%; resolution 7500. MS^n Target Ion Trap = 1e4, 3 µscans, ETD anion AGC target = 2e5, charge dependent ETD reaction time was used. The Thermo Scientific Proteome Discoverer software version 1.2 was used to generate database of glycoproteins and prototype GlycoMaster™ (Bioinformatics Solution) software was used for intact glycopeptides analysis (Figures 2 and 3).

Results

Studies have shown efficient enrichment of neutral and sialylated *N*-linked glycopeptides by ZIC-HILIC[1]; while TiO_2 shows specific binding to sialylated glycopeptides[2]. Graphite columns have shown affinity for glycopeptides with a smaller peptide backbone.[3] In our experiments, TiO_2 and graphite were both used alone and combined for a two-step enrichment approach to ensure all sizes of glycopeptides would be enriched. These were compared against the most commonly used ZIC-HILIC based strategy. To test the enrichment methods, a tryptic digest of human serum was examined. First, enriched digests were incubated with the enzyme PNGase F in the presence of H_2O^{18} to obtain de-glycosylated peptides. Samples were then analyzed using conventional shotgun proteomics to generate a database of glycoproteins in Proteome Discoverer™ software (Figure 3, right column). This database was then brought into GlycoMaster to analyze HCD-PD-ETD data of intact glycopeptides (Figure 2). GlycoMaster software was used to extract the information of glycan fragmentation from the HCD spectrum and peptide fragmentation of the peptide backbone from the ETD spectrum to produce results on the overall glycopeptide structure (Figure 3, left column). Figure 4 summarizes the results from the two different enrichment strategies for human serum. Both approaches show high numbers of unique glycopeptides and glycoproteins with an overlap of only 35%. Though ZIC-HILIC has shown to provide efficient enrichment of sialylated glycopeptides, it also binds with neutral glycopeptides. TiO_2 ,on the other hand, selectively binds to sialylated glycopeptides over neutral glycopeptides, thus providing much more efficient enrichment of these acidic species. Our results confirm this observation as the unique glycopeptides present in the Venn diagram for TiO_2 were N-linked and acidic in nature, while the unique glycopeptides in ZIC-HILIC were mainly O-linked and acidic or *N*-linked and neutral.

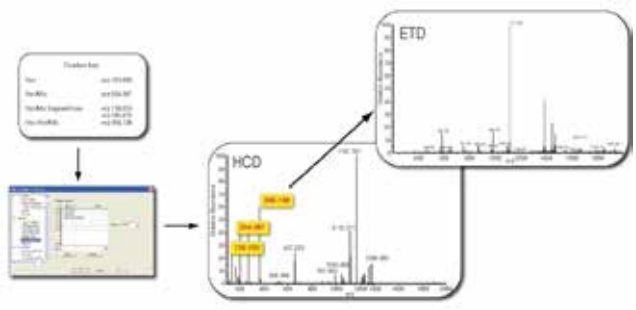

FIGURE 1. Schematic representation of HCD accurate-mass product-dependent ETD (HCD-PD-ETD) acquisition method.

FIGURE 2. User interface of GlycoMaster software.

FIGURE 3. Glycopeptide identification workflow.

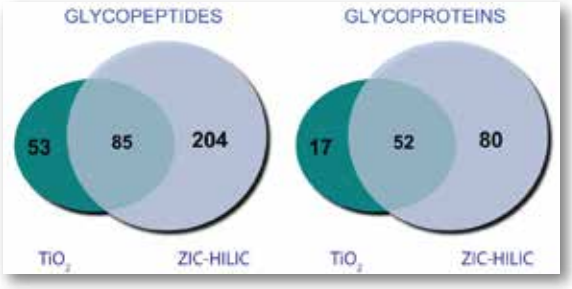

FIGURE 4. Comparison of ZIC-HILIC *vs* TiO_2 for enrichment of human serum glycopeptides.

Table 1 summarizes results for a histidine-rich glycoprotein identified from human serum. This protein contains both *N*-and *O*-linked glycosylation sites. Table 1 and Figures 5-7 show examples of the protein glycopeptides unique to each enrichment. Figure 6 displays the HCD spectrum for a bi-antennary histidine-rich glycoprotein N-linked glycopeptide T340-353 corresponding to *m/z* 958.383 in Figure 5. The corresponding ion trap ETD spectrum is shown in Figure 6. The ETD spectrum at precursor charge +4 generates complete z-ions, enabling unambiguous mapping of the *N*-glycosylation site as Asn_{344}. Figure 7 shows the ETD spectrum for two O-linked glycoforms of peptide T271-284 only identified in the ZIC-HILIC sample. The O-linked glycosylation site(T_{274}) reported here is novel. By employing the strategy outlined above, we were able to identify a total of 149 human serum glycoproteins, with multiple glycopeptides/glycoforms. Overall, ZIC-HILIC and TiO_2 provide complementary enrichment strategies, and their combined use greatly enhanced the human serum glycoproteome coverage compared to a single method.

TABLE 1. Enrichmentof *N*-and *O*-linked sialylated glycopeptides from histidine-rich glycoprotein (P04196) in human serum.

Peptide	# Glycopeptides	
	ZIC-HILIC	TiO_2
HSHNNNSDLHPHK	4	10
SSTTKPPFKPHGSR	3	3
VENTTVYYLVLDVQESDCSVLSRK	2	0
VIDFNCTTSSVSSALANTK	2	0

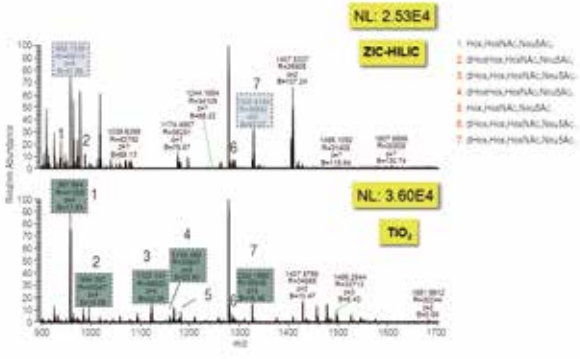

FIGURE 5. Enrichment efficiency of ZIC-HILIC vs TiO_2 for sialylated *N*-linked glycopeptides T340-353 from histidine-rich glycoprotein in human serum.

A novel acquisition strategy called HCD-PD-ETD (Figure 1) has also been implemented and compared to traditional HCD/ETD methods. In this approach, an LTQ Orbitrap Velos™ mass spectrometer equipped with ETD acquires HCD spectra in a data-dependent fashion. The instrument identifies diagnostic glycan oxonium (product) ions at ppm mass accuracy on the fly in the HCD spectra and only

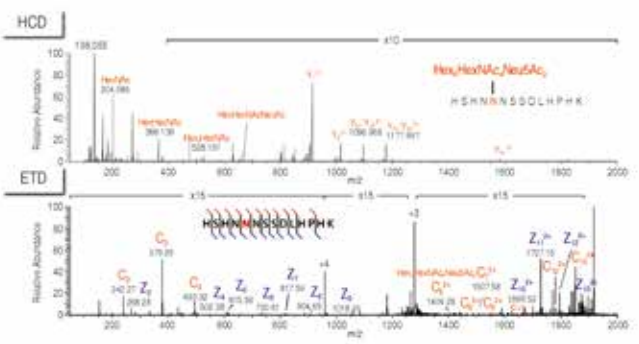

FIGURE 6. Orbitrap HCD spectrum and ion trap ETD spectrum of histidine-rich glycoprotein *N*-linked glycopeptide T340-353 precursor at *m/z* 958.383 (4+)from human serum.

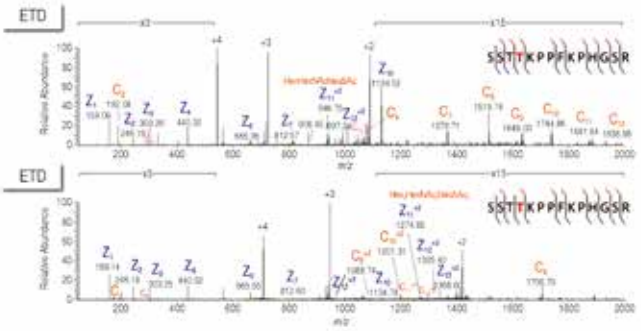

FIGURE 7. LC-MS ion trap ETD spectrum of histidine-rich glycoprotein *O*-linked glycopeptide T271-284 precursor at *m/z* 546.515 (4+) and *m/z* 710.571 (4+) from human serum.

triggers ETD spectra for the glycopeptide precursors. This approach increases overall productivity for MS analysis of glycopeptides by acquiring ETD spectra only when a glycopeptide is detected. Additionally, this approach minimizes overall file size and the number of ETD spectra that are extrapolated to characterize glycopeptides. This automated workflow was applied to analysis of glycopeptides enriched (ZIC-HILIC, TiO_2 and SAX) from human serum. Overall, 706 intact *N*-and *O*-linked glycopeptides were identified in human serum. This novel HCD-PD-ETD acquisition strategy significantly

outperformed the standard HCD/ETD alternating acquisition method. Figure 8 shows the comparison of the HCD-PD-ETD approach to the traditional alternating HCD/ETD strategy for the SAX-enriched sample. Overall, 800 less ETD spectra are acquired in the HCD-PD-ETD approach (Figure 8a), but far more glycopeptides are identified in comparison to alternating HCD/ETD (224 vs 160, Figure 8b). The HCD-PD-ETD approach resulted in more than twice as many ETD spectra identified than the alternating HCD/ETD strategy (Figure 8c).

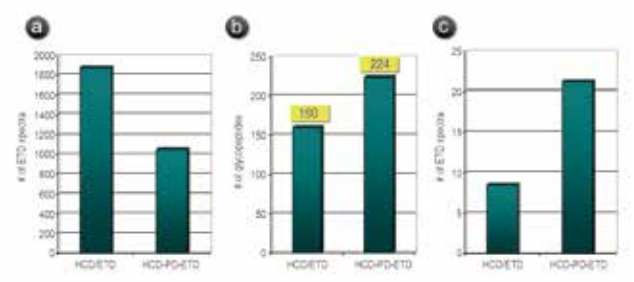

FIGURE 8. Comparison of HCD/ETD *vs* HCD-PD-ETD for SAX enriched sample. (a.) Number of ETD spectra acquired, (b.) number of glycopeptides identified and (c.) percentage of ETD spectra identified.

Conclusion

- A complementary approach of different enrichment strategies for glycopeptide analysis was demonstrated.
- The novel HCD-PD-ETD approach on an LTQ Orbitrap Velos mass spectrometer increased the overall productivity for MS analysis of glycopeptides by streamlining data analysis and improving dynamic range and duty cycle.
- GlycoMaster software automated the analysis of the data acquired from the combined fragmentation techniques.
- The fully automated workflow enabled identification of 149 glycoproteins, 706 *N*-and *O*-linked glycopeptides, and 195 glycosylation sites.

References

1. Takegawa, Y.; Deguchi, K.; Ito, H.; Keira, T.; Nakagawa, H.; Nishimura, S.; Simple separation of isomeric sialylated N-glycopeptides by a zwitterionic type of hydrophilic interaction chromatography, *J Sep Sci*. **2006**, 16, 2533-2540.
2. Larsen, M.R.; Jensen, S.S.; Jakobsen, L.A.; Heegaard, N.H. Exploring the sialiome using titanium dioxide chromatography and mass spectrometry, *Mol. Cell Proteomics*, **2007**, 6, 1778-1787.
3. Larsen, M.R.; Hojrup, P.; Roepstorff, P. Characterization of gel-separated glycoproteins using two-step proteolytic digestion combined with sequential microcolumns and mass spectrometry. *Mol Cell Proteomics*, **2005**, 4, 107-119.

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Analysis of Glycopeptides Using Porous Graphite Chromatography and LTQ Orbitrap XL ETD Hybrid MS

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Introduction

Glycosylation of Asn, Ser or Thr is arguably the most common known post-translational modification (PTM), resulting in a multitude of highly heterogeneous protein isoforms.[1, 2] While the physicochemical differences among the glycosylated protein molecules are often minute, their characterization remains a great analytical challenge. To date, although there are reports of glycoproteome analyses based on electrophoresis techniques, an LC-MS/MS based approach offers advantages in speed, sensitivity, and automation[3]. It remains the most powerful and versatile technique for elucidation of glycopeptide structure. However, in addition to the difficulties in capturing minor glycopeptides efficiently from samples containing large amounts of nonglycosylated peptides produced by proteolytic digestion of complex protein mixtures, commonly used collision-induced dissociation (CID) has limitations for determining the modification site due to the labile nature of the glycan attachment to the peptide ion.[3] CID MS/MS predominantly generates fragment ions from cleavages of glycosidic bonds without breaking amide bonds.[1, 2] In contrast to CID, electron transfer dissociation (ETD) preserves labile PTMs while cleaving peptide bonds, making the identification of modification sites possible.[4, 5] Since glycosylated proteins and the resulting peptides are generally very heterogeneous, their mass spectra are highly complex, consequently highquality liquid chromatography, high mass resolution, and accurate mass measurements of ETD precursors and fragments are essential for glycopeptide analysis. The high-resolution, high-mass-accuracy measurements of the Thermo Scientific LTQ Orbitrap XL ETD hybrid mass spectrometer coupled with additional capabilities such as parallel acquisition, in-source CID, and alternating CID/ETD dissociation can enable a thorough characterization of glycopeptides in a single analysis.

In this study, several glycoproteins: bovine α1-acid glycoprotein, fetuin and human α1-acid glycoprotein, were analyzed using nano LC-MS/MS. The performance of C_8, C_{18}, and porous graphite columns were systematically evaluated and optimized for glycopeptide separation prior to mass spectrometry analysis by an LTQ Orbitrap XL ETD™.

Goal

To demonstrate the advantages of porous graphite chromatography and electron transfer dissociation for analysis of N-glycopeptides on an LTQ Orbitrap XL ETD hybrid mass spectrometer.

Experimental Conditions

Sample Preparation

Glycoproteins purchased from Sigma were denatured in 0.1% SDS 50 mM Tris HCI buffer (pH 8.5), reduced with 5 mM DTT for 1 hr at 60℃ and alkylated with 25 mM iodoacetamide for 2 hr in the dark at room temperature. Then reduced and alkylated proteins were precipitated with acetone, digested, and analyzed by nano-LC-MS[2]. The experiments were conducted on LTQ XL ETD and LTQ Orbitrap XL ETD mass spectrometers using the following conditions:

LC Separation

HPLC System:	Thermo Scientific Surveyor MS Pump with a flow splitter
Columns:	Agilent ZORBAX 300SB C8 column (75 µm × 5 cm); Microtech, C18 column (150 µm × 10 cm) Thermo Scientific Hypercarb porous graphite column (75 µm × 5 cm)
Mobile Phases:	A: 0.1% Formic acid; B: 0.1% Formic acid in acetonitrile
Gradient:	For Hypercarb™ column 5–50% B in 30 minutes For RP columns 5–35% B in 30 minutes

MS Analysis

Mass Spectrometer:	LTQ XL™ linear ion trap mass spectrometer with ETD and nano-ESI source
Spray Voltage:	2 kV
Capillary Temp:	160 ℃
Capillary Voltage:	35 V
Tube Lens:	125 V
MS^n Target:	1e4
Mass Range:	50–2000 *m/z* or 100–4000 *m/z*
Anion Reagent:	Fluoranthene
Anion Reagent Isolation:	On
Anion Target:	2e5
Max Anion Injection Time:	50 ms
ETD Reaction Time:	75–150 ms
Mass Spectrometer:	LTQ Orbitrap XL ETD hybrid MS
Mass Range:	400–2000 *m/z*, resolution 60,000–100,000 @ *m/z* 400
FT MS AGC Target:	5e5
FT MS/MS AGC Target:	1e5, 3 amu isolation width
MS/MS Resolution:	7,500 FWHM at *m/z* 400, 3 microscans
Monoisotopic Precursor Selection:	On
Exclusion Mass Tolerance:	10 ppm
Max Ion Time FT MS:	500 ms
Max Ion Time FT MS/MS:	500 ms
Full MS Range:	400–2000 *m/z*
MS/MS Mass Range:	100–2000 *m/z*
Survey Scan:	Source CID at 65 V for *m/z* 204

Data Processing

Thermo Scientific Xtract software was used for deconvolution of multiply charged precursors and MS/MS spectra. The GlycoMod tool from the Swiss-Prot website was used to assign possible oligosaccharide structures and compositions.

Results and Discussion

LTQ Orbitrap XL ETD Instrument

The LTQ Orbitrap XL ETD mass spectrometer is a high-resolution, accurate-mass hybrid mass spectrometer equipped with an ETD source.[6] Figure 1 is the schematic of the LTQ Orbitrap XL ETD MS. The ETD anion reagent ions travel from the ETD source through the HCD collision cell and C-trap into the linear ion trap. There they are isolated and reacted with the precursor peptides ions in the ion trap. The resulting fragments can be measured in either the ion trap or Orbitrap™ mass analyzers. There are three additional fragmentation modes available on this instrument: higher-energy collisional dissociation (HCD), CID, and pulsed-Q dissociation (PQD).

Since CID and ETD can provide complementary glycopeptide structural information, coupling high-resolution, high-mass-accuracy full MS with alternating CID and ETD MS/MS makes comprehensive glycopeptide analysis achievable (Figure 2).[4, 5]

Choosing a Stationary Phase for Glycopeptide Analysis

We evaluated the performance of three stationary phases for LC analysis of glycosylated peptides. Figure 3 shows analysis of bovine α1-acid glycoprotein on C_{18} and porous graphite columns. One pmol of protein digest was injected into C_{18} column versus 500 fmol on porous graphite column. The profiles are the extracted ion chromatograms of *m/z* 1706.3, 1138.5 and 867.3 belonging to 2+, 3+ and 4+ molecular ions of the bi-antennary glycopeptide $_{91}$CVYNCSFIK$_{99}$. The intensities of the 2+ and 3+ glycopeptide precursor ions from analysis with the porous graphite column were similar to the intensities from analysis with the C_{18} column despite 50% lower load on-column. This was likely due to the higher affinity of

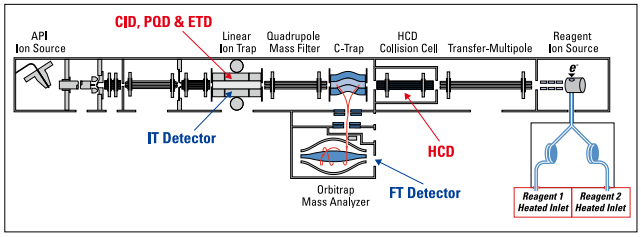


Figure 1. Schematic diagram of an LTQ Orbitrap XL ETD hybrid mass spectrometer

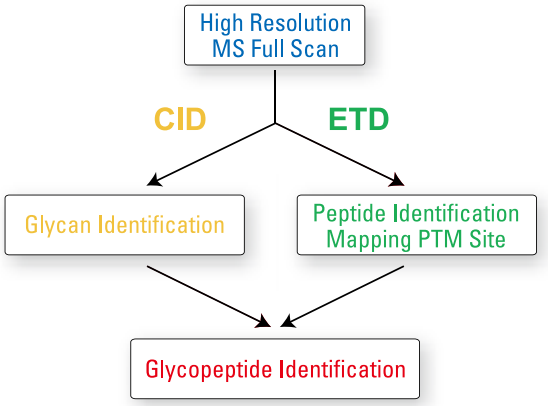


Figure 2. Glycopeptide analysis flow chart using an LTQ Orbitrap XL ETD hybrid mass spectrometer

porous graphite for hydrophilic peptides. In addition, chromatography using a porous graphite stationary phase promoted formation of abundant higher-charge-state metal-adducted precursor ions, which improved detection limits for all observed charge states of glycopeptides.

Figure 4 shows a high-resolution deconvoluted spectrum of these glycopeptides. At least one potassium adduct was observed for each glycoform. As demonstrated in Figure 3, formation of a metal adduct is likely responsible for producing higher-charge-state ions: $(M+K+3H)^{4+}$ precursor at *m/z* 867.3 was the dominant peak for glycopeptide 5 (Figure 4) while $(M+3H)^{3+}$ precursor at *m/z* 1138.6 was the dominant signal for glycopeptide 2 (Figure 4). Formation of higher-charge precursors can be explained by partial neutralization of sialic acid negative charges by metal cations.[7, 8]

Bovine and human α1-acid glycoproteins contains five N-glycosylation sites with hybrid-type glycan structures.[9, 10] Four out of five glycopeptides were detected and identified using a porous graphite column compared to two out of five peptides on C_{18} and three out of five peptides on C_8

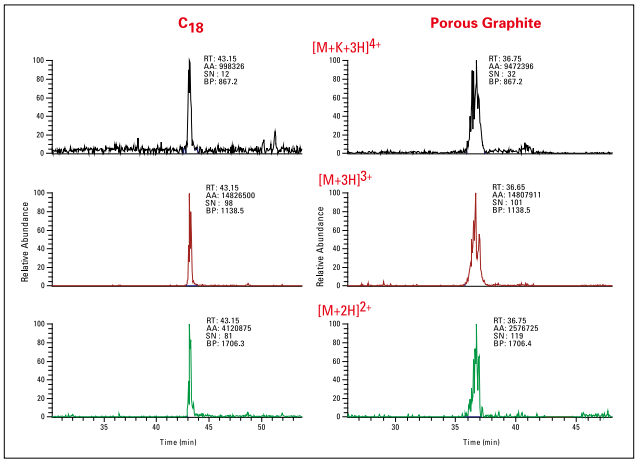


Figure 3. Comparison of different charge state extracted ion chromatograms of bovine α1-acid glycoprotein bi-antennary peptide $_{91}$CVYNCSFIK$_{99}$ using C18 and porous graphite columns

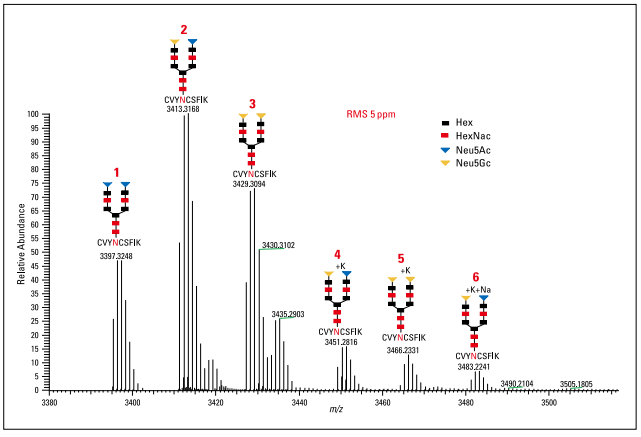


Figure 4. Deconvoluted full MS spectrum of bovine α1-acid glycoprotein bi-antennary peptide $_{91}$CVYNCSFIK$_{99}$

columns (Table 1) without any prior enrichment. Only the largest and most hydrophobic peptides were not detected using any of the phases. Similar results were obtained for bovine fetuin digest. All bovine fetuin glycopeptides could be detected using C_{18}[2] or C_8 chromatography. However, they were mostly present as lower-charge species, resulting in poorer MS/MS ETD spectra without enough information for their unambiguous identification. Previously, Alley and co-workers reported that fetuin glycopeptides could not be observed after separation on a graphite HPLC Glycan Chip.[11] Our studies indicated that larger hydrophobic peptides elute much later in the gradient. Nevertheless, three out of four fetuin peptides were identified after separation on the porous graphite column. Only the largest O-glycosylated 246-306 peptide was not detected. Figure

5 shows an example of an ETD spectrum of 5+ precursor at *m/z* 1307 of bovine fetuin tri-antennary peptide $_{72}$RPTGEVYDIEIDTLETTCHVLDPTPLANCSVR$_{103}$, which was successfully identified after separation on porous graphite column. After optimization, all nano LC-MS/MS analyses of glycopeptides were performed using a porous graphite column and 1 pmol of glycoprotein digest.

Table 1. Bovine α1-acid glycoprotein glycopeptides detected by nano LC-MS/MS

	Type of LC Column		
Peptides	**Graphite**	**C18**	**C8**
$_{103}$QNGTLSK$_{109}$	•		
$_{53}$NPEYNK$_{58}$	•		
$_{91}$CVYNCSFIK$_{99}$	•	•	•
$_{128}$TFMLAASWNGTK$_{139}$	•	•	•
$_{19}$QSPECANLMTVAPITNATMDLLSGK$_{43}$			•

MS /MS Analysis of Glycopeptides

To identify glycopeptides, we utilized a strategy described by Peterman and Mulholland[2], where in-source CID generated characteristic oxonium ions at *m/z* 204 and/or 366 and were further fragmented by a dedicated MS^3 event. This allowed for a highly sensitive and selective detection of the eluting glycosylated peptide ion(s) at a given retention time. High-resolution, accurate-mass full-scan MS was measured in the Orbitrap mass analyzer, as shown in Figure 6, followed by data-dependent MS/MS alternating CID and ETD scans with fragments analyzed in either the ion trap or Orbitrap mass analyzers. The resulting CID spectra of the 3+ precursors (Figure 6c) were used for glycan structure elucidation and the ETD spectra of the 4+ precursors (Figure 6b) were used for glycopeptide identification as demonstrated in Figures 7 and 8 for the bovine α 1-acid glycoprotein peptide $_{103}$QNGTLSK$_{109}$. Figure 7, inset, shows the CID spectrum of the precursor $[M+3H]^{3+}$ at *m/z* 990.395 measured in the Orbitrap mass analyzer. The oxonium fragment ion at *m/z* 366 confirmed this precursor as a glycopeptide. The spectrum was deconvoluted and glycan composition was assigned as shown in Figure 7. This glycoform contains one each of N-acetylneuraminic (Neu5Ac) and N-glycolyeuraminic (Neu5Gc) acids at the glycan branch end as determined

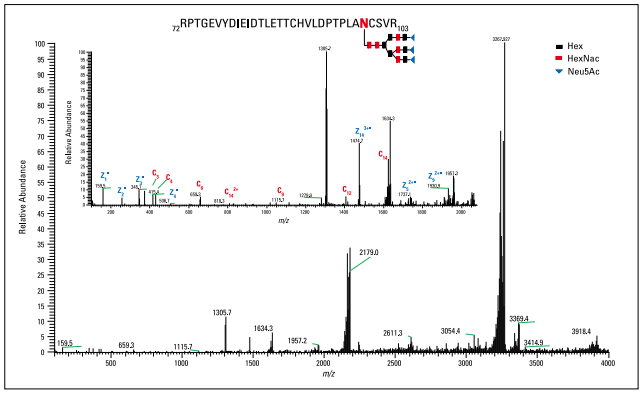
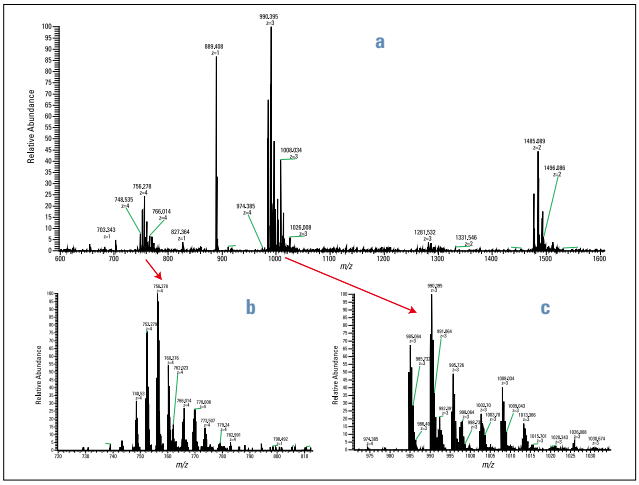


Figure 5. Ion trap MS/MS ETD spectrum of bovine fetuin tri-antennary peptide $_{72}$RPTGEVYDIEIDTLETTCHVLDPTPLANCSVR$_{103}$ (5+, *m/z* 1307). Insert: Magnified region for scan range 100-2000 *m/z*.



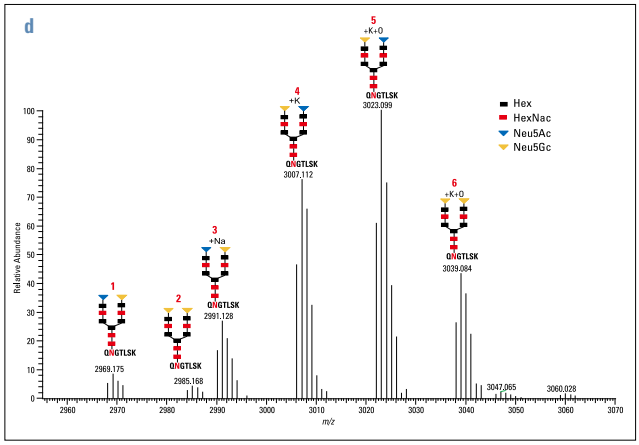


Figure 6. a) MS full scan; b, c) zoomed in charge states 4+, 3+ showing highly heterogeneous glycoforms; d) deconvoluted full MS spectrum of bovine α1-acid glycoprotein bi-antennary peptide $_{103}$QNGTLSK$_{109}$.

by the presence of 657/673 fragment pair. The product ion at *m/z* 950.4805 corresponds to $[M\text{-}GlcNAc+H]^+$. All measured masses were within 5 ppm of their theoretical values which helped unambiguously assign the glycan portion as a bi-sialated glycopeptide structure.

Higher-charged 4+ metal-adducted precursor ions were further selected for ETD analysis. From the ETD MS/MS spectrum of the precursor $[M+K+O+3H]^{4+}$ as shown in Figure 8, the glycosylation site was clearly identified at Asn 104 based on an almost complete series of *c/z•* ions. No significant loss of carbohydrate was detected and, as expected, several of the observed glycan-containing fragments retained potassium ions. The high-resolution, high-mass-accuracy and low chemical noise of the Orbitrap mass analyzer significantly benefited ETD analysis of glycopeptides with charge states 4+ and above, allowing straightforward deconvolution and interpretation of spectra (Figure 8).

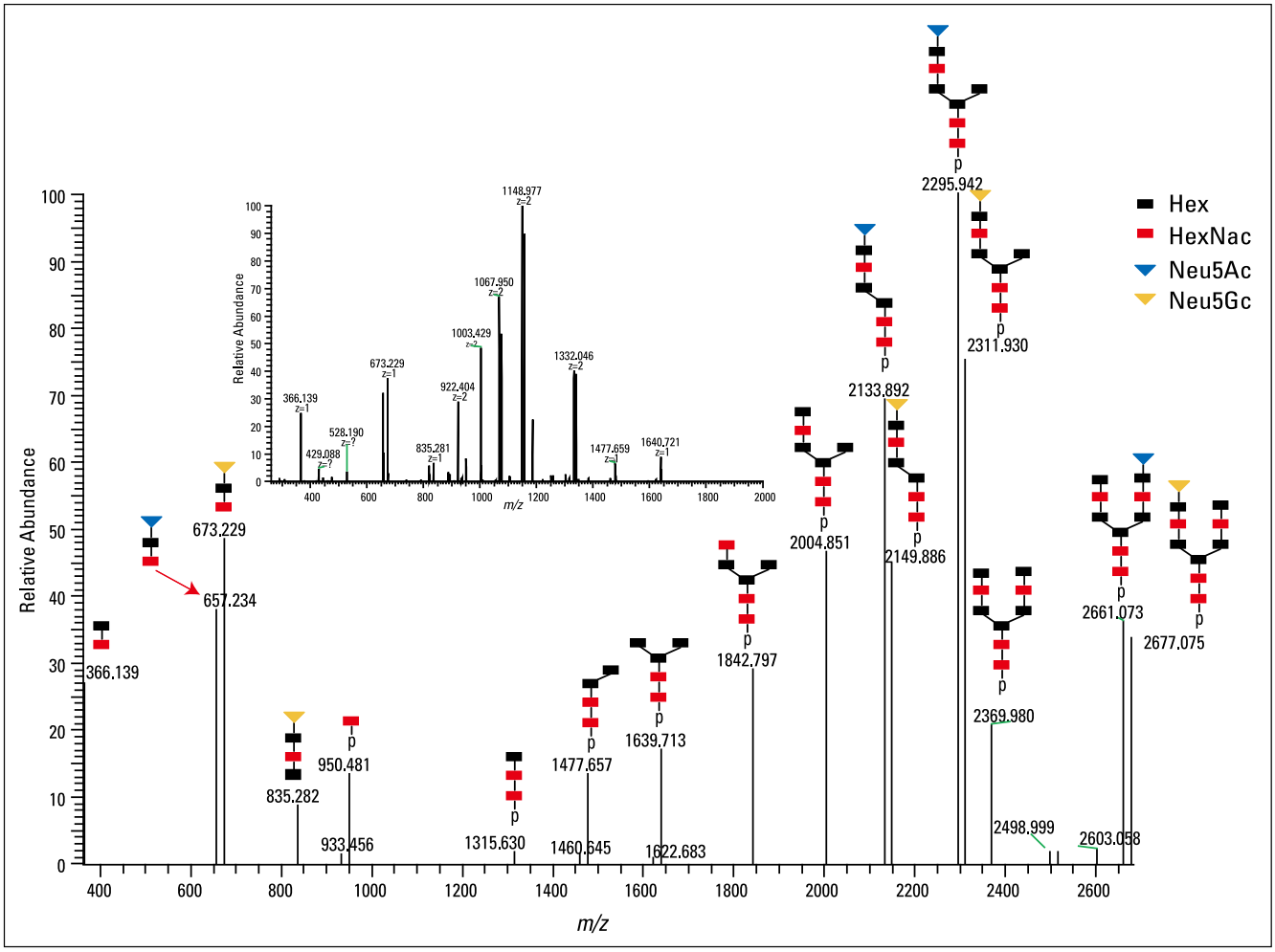


Figure 7. Deconvoluted Orbitrap CID MS/MS spectrum of bovine α1-acid bi-antennary glycopeptide $_{103}$QNGTLSK$_{109}$. Insert shows original Orbitrap CID spectrum of 3+ parent at *m/z* 990.395 (Figure 6d, structure 1).

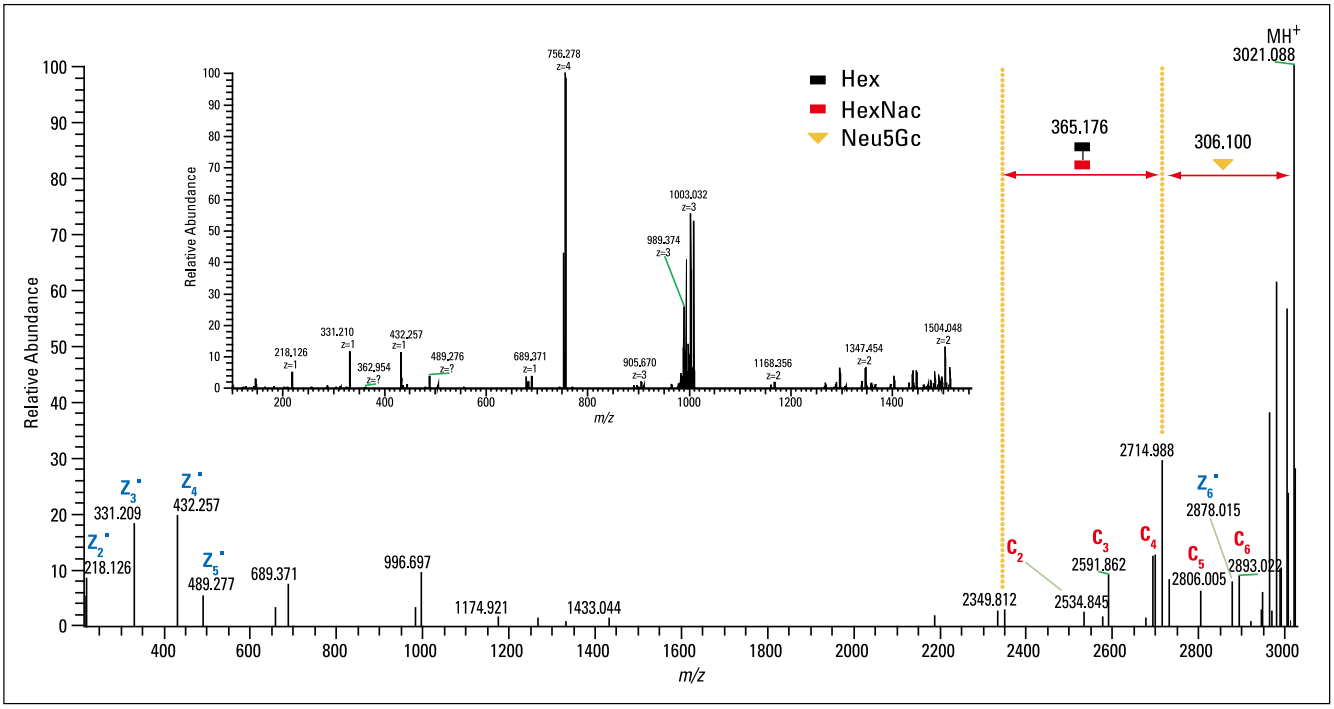


Figure 8. Deconvoluted Orbitrap ETD MS/MS spectrum of bovine α1-acid bi-antennary glycopeptide $_{103}$QNGTLSK$_{109}$. Insert shows original Orbitrap ETD spectrum of [M+K+O+3H] $^{4+}$ at *m/z* 756.278 ion (Figure 6d, structure 5).

Conclusions

- The Hypercarb porous graphite column demonstrated excellent separation for glycopeptide analysis, especially for short, hydrophilic peptides containing bi- or triantennary glycan chains. It allowed for their sensitive detection without any prior enrichment.
- Formation of metal adducts promoted evolution of higher-charge species, aiding ETD fragmentation of glycopeptides.
- ETD preserved labile glycans, facilitating the identification of both the peptide of interest and the site of modification.
- Combining peptide structural information obtained by ETD and the glycan composition information obtained by CID enabled confident identification and characterization of glycopeptides within a single LC-MS analysis using an LTQ Orbitrap XL ETD mass spectrometer.

References

1. Morelle, W., Canis, K., Chirat, F., Faid, V. and Michalski, J-C. (2006) The use of mass spectrometry for the proteomic analysis of glycosylation. *Proteomics*, 6, 3993-15.

2. Peterman, S.M. and Mulholland, J.J.(2006) A novel approach for identification and characterization of glycoproteins using a hybrid linear ion trap/FT-ICR mass spectrometer. *J. Am. Soc. Mass Spectrom.*, 17(2), 168-79.

3. Kaji,H, Isobe, Toshiaki, (2008), Liquid Chromatography/Mass Spectrometry (LC-MS)-Based Glycoproteomics Technologies for Cancer Biomarker Discovery. *Clinical Proteom*, 4:14-24

4. Syka J.E., Coon J.J., Schroder M.J., Shabanowitz J, Hunt D.F. (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. USA*; 101, 9528-33.

5. Alley Jr., W.R., Merchref, Y. and Novotny, M.V. (2009) Characterization of glycopeptides by combining collision-induced dissociation and electron-transfer dissociation mass spectrometry data. *Rapid Commun. Mass Spectrom.*, 23, 161-170.

6. McAlister, G.C., Phanstiel, D., Good D.M., Berggren,

W.T. and Coon, J.J. (2007) Implementation of electron-transfer dissociation on a hybrid linear ion trap-orbitrap mass spectrometer. Molecular & Cellular Proteomics, 2007, 6, 1942-1951.

7. Newton, K.A., Amunugama, R. and McLuckey, S.A. (2005) Gas-phase ion/ion reactions of multiply protonated polypeptides with metal containing anions. *J. Phys. Chem. A*., 109(16), 3608-16.

8. Medzihradszky, K.F., Guan, S., Maltby, D.A. and Burlingame, A.L. (2007) Sulfopeptide fragmentation in electron-capture and electron-transfer dissociation. *J. Am. Soc. Mass. Spectrom*., 18(9), 1617-24.

9. Snovida, S.I., Chen, V.C., Krokhin, O. and Perreault, H. (2006) Isolation and identification of sialylated glycopeptides from bovine α1-acid glycoprotein by off-line capillary electrophoresis MALDI-TOF mass spectrometry. *Anal. Chem.*, 78, 6556-635

10. Treuheit, M.J., Costello, C.E. and Halsall, H.B (1992) Analysis of the five glycosylation sites of human α1-acid glycoprotein. *Biochem. J*., 283, 105-12.

11. Alley Jr., W.R., Merchref, Y. and Novotny, M.V. (2007) Using graphitized carbon for glycopeptides separations prior to mass spectral detection. *Proceedings of the 55th ASMS conference*, Indianapolis.

A Complete Workflow Solution for Monoclonal Antibody Glycoform Characterization Combining a Novel Glycan Column Technology and Bench-Top Orbitrap LC-MS/MS

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Overview

Purpose: To develop a complete workflow solution for monoclonal antibody (mAb) glycoform characterization using a unique glycan column technology and a Thermo Scientific™ bench-top Orbitrap™ LC-MS/MS.

Methods: Glycans are separated using a recently developed high-performance HPLC/UHPLC column, a Thermo Scientific™ GlycanPac™ AXH-1 column. A datadependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans.

Results: The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity. A complete workflow solution was developed for glycan profiling combining the unique column technology and a bench-top Orbitrap LCMS/MS (Figure 1). This workflow was applied to antibody glycoform characterization. Confident identification and structural confirmation were achieved for released glycans from a standard glycoprotein and a monoclonal antibody.

Introduction

Because glycosylation is critical to the efficacy of antibody therapeutics, the FDA requires that a consistent human-type glycosylation be maintained for recombinant monoclonal antibodies (mAb), irrespective of the system in which they are produced. The complex branching and isomeric nature of glycans pose significant analytical challenges for their identification and characterization. Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans.

The recently developed GlycanPac AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural, qualitative and quantitative analysis of glycans. It has a unique selectivity for biologically relevant glycans including glycans from antibodies, either labeled or native and is designed for highresolution, high-throughput analysis by LC-fluorescence or LC-MS methods. Because glycans are very hydrophilic and polar, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine or zwitterionic packing materials are often used for their analysis. HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. Identification of the glycan charge state is not possible by HILIC. The GlycanPac AXH-1 column overcomes these limitations and can separate glycans based on charge, size and polarity configuration. It provides both greater selectivity and higher resolution. In this study, we characterized N-linked glycans released from a glycoprotein standard and a monoclonal antibody by LC-MS/MS methods using the new column technology and high-resolution Orbitrap mass spectrometry.

Methods

Sample preparation

Native glycans are released from glycoproteins or mAb with PNGase F enzyme. The released glycans are conjugated with 2-amino benzamide (2-AB) label group with reported procedure of Bigge *et. al*.[1]

Liquid chromatography

All the glycans are separated using a recently developed high-performance HPLC/UHPLC column, GlycanPac AXH-1, on a Thermo Scientific™ Dionex™ Ultimate 3000 UHPLC with either s fluorescence or MS detector.

For intact antibody, a Thermo Scientific™ ProSwift RP-10R monolithic column (1 × 50 mm) was used for desalting. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80 ℃ during analysis. Flow rate was 60 µL/min. After injection of 1 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0 min, 20%B; 1.0 min, 35%B; 3.0 min, 55%B; 4.0 min, 98%B; 7.0 min, 98% B; 7.1 min, 20%B; 15.0 min, 20%B).

Mass spectrometry

A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans. The following MS and MS/MS settings were used: MS scan range 380-2000 *m/z*. FT-MS was acquired at 70,000 resolution at m/z 200 with AGC target of 1×10^6 and DDA MS2 acquired at 17,500 resolution at m/z 200 with AGC target of 2×10^5. Intact mAbs were analyzed by ESI-MS for intact molecular mass. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 ℃ . S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb. The AGC target was set at 3×10^6. Maximum IT was set at 250 ms.

Data analysis

SimGlycan® software from PREMIER Biosoft was used for glycan identification and structural elucidation[2]. SimGlycan software accepts raw data files from Thermo Scientific mass spectrometers and elucidates the associated glycan structure by database searching and scoring techniques.

Full MS spectra of mAb were analyzed using Thermo Scientific™ Protein Deconvolution™ 2.0 software. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input *m/z* spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses of various combinations of commonly found glycoforms

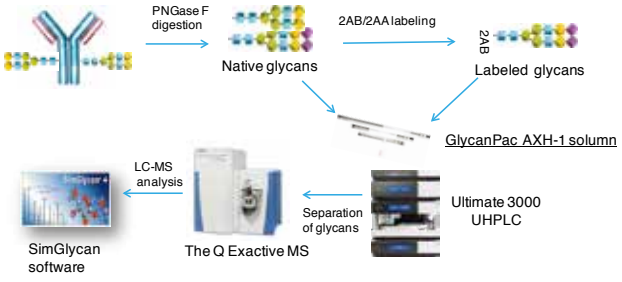

Figure 1. A complete LC-MS/MS workflow solution for monoclonal antibody glycan profiling

Results

Separation of Glycans Based on Charge, Size and Polarity
The GlycanPac AXH-1 column can be used for qualitative, quantitative, structural analysis and characterization of uncharged (neutral) and charged glycans present in proteins. The separation and elution of glycans are based on charge; the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated, di-sialylated, tri-sialylated, tetra-sialylated and finally penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. In this study, the structure of glycans present in each peak was determined using high resolution LC-MS/MS. As shown in Figure 2, the detailed structural information obtained from the MS/MS data validated the ability of GlycanPac AXH-1 column to separate labeled N-glycans based on charge, size and polarity. However, co-elution of different charge state glycans is common with other commercially available HILIC column as shown in Figure 3.

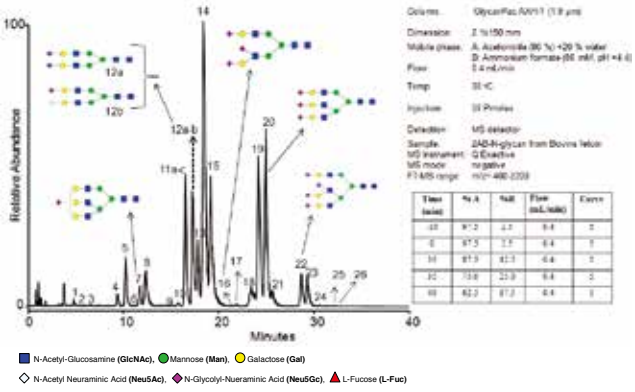

Figure 2. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by GlycanPac AXH-1 (1.9 µm) column with MS detection.

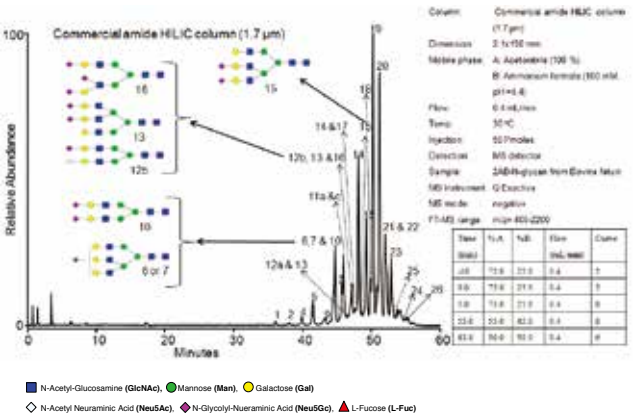

Figure 3. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by a commercial amide HILIC column (1.7 µm) with MS detection.

The GlycanPac AXH-1 column is also well suited for high performance LC/MS separation and analysis of native glycans from proteins (data not shown). Analyzing unlabeled glycans not only eliminates the extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Monoclonal antibody (mAb) glycan profiling using GlycanPac AXH-1 column and high resolution LC-MS/MS

Intact mass measurement of a monoclonal antibody identified glycoforms derived from the combination of any two of the three N-glycans, G0F, G1F and G2F. However, the mass errors for some of the intact glycoforms of this antibody ranged from 20-60 ppm (Figure 4A) which is larger than the <10 ppm observed for other samples (data not shown). Furthermore, the intact mass error for the deglycosylated form of this antibody was within 10 ppm (Figure 4B), suggesting that some minor glycosylation forms of this molecule that were not detected at the intact level had interfered with the observed intact mass of the major glycoforms. To further characterize this antibody, released glycans from this protein were separated using the GlycanPac AXH-1column. The separation and elution of glycans from GlycanPac AXH-1 column are based on charge with neutral glycans eluting first, followed by the acidic sialylated species. Glycans of each charge state are further separated based on their size and polarity (Figure 5).

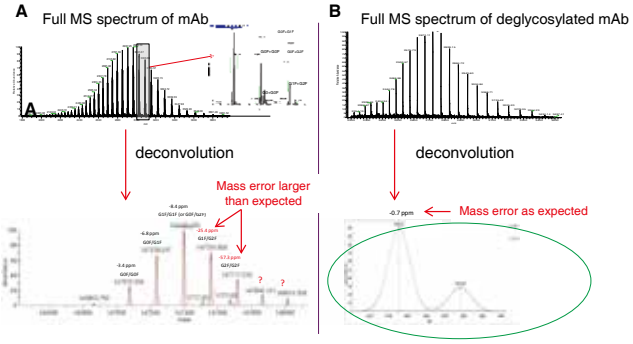

Figure 4. Observed molecular mass of glycosylated (A) and deglycosylated (B) forms of a intact monoclonal antibody. Some of the intact antibody major glycoforms have an observed mass error larger than expected. There are also two potentially double fucosylated peaks that need to be confirmed.

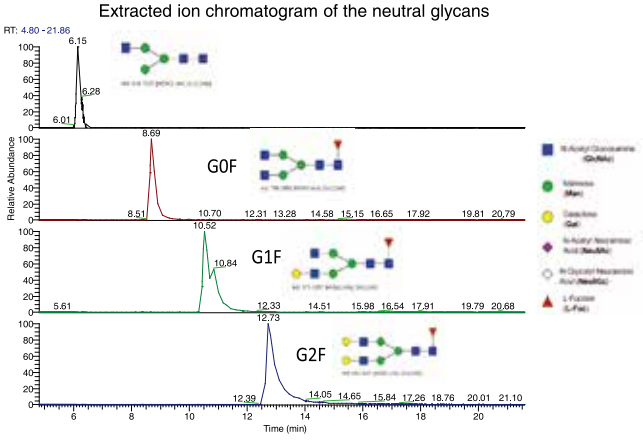

Figure 5. Separation of the major, neutral N-glycans on GlycanPac AXH-1 column

Characterization of glycans in each peak was performed by Full MS and data dependent MS/MS using HCD. The information-rich HCD spectra contain fragment ions that were generated from both cross-ring and glycosidic bond fragmentations (Figure 6). Three different types of glycans were found from this monoclonal antibody, the majority of glycans identified were neutral, including G0F, G1F and G2F which were also the major glycoforms identified at the intact protein level for this antibody (Figure 4A). Also identified were less abundant, non-fucosylated forms of G1 and G2, minor amounts of mono-sialylated and di-sialylated species with and without fucosylation, as well as double fucosylated species that were not identified at the intact protein level (Figure 7).

Figure 6. Identification and structural confirmation of released glycan using high resolution HCD MS/MS

Fragment ion type	Percentage match (%) of theoretical fragments
Single glycosidic	32.14
Glycosidic/glycosidic	30.95
Single cross ring	20.21
Cross ring/glycosidic	14.95

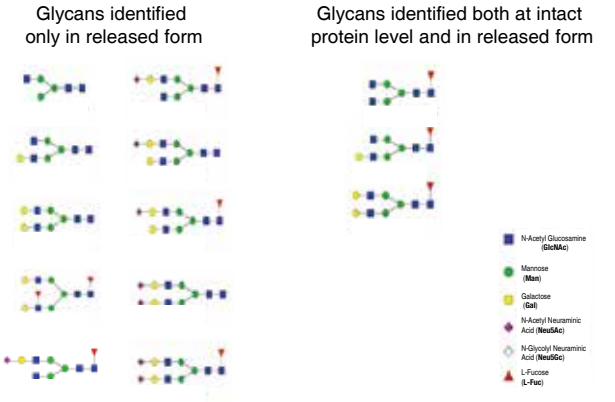

Figure 7. Identified glycans from monoclonal antibody

These results explain that the unexpected mass error observed previously is due to the interfering minor glycoforms that have a molecular mass close to the major ones. In the deconvoluted MS spectrum, the base of the antibody major glycoform peaks covers a mass range of about 40 Da due to the distribution of the unresolved isotopic peaks of a large protein of this size. Therefore any interfering species within 20 Da of mass difference would cause a mass shift of the major glycoform peaks, rather than forming a separate peak. For example, in this case, the replacement of a Fuc and a Gal by Neu5Ac, which would have a mass difference of -17Da, could cause the negative mass shift observed in this study, especially when the interfering species is relatively low in abundance (Figure 8). Results in this study indicate that rapid and sensitive antibody glycan profiling can be achieved using GlycanPac AXH-1 column and HR/AM Orbitrap LC-MS/MS.

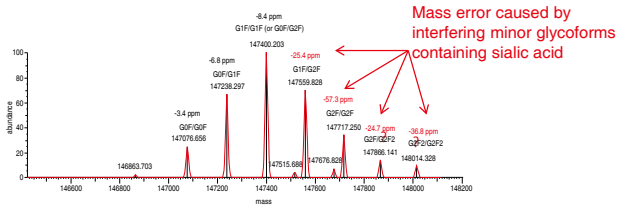

Figure 8. Annotated glycoforms of a monoclonal antibody

Conclusion

- GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity.
- The GlycanPac AXH-1 columns are compatible with MS instruments. LC-ESI-FTMS or FT-MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.
- Confident identification and structural confirmation of glycans can be achieved using high-resolution HCD MS/MS which produces an informative spectrum containing glycosidic and cross ring fragment ions.
- A complete workflow solution was developed for glycan profiling combining the unique GlycanPac AXH-1 column technology and a bench-top Orbitrap LC-MS/MS.
- This workflow was applied to characterize a monoclonal antibody glycoforms. Confident identification and structural confirmation was achieved for released glycans from the monoclonal antibody.

References

1. Bigge, J. C. et al., Non-selective and efficient fluroscent labeling of glycans using 2-amino benzamide and anthranilic acid. Analytical Biochemistry, **1995**, *230*, 229- 238.
2. Apte, A and Meitei, N.S., Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan, **2010**, *600*, 269-81.

Novel Glycan Column Technology for the LC-MS Analysis of Labeled and Native *N*-Glycans Released from Proteins and Antibodies

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Overview

Purpose: Development of novel high-performance liquid chromatography (HPLC) and ultra high-performance liquid chromatography (UHPLC) columns for highresolution separation and structural characterization of native and fluorescently labeled *N*-glycans released from various proteins, including antibodies.

Methods: UHPLC with fluorescence detection (FLD) for the chromatographic analysis of labeled *N*-glycans. LC with mass spectrometry (MS) and LC-MS/MS analysis for structural characterization of both labeled and native *N*-glycans from proteins by MS detection.

Results: We have developed a high-performance, silica-based HPLC/UHPLC column (Thermo Scientific™ GlycanPac™ AXH-1) specifically designed for simultaneous separation of glycans by charge, size, and polarity. It is designed for high-resolution, and high-throughput analysis, with unique selectivity for biologically relevant glycans, either labeled or native, by LC-FLD and LC-MS methods.

Introduction

Glycans are widely distributed in biological systems in ‘free state’ and conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They are involved in a wide range of biological and physiological processes.[1] The functions, including efficacy and safety of protein-based drugs such as recombinant proteins and monoclonal antibodies (MAbs), are often dependent on the structure and types of glycans attached to the proteins.[2] The structures of glycans are quite diverse, complex, and heterogeneous due to post-translational modifications and physiological conditions. The structural characterization and quantitative estimation of glycans is highly essential in biotherapeutics and biopharmaceutical projects.[3] However, it is tremendously challenging to comprehensively characterize glycan profiles and determine the structures of glycans.

The GlycanPac AXH-1 columns are high-performance HPLC/UHPLC columns specifically designed for structural, qualitative, and quantitative analysis of glycans. They are designed for high-resolution and high-throughput analysis, with unique selectivity for biologically relevant glycans, including glycans from antibodies—either labeled or native—by LC-fluorescence or LC-MS methods. Because glycans are highly hydrophilic and polar substances, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterion packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size- and composition-based separation. However, identification of the glycan charge state is not possible with these types of columns because glycans of different charge states are intermingled in the separation envelope, making this approach limited. The GlycanPac AXH-1 column, which is based on advanced mixed-mode chromatography technology, overcomes these limitations and can separate glycans based on charge, size, and polarity configuration. In addition, each glycan charge

state can be quantified. The GlycanPac AXH-1 column provides both greater selectivity and higher resolution, along with faster quantitative analysis.

Methods

Sample Preparation

Release native glycans from glycoproteins with PNGase F enzyme. Conjugate the released glycans with 2-amino benzamide (2AB) label group using the reported procedure of Bigge et al.[4] Here, 2-AB A1 (P/N GKSB 311), 2-AB A2 (P/N GKSB 312), and 2-AB A3 (P/N GKSB 314) were purchased from Prozyme® (Hayward, CA). Prior to analysis, dissolve samples in 100% buffer (100 mM ammonium formate, pH = 4.4) and dilute further with acetonitrile to make 30% buffer and 70% acetonitrile.

Liquid Chromatography

All the glycans were separated using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system and either a Thermo Scientific™ Dionex™ UltiMate™ FLD-3400RS Rapid Separation Fluorescence Detector or MS detector.

Mass Spectrometry

MS analysis was performed with a Thermo Scientific™ Q Exactive™ Benchtop LC-MS/MS in negative ion mode at the following settings: MS scan range 380–2000. FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of $1e^6$; and DDA MS2 acquired at 17,500 resolution at *m/z* 200 with AGC target of $2e^5$.

Data Analysis

SimGlycan® software (PREMIER Biosoft) was used for glycan identification and structural elucidation data analysis.[5] SimGlycan software accepts raw data files from Thermo Scientific™ mass spectrometers and elucidates the associated glycan structure by database searching and scoring techniques.

Results

Separation of Labeled Glycans Based on Charge, Size, and Polarity

The GlycanPac AXH-1 column can be used for qualitative, quantitative, and structural analysis as well as characterization of uncharged (neutral) and charged glycans present in proteins. Figure 1 shows bovine fetuin on the GlycanPac AXH-1 (1.9 µm, 2.1 × 150 mm) column using fluorescence detection. The separation and elution of glycans are based on charge: the neutral glycans elute first, followed by the separation of acidic 2AB labeled *N*-glycans from monosialylated, disialylated, trisialylated, tetrasialylated and finally pentasialylated species. Glycans of each charge state are further separated based on their size and polarity. The retention time of each glycan charge state was confirmed using 2AB labeled glycan standards (as shown in Figure 2). Separation of glycans is based on charge, size, and polarity, which provides significant structural and quantitative information. The chromatographic profiles shown in Figures 1 and 2, detected by fluorescence detection, provide qualitative information about the separation of *N*-glycans. The structure of glycans present in each peak was determined from the LC-MS study using the GlycanPac AXH-1 (1.9 µm) column as shown in the following section.

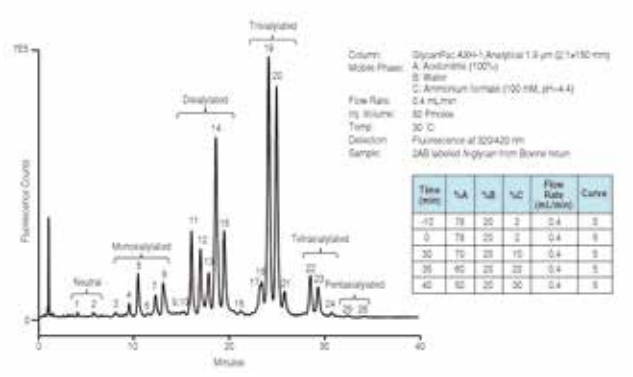


FIGURE 1. Separation of 2AB labeled *N*-glycans from Bovine fetuin by charge, size and polarity.

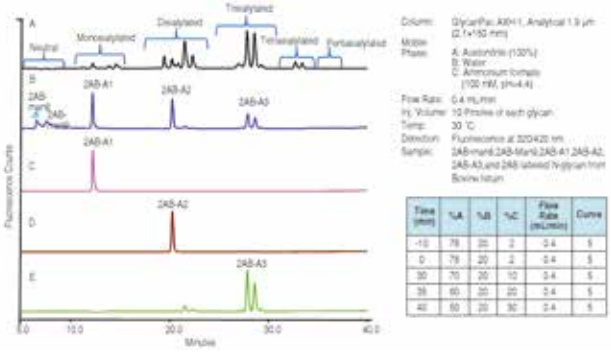


FIGURE 2. Comparison of 2AB labeled *N*-glycans standards and 2AB-*N*-glycans from fetuin.

LC-MS and LC-MS/MS Analysis of 2AB Labeled *N*-Glycan Using GlycanPac AXH-1 Column

The coupling of the GlycanPac AXH-1 column to MS was also explored. This is particularly attractive as MS, with it's ability to provide structural information, enables in-depth analysis of complex glycans. 2AB labeled *N*-glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer. Datadependant MS/MS spectra were acquired on all precursor ions ($z \leq 2$) and SimGlycan software was used for glycan structural elucidation. A representative example of the analysis is shown in Figure 3. The detailed structural information obtained from the MS/MS data further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity. However, coelution of different charge state glycans (Figure 4) is common with other commercially available HILIC columns.

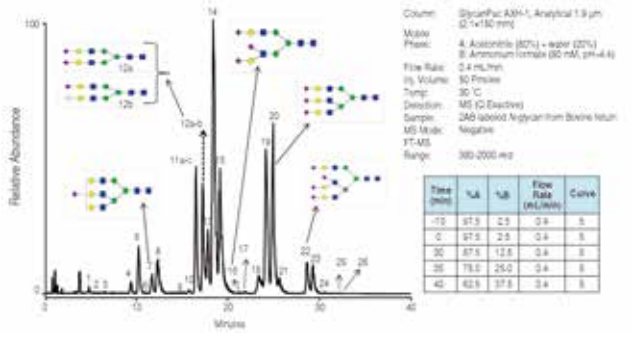


FIGURE 3. LC-MS analysis of 2AB labeled *N*-glycans from Bovine fetuin by the GlycanPac AXH-1 (1.9 µm) column with MS detection.

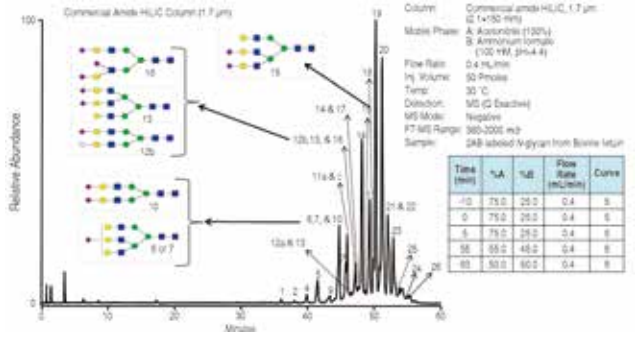


FIGURE 4. LC-MS analysis of 2AB labeled *N*-glycans from Bovine fetuin by a commercial amide HILIC column (1.7 µm) with MS detection.

LC-MS Analysis of Native Glycans Released from Proteins

The GlycanPac AXH-1 column is well suited for high-performance LC/MS separation and analysis of native glycans from MAbs and other proteins. Analyzing unlabeled glycans not only eliminates the extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction. Figure 5 shows the LC/MS analysis of native *N*-glycans from Bovine fetuin using the GlycaPac AXH-1 column (1.9 µm). The native glycans were separated based on charge, size, and polarity. Using an ammonium formate/ acetonitrile gradient highly compatible with MS detection, the separation enables excellent MS and MS/MS fragmentation data for accurate confirmation of the glycan structure of each chromatographic peak. Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially higher sialic acid glycans. However, fluorescently labeled glycans generally provide better and more MS/MS fragmentation peaks. The GlycanPac AXH-1 column is useful for the analysis of both native and labeled *N*-glycans, depending on the amount of sample available. If the amount of the sample is not extremely limited, analysis of unlabeled glycans using the GlycanPac AXH-1 is highly feasible.

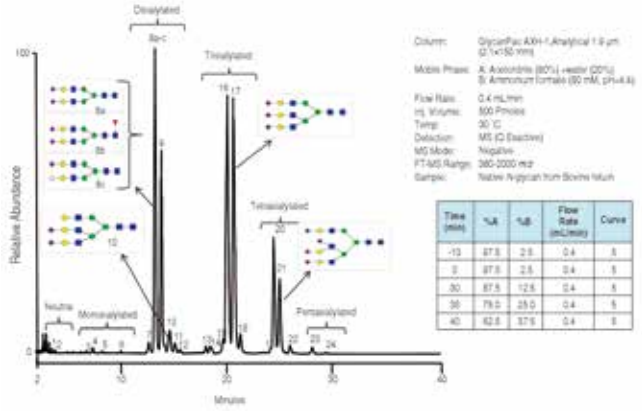


FIGURE 5. LC-MS analysis of native *N*-glycans from Bovine fetuin. All the peaks are detected by MS detection in negative ion mode.

Structural Analysis of *N*-Glycans from Antibodies by LC-MS Using GlycanPac AXH-1 Column

Antibody research has gained significant interest as a part of the development of protein biotherapeutics. Glycosylation of antibodies is a prime source of product heterogeneity with respect to both structure and function. Variation in glycosylation is one of the main factors in product batch-to-batch variation,[2-3] affecting product stability *in vivo* and significantly influencing Fc effector functions *in vivo*. A representative example of the chromatographic separation of antibody glycans is shown in Figure 6, where 2AA labeled *N*-glycans from IgG were separated using the GlycanPac AXH-1 column (1.9 µm). Characterization of glycans in each peak was performed by LC-MS/MS and results are shown in Figure 7. Three different glycan charge states were found in this human IgG; the majority of glycans are neutral or monosialylated, with minor amounts of disialylated glycans. Separation of glycans based on charge provides advantages compared to other commercially available HILIC columns.

Quantitative Determination of Glycans Based on Charge

Quantitative analysis of each glycan is essential for quick assessment of glycan variation in protein batch comparisons and for comparison of diseased to normal cell glycosylation profiles. In addition, quantitative analysis of glycans separated based on charge state also provide a tool for calculating the relative amounts of different sialic acid linkages after enzymatic digestion with silidase S and sialidase A. Figure 8 shows the quantitative analysis of 2AB labeled *N*-glycans based on charge the using GlycanPac AXH-1 column (1.9 µm) with fluorescence detection. The relative amount of each charge state glycan was estimated using a standard curve. A standards curve was drawn using the data from the chromatographic analysis of 2AB labeled A2 glycan standard, with the injection of different amount of samples starting from 0.1 to 5 pmole).

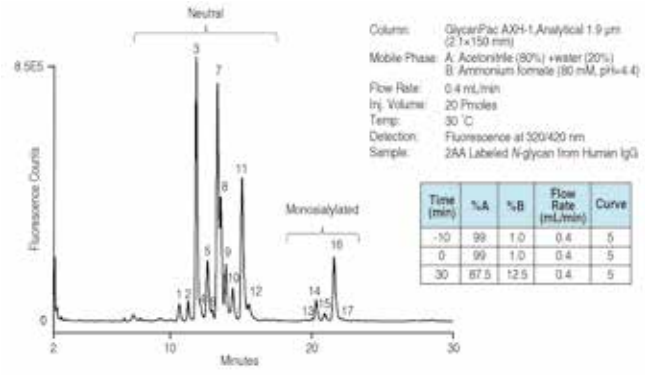


FIGURE 6. Analysis of 2AA labeled *N*-glycans from human IgG.

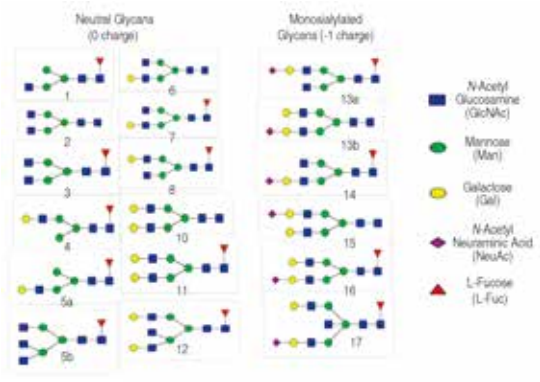


FIGURE 7. Mass spectroscopic characterization of glycans in each Figure 6 peak.

Quantitative Determination of Glycans Based on Charge

Quantitative analysis of each glycan is essential for quick assessment of glycan variation in protein batch comparisons and for comparison of diseased to normal cell glycosylation profiles. In addition, quantitative analysis of glycans separated based on charge state also provide a tool for calculating the relative amounts of different sialic acid linkages after enzymatic digestion with silidase S and sialidase A. Figure 8 shows the quantitative analysis of 2AB labeled *N*-glycans based on charge the using GlycanPac AXH-1 column (1.9 µm) with fluorescence detection. The relative amount of each charge state glycan was estimated using a standard curve. A standards curve was drawn using the data from the chromatographic analysis of 2AB labeled A2 glycan standard, with the injection of different amount of samples starting from 0.1 to 5 pmole).

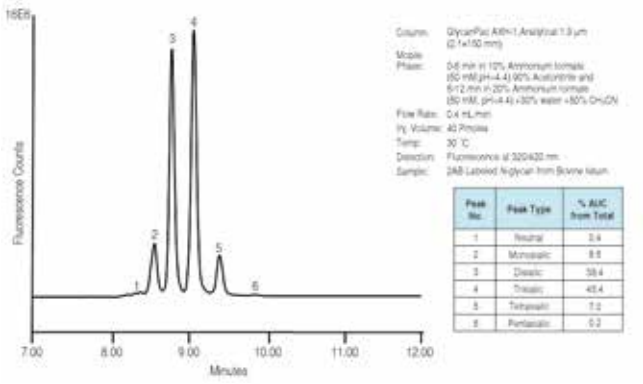


FIGURE 8. Quantitative estimation of each charge state glycan in 2AB labeled *N*-glycan from Fetuin

Conclusion

- The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size, and polarity not possible with commercial HILIC columns.
- LC-ESI-FTMS or FT-MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.
- The GlycanPac AXH-1 column is useful for both high-resolution charge-based separation and easy quantification of glycans.
- The GlycanPac AXH-1 columns are compatible with various MS instruments. These new columns have high-chromatographic efficiency and excellent column stability.
- The GlycanPac AXH-1 column is also useful for the separation of reduced *O*-glycans from proteins and mucins.
- The GlycanPac AXH-1 column is useful for the analysis of charged and neutral glycosylaminoglycans and glycolipids.

References

1. Varki, A. Biological Roles of Oligosaccharides: All the Theories Are Correct. *Glycobiology* **1993**, *3*, 97–130.
2. Bertozzi, C.R.; Freeze, H.H.; Varki, A.; Esko, J.D. *Glycans in Biotechnology and the Pharmaceutical Industry, Essentials of Glycobiology, Second Edition*; Cold Spring Harbor Laboratory Press: New York, 2009; Chapter 51.
3. *Guidance for Industry, Scientific Considerations in Demonstrating Biosimilarity to a Reference Product, Draft Guidance*; U.S. Department of Health and Human Services Food and Drug Administration, February 2012 [Online] www.fda.gov/downloads/Drugs/ GuidanceComplianceRegulatoryInformation/Guidances/UCM291128.pdf (accessed Jan. 18, 2013).
4. Bigge, J.C. et al., Non-Selective and Efficient Fluroscent Labeling of Glycans Using 2-Amino Benzamide and Anthranilic Acid. *Anal. Biochem.* **1995**, *230*, 229–238.
5. Apte, A; Meitei, N.S. Bioinformatics in Glycomics: Glycan Characterization with Mass Spectrometric Data Using SimGlycan. *Methods Mol. Biol.* **2010**, *600*, 269–81.

Acknowledgements

We would like to thank Mark Tracy, Yoginder Singh, Jessica Wang, and Patrick K. Bennett from Thermo Fisher Scientific for their help and permission to use their UHPLC and MS instruments.

应用 Target MS2 方法对大鼠血浆中人重组干扰素进行绝对定量的测试方案

顾培明
赛默飞世尔科技（中国）有限公司

1. 前言

随着蛋白质组学的不断发展，蛋白质组学已广泛深入到生物学研究的各个领域，目前，蛋白质组学研究已经由传统的大规模鉴定逐步转向了靶向蛋白质组学，研究内容已经由蛋白质鉴定扩展到了蛋白质相对和绝对定量，对复杂样本中蛋白质的相对与绝对定量有助于发现疾病的生物标志物，对于研究各类疾病的发生与发展有着重要的意义。

基于质谱的蛋白质组学定量方法主要有 SILAC，TMT，iTRAQ 以及应用三重四级杆的 MRM 等，本文主要介绍了赛默飞世尔新一代台式高分辨质谱仪 Q-Exactive 在目标蛋白质定量方面的应用。结果显示 Q-Exactive 不仅可以在 ng 级上样量情况下达到 98% 以上的蛋白质氨基酸序列匹配度，同时应用 target ms2 方法，可以很好的对复杂样本中目标蛋白质进行绝对定量，其定量下限达到了 amol，动态范围达到了 6 个数量级，且线性相关系数在 0.99 以上。

2. 实验部分

2.1 样品信息

人重组干扰素 α-2b 标准品（浓度为 1mg/ml），注射人重组干扰素 α-2b 后的处理组大鼠血浆样本以及空白大鼠血浆样本。

2.2 实验流程

1. 使用 Full MS/ddMS2 方法对人重组干扰素 α-2b 标准品酶解的肽段进行鉴定，筛选其 unique peptide；

2. 将人重组干扰素 α-2b 标准品酶解肽段稀释成不同浓度的标准品，其浓度分别为 10^4ng/ml，10^3ng/ml，10^2ng/ml，10^1ng/ml，10^0ng/ml，10^{-2}ng/ml，并掺入至空白大鼠血浆样本酶解肽段中，分别上样 18ul，使用

Target MS2 方法根据其 unique peptide 的 fragment ion 峰面积，绘制标准曲线；

3. 处理组大鼠血浆酶解肽段上样 18ul，根据其 unique-peptide 的 fragment ion 峰面积计算出大鼠血浆中人重组干扰素 α-2b 的浓度。

2.3 仪器条件

2.3.1 高效液相色谱条件

高效液相色谱仪：Thermo Scientific Easy-nLC 1000

色谱柱：C18 ,3μm, 100Å ,75μm × 15 cm

上样量：均 18 ul

流动相：A：0.1% Formic acid in water；

B：0.1% Formic acid in Acetonitrile

色谱梯度：

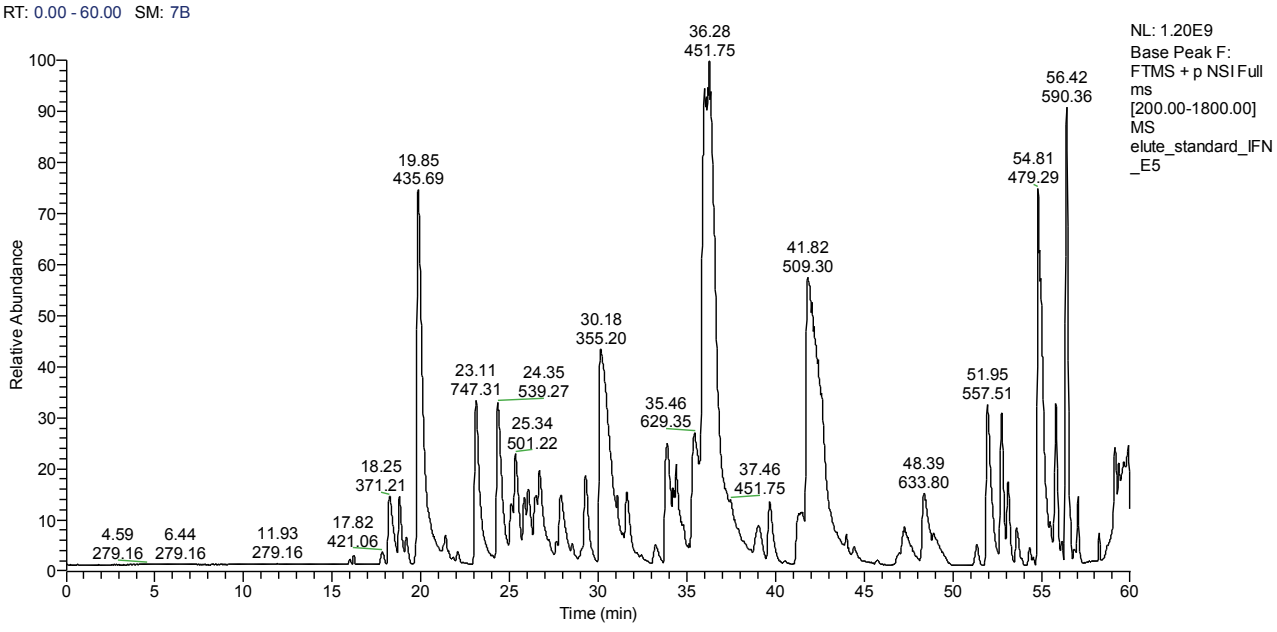
Time [mm:ss]	Duration [mm:ss]	Flow [nl/min]	Mixture [%B]
00:00	00:00	350	3
05:00	05:00	350	8
42:00	37:00	350	20
47:00	05:00	350	30
50:00	03:00	350	90
60:00	10:00	350	90

2.3.2 质谱条件
标准品：
质谱仪：Thermo Scientific Q Exactive
喷雾电压：2.6 kV
毛细管温度：250 ℃
S-lens：50%
碰撞能量：27% HCD
分辨率设置：一级 70,000@m/z 200，二级 17,500@m/z 200 母离子扫描范围：m/z 200-1800；子离子扫描范围：start from m/z 100
Data-dependent MS/MS：Top 20
标准曲线与正式样本：
质谱仪：Thermo Scientific Q Exactive
喷雾电压：2.6 kV
毛细管温度：250 ℃
S-lens：50%
碰撞能量：27% HCD
分辨率设置：35,000@m/z 200
Isolation Window：4 M/Z
Inclusion List：M/Z 451.75

3 结果分析

3.1 筛选定量肽段

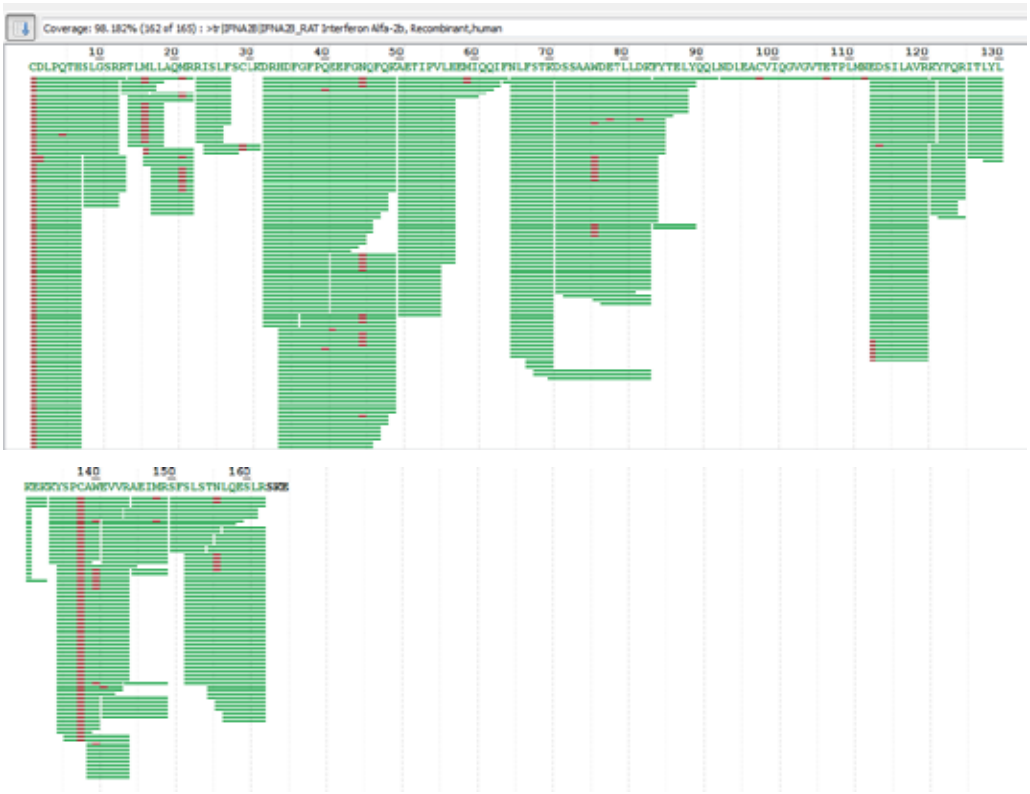
首先使用 Q-Exactive 对人重组干扰素 α-2b 标准品的酶解肽段进行 Full MS/ddMS2 扫描，上样量为 500ng（不计酶解损失），筛选人重组干扰素 α-2b 的 unique peptide，该肽段必须为人重组干扰素 α-2b 所特有的肽段，才可以用于表征人重组干扰素 α-2b 的量。
下图展示的为人重组干扰素 α-2b 色谱质谱的原始图，图中可见，整个色谱峰分离比较均一，强度比较高。



将上述得到的原始文件使用 Byonic 软件进行搜库处理，观察蛋白质的序列匹配度，数据库使用 DrugBank 下载的人重组干扰素 α-2b 的氨基酸序列。

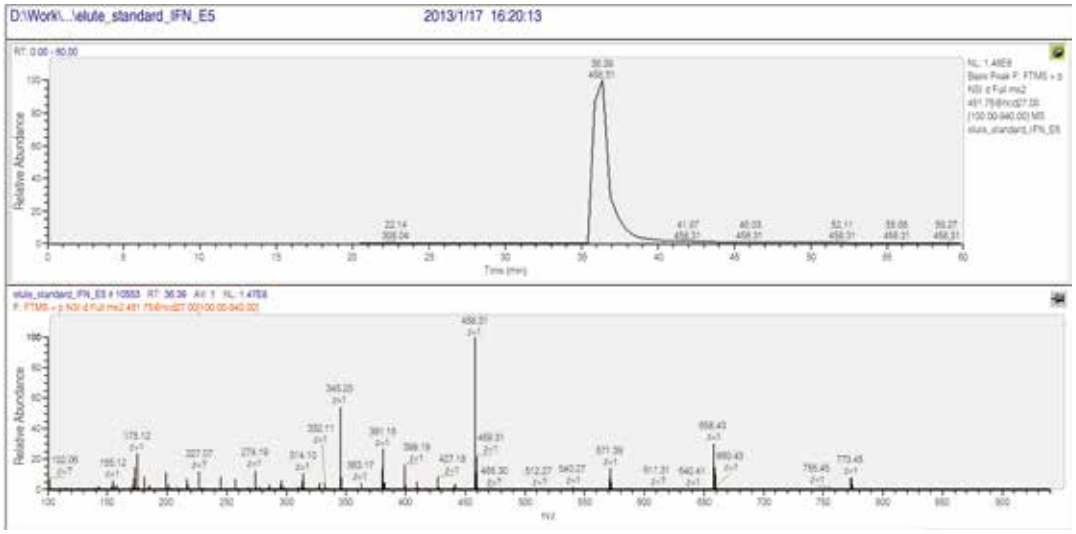
Protein Name	Log Prob	Best LogProb	Best score	Total Intensity	# of spectra	# of unique peptides	# of mod peptides	Coverage%	# AA's in protein
IFN	-314.48	-13.80	906.90	7312135064.4	929	137	39	98.18	165

从上述表格可见，人重组干扰素 α-2b 的氨基酸序列匹配度达到了 98.18%，说明 Q-Exactive 的超高灵敏度，在不到 500ng 的上样量的情况下可以对蛋白质进行高氨基酸序列匹配度的分析，对于后续数据分析的可靠性非常重要。下图展示的是人重组干扰素 α-2b 的具体氨基酸序列匹配情况。图中绿色标记的氨基酸序列为鉴定到的氨基酸，红色标记的为鉴定到的带有修饰的氨基酸，黑色标记的为实验中没有鉴定到的氨基酸。

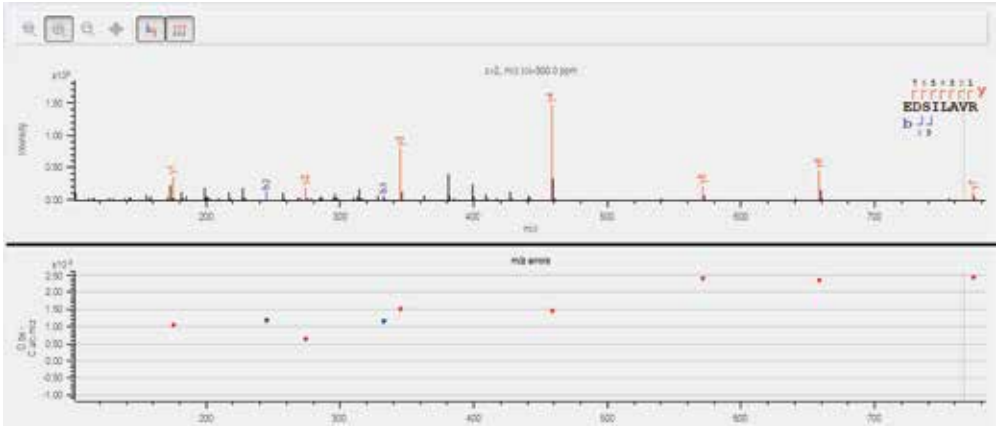


从上述图中可以看出，165 个氨基酸中只有最后三个氨基酸没有被鉴定到，分析发现 162 位的氨基酸为 R，因而在 Trypsin 的酶解中会被切割，产生的肽段为最后三个氨基酸，该肽段过短，不适于质谱检测。

从上述的色谱质谱原始图中可以看出在洗脱时间为 36.28min，m/z 为 451.75 的色谱峰其强度最高，搜库结果显示该色谱峰为人重组干扰素 α-2b 的 unique peptide“EDSILAVR”，下图展示的是 EDSILAVR（m/z 451.75）的母离子色谱质谱抽提峰及其二级谱图，

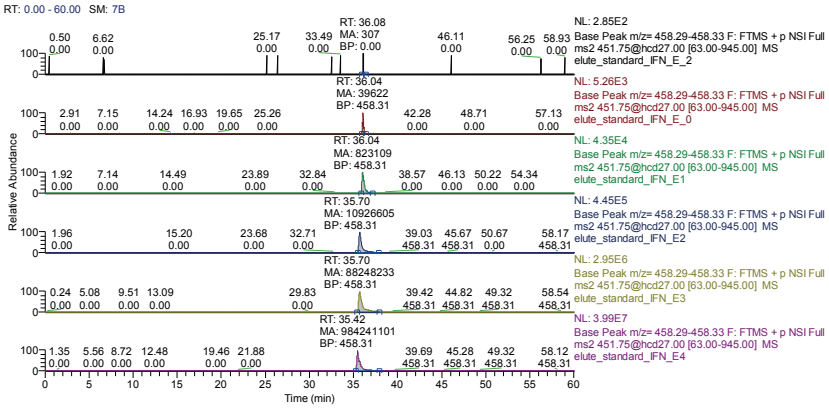


下图为上述的肽段 EDSILAVR 二级谱图的解析情况，图中可见所有的 y 系列离子均被鉴定到，子离子的实测 m/z 与理论 m/z 偏差均在 10^{-3} 水平，谱图质量非常高，结果非常可信。另外，从二级谱图中可以看出，二级碎片子离子 y4 ，m/z 458.31，其强度最高，因而在随后的数据分析中选取 451.75- 458.31 这一母离子 - 子离子对来表征 EDSILAVR 的含量，进而表征人重组干扰素 α-2b 的含量（该方法即为选择反应监测技术：SRM Selected Reaction Monitoring）。



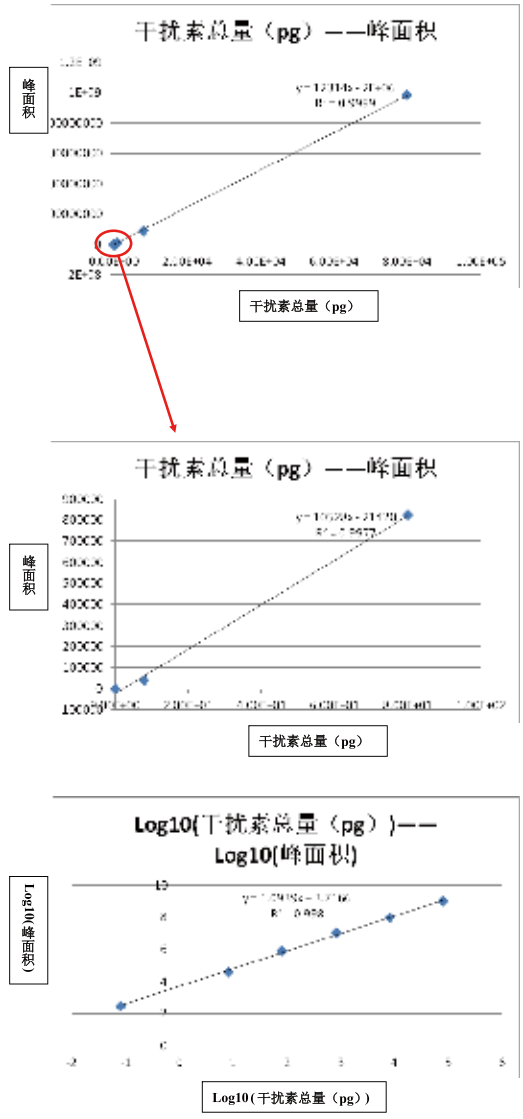
3.2 标准曲线绘制

人重组干扰素 α-2b 酶解的标准肽段稀释成以下几个浓　度：10^{4}ng/ml，10^{3}ng/ml，10^{2}ng/ml，10^{1}ng/ml，10^{0}ng/ml，10^{-2}ng/ml，各自吸样 8ul，与 10ul 空白血浆酶解的肽段样本混匀，上样 18ul，质谱检测，检测方法使用 Target MS2，inclusion list 为 m/z 451.75，isolation window 为 4 m/z。对子离子 458.31 进行抽提，结果见下图。



从上图可以看出，所有 6 个浓度的标准品均在 36min 左右有一个色谱洗脱峰出现，与上述标准品的洗脱时间一致，该色谱峰为 451.75-458.31 母离子 - 子离子对的色谱峰，即 EDSILAVR 的洗脱峰，分别对各个色谱峰的峰面积（即上图中色谱峰上显示的 MA 数值）进行积分，结果见下述表格。

浓度（ng/ml）	浓度（pg/ul）	上样体积（ul）	干扰素总量（pg）	峰面积
1.00E-02	1.00E-02	8	8.00E-02	307
1.00E+00	1.00E+00	8	8.00E+00	39622
1.00E+01	1.00E+01	8	8.00E+01	823109
1.00E+02	1.00E+02	8	8.00E+02	10926605
1.00E+03	1.00E+03	8	8.00E+03	88248233
1.00E+04	1.00E+04	8	8.00E+04	984241101



从上述结果中可以看出，在 6 个数量级的浓度范围内，浓度与峰面积的线性相关系数在 0.99 以上，说明 Q-Exactive 的检测动态范围足够大，可以很好的应用于蛋白质药物代谢动力学研究。

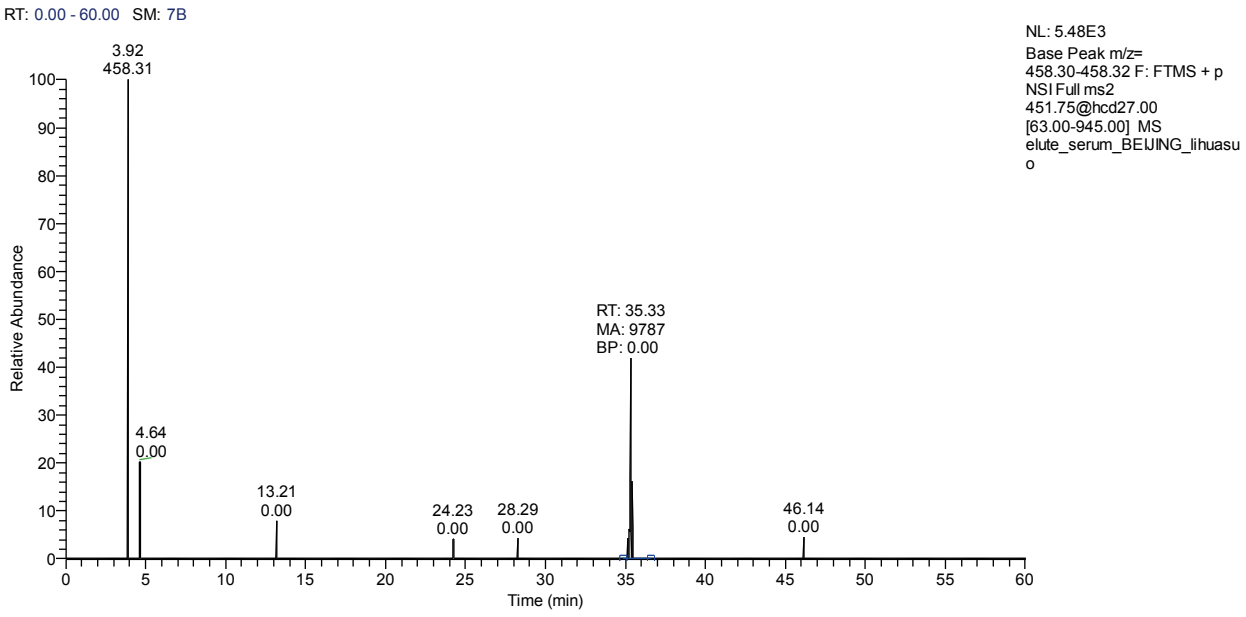
3.3 大鼠血浆样本中人重组干扰素 α-2b 浓度的测定

大鼠血浆样本上样 18ul，质谱检测，原始结果见下图，图中可见在 35.33min 有一个明显的色谱洗脱峰出现，洗脱时间与上述的标准品的洗脱时间相一致，为血浆样本中的人重组干扰素 α-2b 的肽段 EDSILAVR 的洗脱峰，对 m/z 458.31 这一子离子的面积进行积分。

峰面积为 9787, 代入上述的标准曲线中进行计算，得出原始血浆中人重组干扰素 α-2b 浓度为 26.9185 ng/ml。

4 总结

综上所述，Q-Exactive 拥有超高的灵敏度，在不到 500ng 上样量的情况下对人重组干扰素 α-2b 的氨基酸序列匹配度达到了 98.2%；Q-Exactive 拥有足够高的动态范围，在对人重组干扰素 α-2b 的标准品检测中，其动态范围达到了 6 个数量级，并且线性相关系数达到了 0.99 以上。简而言之，Q-Exactive 的超高性能可以很好的应用于大分子药物代谢的研究中去。



Simultaneous Quantitation of a Monoclonal Antibody and Two Proteins in Human Plasma by High Resolution and Accurate Mass Measurements

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Introduction

The quantitation of proteins in serum is essential for the assessment of the immune response to therapeutic proteins such as monoclonal antibodies and for understanding the immunological properties of these proteins. If therapeutic and endogenous proteins have to be distinguished or several proteins need to be monitored in parallel the use of ligand binding assays such as ELISAs is limited. Liquid chromatography coupled with mass spectrometry (LC-MS) is an emerging technology for this application. Whereas triple quad type mass spectrometers have been the instrument of choice for high throughput quantitation assays in the past, instruments providing high resolution and accurate mass have started to show their benefits in this application area.

Here we have developed a rapid and robust quantitation strategy of Cetuximab (Erbitux, a therapeutic monoclonal antibody for the treatment of colorectal cancer) and two additional protein standards (bovine serum albumin and bovine transferrin) spiked into human plasma with a LLOQ at the low pmol/mL level. Following the addition of isotope labeled standard peptides as internal standards, the plasma samples were reduced, alkylated and digested with trypsin. One of the key challenges in the sample preparation workflow, the control of the digestion step, was addressed by optimizing digest conditions such as time, temperature and amount of trypsin used. Samples were analyzed using a high resolution non-targeted full-scan-MS method in comparison with a targeted SIM and targeted MS/MS methods selectively focusing on proteotypic peptides and their corresponding isotope labeled analogs.

Experimental

General: Samples were denaturated, reduced and alkylated followed by overnight digestion with trypsin. After peptide clean-up and evaporation to dryness, the samples were reconstituted in acidified water/ACN (95:5, v/v) and analyzed by LC-MS resp. LC-MS/MS in high-resolution mode. The method covers a quantitation range of 6.60 to 660 pmol/mL for Cetuximab (19.8 to 1980 fmol on column), a concentration range of 13.0 to 1300 pmol/mL for bovine transferrin (39 to 3900 fmol on column), and a concentration range of 15.0 to 1500 pmol/mL for bovine serum albumin (45 to 4500 fmol on column).

Working-solutions and Sample Preparation: The target proteins were dissolved separately in denaturing buffer at concentrations of 2.00 mg/mL. Aliquots of 10.0 µL of each solution were spiked into 170 µL human plasma and subsequently diluted with human plasma containing the labeled internal standards of the target peptides. 20.0 µL reducing agent were added and the samples were incubated at 37C and 500 rpm for 1 hour. After the addition of 40.0 µL alkylation agent and incubation at room temperature for 30.0 minutes, 480 µL dilution buffer (pH ~ 8) and 36.0 µL trypsin were added and the samples were incubated overnight at 37C and 1000 rpm. Digestion was stopped by addition of

80.0 µL 10% TFA solution (pH < 2). After clean-up using a C18 spin plate, the combined eluates were evaporated to dryness and reconstituted in 100 µL acidified water/ACN (95:5, v/v).

Chromatography: Separations were performed on a Thermo Scientific Hypersil Gold (2.0 × 50 mm, 1.9 µm) column. Column temperature was kept at ambient temperature, flow rate was 0.3 mL/min. Aliquots of 10.0 µL were injected onto the column. Eluent A consisted of 0.1% aq. formic acid, eluent B of 0.1% formic acid in ACN. The eluent composition was changed linearly from 95% to 65% A in 7 minutes and then ramped to 5% A in 0.1 minutes. Eluent composition was kept at 5% A for 2.9 minutes and then went back to starting conditions.

Instrumentation and MS-Method : A HTS PAL autosampler (CTC Analytics) in combination with a Thermo Scientific Accela 600 quarternary pump were coupled to a Thermo Scientific Q-Exactive benchtop Quadrupole-Orbitrap mass spectrometer. Three MS data acquisition methods were applied: 1) *Full-scan-MS (non-targeted approach)*, using a mass resolution of 140.000 (@m/z 200), 2) *targeted and timed SIM* using a targeted precursor list in combination with retention time limits, using a window of 2 Da for each SIM scan and a resolution of 140.000. In order to increase the number of scans per peptide the multiplexing feature was used. 3) *targeted MS/MS*, acquiring full MS/MS spectra based on a precursor inclusion list in combination with retention times limits using an isolation window of 1.5 Da and a resolution of 17.500.

Results

Here we present a high resolution and accurate mass LC-MS method for the quantitation of the monoclonal antibody Cetuximab, also known as Erbitux, and two standard proteins bovine serum albumin (BSA) and bovine transferrin, spiked into human plasma. The benchtop Quadrupole-Orbitrap mass spectrometer used in these experiments offers a variety of acquisition options for quantitation [1]. Here we have used three different methods:

1) a Full-scan-MS method which is the easiest method and does not require any selection of precursors or fragment ions before data acquisition is performed.
2) a targeted SIM method comprising of the acquisition of narrow slices (≤ 10 Da) of a Full-MS scan. The method works in a targeted approach using a precursor ion list, plus in a timed approach, monitoring the parent ions each only in a distinct retention time window. In addition we have made use of the multiplexing option which provides a higher duty cycle by simultaneous detection of non-overlapping SIM windows in the Orbitrap mass analyzer.
3) a timed and targeted MS/MS method that selects precursor ions from an inclusion list only for a limited time provided as a retention time window. Full-MS/MS spectra are acquired (also called parallel reaction monitoring, PRM) allowing for selection of one or more fragment ions used for quantitation post-acquisition.

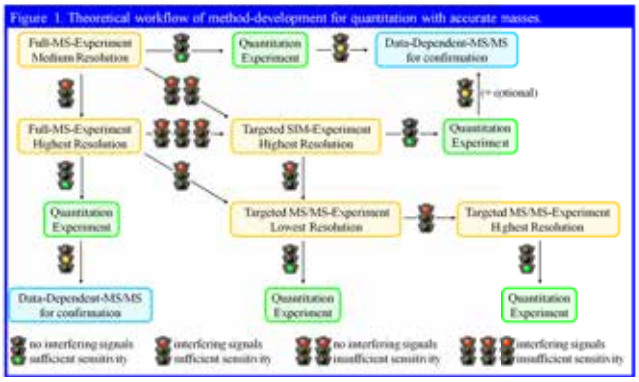
For the development of an absolute quantitation assay, even when a Full-scan-MS acquisition method is used not requiring the selection of precursor or fragment masses, the selection of proteotypic peptides being targeted in the quantitation experiment is essential for two reasons: in order to spike the isotope analogs into the sample as internal standard for quantitation but also as a reference for the retention time. Due to identical behavior during chromatography as their endogenous partners heavy labeled peptides are important indicators at what RT the peptide of interest elutes which is the key to a successful quantitation when analyzing very complex mixtures such as serum or plasma.

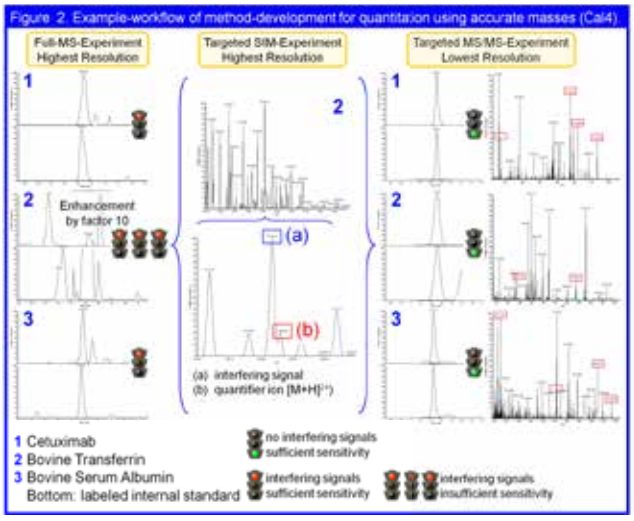
Here we have selected three peptides per protein and included each peptide as stable isotope labeled peptide in the assay. Eight calibration levels (CALs) and quality control (QC) samples were prepared to cover a concentration range of 6.6 to 660 pmol/mL for the monoclonal antibody, 13.0 to 1.300 pmol/mL for transferrin and 15.0 to 1500 pmol/mL for albumin. Peptide sequences, masses and fragment masses used for quantitation for all light and heavy peptides are listed in Table 1. Figure 1 summarizes the theoretical workflow in a decision tree for method development for a quantitation experiment using high resolution and accurate mass instrumentation. Figure 2 highlights an example for the data obtained for the peptides from the three proteins using a Full-scan-MS method (left) versus a targeted SIM

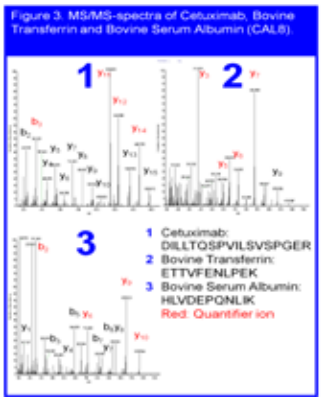
method (middle) versus a targeted MS/MS experiment (right).

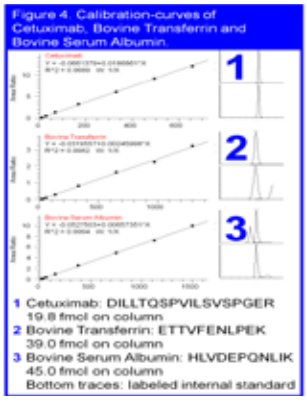
Data obtained from the Full-scan-MS method show a mixed result. For some peptides sensitivity and selectivity are sufficient, for some sensitivity is sufficient but quantitation is disturbed by interfering signals and for some peptides sensitivity is not sufficient and interfering signals are present. The sensitivity is significantly enhanced using the targeted SIM method, however the interfering signals even at the highest resolution setting are jeopardizing the quantitation. Results obtained using the targeted MS/MS method in contrast show sufficient sensitivity and no interfering signals due to the selectivity gain inherent with the isolation by the quadrupole. The benefit of using the targeted MS/MS method over the acquisition of SRMs on a triple quad type instrument is the possibility to decide post-acquisition which and how many fragment ions to use for quantitation. Figure 3 shows the MS/MS spectra for one of the peptides used for each of the three proteins, demonstrating the excellent spectral quality and richness in b- and y-ions detected. Calibration curves obtained from these three examples of peptides are shown in Figure 4, highlighting the excellent correlation coefficients. All samples were analyzed in triplicate. Intra-run precision and accuracy was determined at the LLOQ and above as listed in Table 2.

The scan speed and resulting number of scans per peptide and time scheduling of the targeted MS/MS experiments are shown in Figure 5 (right). In average 12-15 Full MS/MS spectra were obtained for each peptide (Figure 5, left).









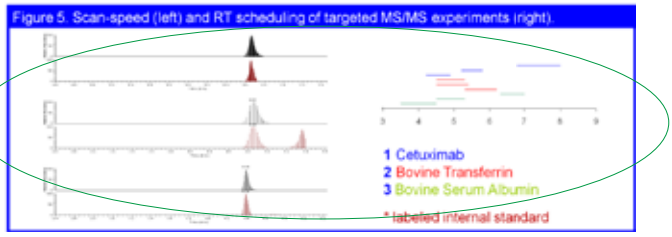


Table 1. MS-parameter

Protein	Peptide Sequence	Molecular formular $[M+H]^{2+}$	Parent ion mass [Da]	Product ion mass [Da] used for quantitation (post-acquisition)
Cetuximab	YASESISGIPSR	$C_{54}H_{89}N_{15}O_{20}$	633.8199	359.204, 616.341, 816.457, 1032.532
	YASESISGIPS[R($^{13}C_6{}^{15}N_4$)]	$C_{48}H_{89}N_{11}O_{20}{}^{13}C_6{}^{15}N_4$	638.8240	369.212, 626.350, 826.466, 1042.540
	ASQSIGTNIHWYQQR	$C_{78}H_{119}N_{25}O_{24}$	894.9424	594.299, 780.379, 917.438, 1302.634
	ASQSIGTNIHWYQQ[R($^{13}C_6{}^{15}N_4$)]	$C_{72}H_{119}N_{21}O_{24}{}^{13}C_6{}^{15}N_4$	899.9466	604.308, 790.387, 927.456, 1312.642
	DILLTQSPVILSVSPGER	$C_{85}H_{148}N_{22}O_{28}$	962.5411	342.202, 1153.658, 1240.690, 1469.796
	DILLTQSPVILSVSPGE[R($^{13}C_6{}^{15}N_4$)]	$C_{79}H_{148}N_{18}O_{28}{}^{13}C_6{}^{15}N_4$	967.5453	342.202, 1163.666, 1250.698, 1479.804
Bovine Transferrin	EPYFGYSGAFK	$C_{62}H_{82}N_{12}O_{17}$	633.2955	509.272, 729.357, 876.425, 1039.488
	EPYFGYSGAF[K($^{13}C_6{}^{15}N_2$)]	$C_{56}H_{82}N_{10}O_{17}{}^{13}C_6{}^{15}N_2$	637.3026	517.286, 737.371, 884.439, 1047.503
	ETTVFENLPEK	$C_{58}H_{93}N_{13}O_{21}$	653.8299	373.208, 600.335, 729.378, 876.446
	ETTVFENLPE[K($^{13}C_6{}^{15}N_2$)]	$C_{52}H_{93}N_{11}O_{21}{}^{13}C_6{}^{15}N_2$	657.8370	381.222, 608.349, 737.392, 884.460
	GSNFQLDQLQGR	$C_{57}H_{93}N_{19}O_{20}$	681.8417	601.342, 716.369, 829.453, 957.511
	GSNFQLDQLQG[R($^{13}C_6{}^{15}N_4$)]	$C_{51}H_{83}N_{15}O_{20}{}^{13}C_6{}^{15}N_4$	686.8458	611.350, 726.377, 839.461, 1114.588
Bovine Serum Albumin	HLVDEPQNLIK	$C_{66}H_{98}N_{16}O_{18}$	653.3617	251.150, 712.435, 1055.573, 1168.657
	HLVDEPQNLI[K($^{13}C_6{}^{15}N_2$)]	$C_{62}H_{98}N_{14}O_{18}{}^{13}C_6{}^{15}N_2$	657.3688	251.150, 720.449, 1063.587, 1176.671
	LVNELTEFAK	$C_{63}H_{88}N_{12}O_{17}$	582.3190	595.309, 708.393, 837.435, 951.478
	LVNELTEFA[K($^{13}C_6{}^{15}N_2$)]	$C_{47}H_{88}N_{10}O_{17}{}^{13}C_6{}^{15}N_2$	586.3261	603.323, 716.407, 845.450, 959.792
	TVMENFVAFVDK	$C_{64}H_{100}N_{14}O_{19}S$	700.3499	579.314, 678.382, 939.493, 1199.577
	TVMENFVAFVD[K($^{13}C_6{}^{15}N_2$)]	$C_{58}H_{100}N_{12}O_{19}S{}^{13}C_6{}^{15}N_2$	704.3570	587.328, 686.396, 947.507, 1207.591

MS-Parameter: ESI positive, spray voltage 4.0 kV, capillary temperature 300 C
Full-Scan-MS-Experiment: AGC 3e6, Max IT 100 msec, resolution 140.000, scan-range m/z 500 - 1000
Targeted SIM-Experiment: AGC 1e5, Max IT 120 msec, resolution 140.000, isolation window 2.0, scan-range m/z 400 - 1000
Targeted MS/MS-Experiment: AGC 1e5, Max IT 100 msec, resolution 17.500, isolation window 1.5 , norm. collision energy 27%

Table 2. Intra-run precision and accuracy.

Analyte		at LLOQ	> LLOQ
Cetuximab	$Prec_{Intra}$	6.8%	5.2 - 8.5%
	Acc_{Intra}	83.1%	93.6 - 112.8%
Bovine Transferrin	$Prec_{Intra}$	7.5%	3.0 - 12.6%
	Acc_{Intra}	117.3%	88.2 - 106.3%
Bovine Serum Albumin	$Prec_{Intra}$	3.4%	3.8 - 8.5%
	Acc_{Intra}	110.7%	98.3 - 111.0%

Conclusions

- A sensitive, robust and rapid HR/AM LC-MS/MS method for the simultaneous quantitation of a monoclonal antibody (Cetuximab) and two proteins (bovine transferrin and bovine serum albumin) in human plasma has been established.
- Targeted MS/MS provided best selectivity combined with sensitivity over Full-scan-MS and targeted SIM methods and provides qualitative (confirmation of correct peptide sequence) and quantitative information at the same time.
- Calibration curves with excellent correlation coefficients were obtained with very good intra-assay accuracy and precision.

References

[1] Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer. Gallien S, et al. 2012, Mol Cell Proteomics. Sep 7.

Multiplexing SIMs on a Novel Benchtop Orbitrap MS with a Quadrupole Mass Filter to Increase Sensitivity for Peptide Quantitation

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Overview

Purpose: To develop multiplexed selected ion monitoring (SIM) methods on a novel benchtop quadrupole-Orbitrap™ mass spectrometer for biopharmaceutical applications.

Methods: We employed a ten-minute LC method at 150 µL.min.$^{-1}$ Mass spectrometry analysis was performed using a quadrupole-Orbitrap LC-MS/MS systems equipped with a heated electrospray ionization (HESI) source. Wide SIM was used.

Results: A generic method development approach was used to analyze Insulin, Exendin-4 and GLP-1 peptides, between 10 pg.mL^{-1} and 10 µg.mL,$^{-1}$ with good linearity and reproducibility.

Introduction

In bioanalysis, triple quadrupole MS systems have been predominantly used for peptide quantitation. Although triple quadrupole systems generally offer the most sensitive and robust quantitation, the poor fragmentation and multiple charge states of large peptides can result in weak selected reaction monitoring (SRM) signals.

In this investigation, we demonstrate the accurate and precise quantification of peptides in a biological matrix suing a new approach that utilizes multiplexed SIMs capability provided on a novel benchtop quadrupole-Orbitrap mass spectrometer.

Multiplexed SIMs refers to multiple fills of the instrument's C-trap with quadrupolefiltered ions, prior to a Orbitrap mass analyzer scan.

Methods

Sample Preparation

Calibration curves were prepared in rat plasma.

Liquid Chromatography (Generic Separation)

A Thermo Scientific Accela UHPLC and Thermo Scientific Open Accela Autosampler were used to reproduce linear gradients on a 100 × 1mm, 1.9 µm particle size Thermo Scientific Hypersil GOLD column at 150 µL.min.$^{-1}$ The mobile phase was 0.1% formic acid in water and acetonitrile.

Mass Spectrometry

MS experiments were conducted on a Thermo Scientific Q Exactive high-performance benchtop quadrupole-Orbitrap system (Figure 1) operated in multiplexed SIM mode using 70,000 resolution (maximum 140,000 resolution at *m/z* 200). Targeted multiplexed SIM fills the C-trap with multiple packages of ions from the selected scan ranges. The C-trap collects the ions, and the Orbitrap mass analyzer analyses the ions previously collected. The isolation window used for the three peptides was 20 amu. To achieve maximum sensitivity, the AGC target values were optimized for each peptide.

Source conditions: heater temperature 200 ºC, capillary temperature 275 ºC, spray voltage 3.5kV, sheath gas 40, aux gas 10.

Data Analysis

The data were processed with Thermo Scientific LCQUAN quantitation software revison 2.7 using a 5 ppm mass window.

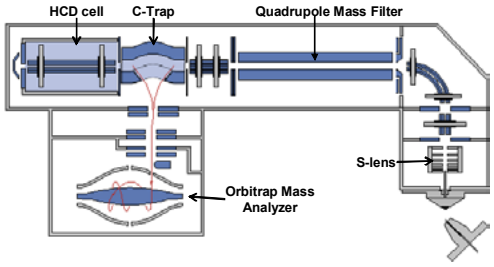


FIGURE 1. Schematic of the Q Exactive benchtop quadrupole-Orbitrap mass spectrometer.

Results

As shown in Figures 2 through 10, and Tables1 through 4, all three peptides showed excellent sensitivity and reproducibility, over large dynamic ranges.

Insulin

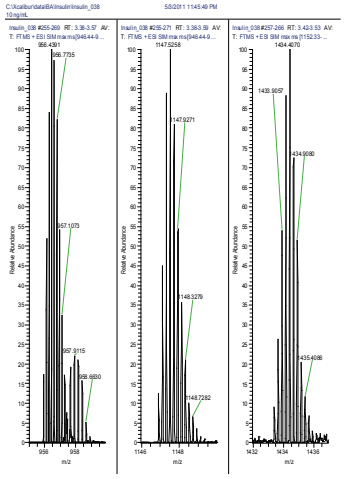


FIGURE 2. Spectra of the acquired mass peaks of human insulin at 70,000 resolution.

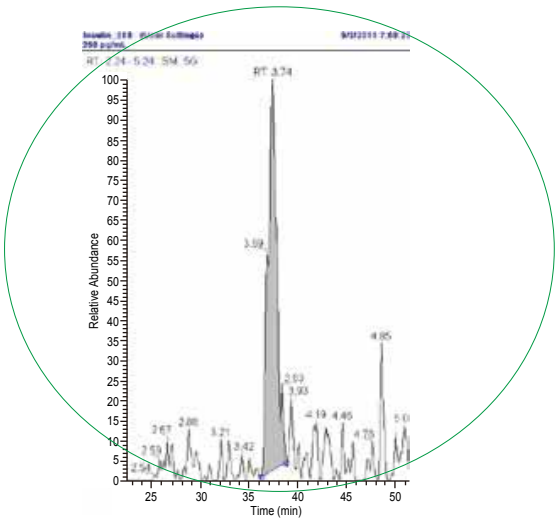


FIGURE 3. Chromatogram of 250 pg.mL^{-1} human insulin (LLOQ).

TABLE 1. Insulin summary table; dynamic range from 250 pg.mL^{-1} to 1000 ng.mL.$^{-1}$

Nominal Concentration (ng/mL)	Replicate #	Mean Calculated Concentration	Stdev	% CV
0.25	4	0.260	0.0300	11.5
0.5	4	0.434	0.0501	11.5
1	4	0.906	0.0540	5.96
2.5	4	2.81	0.0568	2.02
5	4	4.79	0.112	2.33
10	4	10.3	0.284	2.77
25	4	27.9	0.247	0.89
50	4	44.0	1.35	3.08
100	4	92.8	4.29	4.62
250	4	276	9.99	3.62
1000	4	979	36.7	3.75

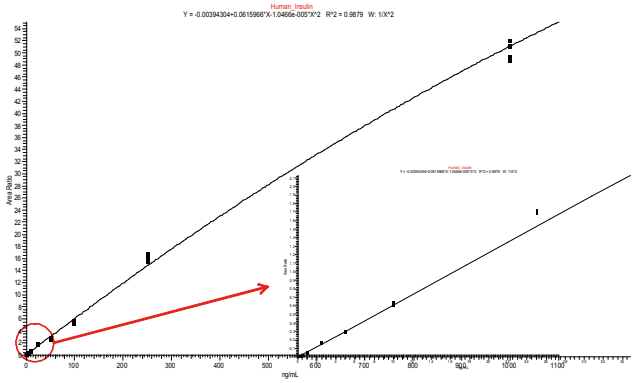


FIGURE 4. Insulin calibration curve, dynamic range from 250 pg.mL^{-1} to 1000 ng.mL^{-1}

Exendin-4

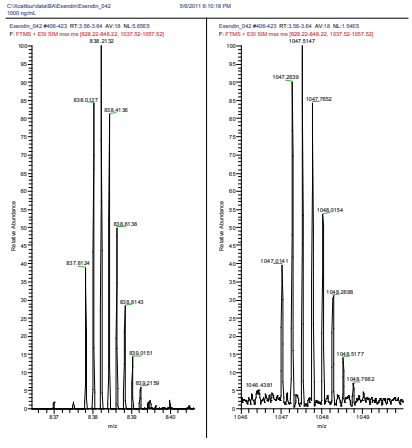


FIGURE 5. Spectra of the acquired mass peaks of Exendin-4 at 70,000 resolution.

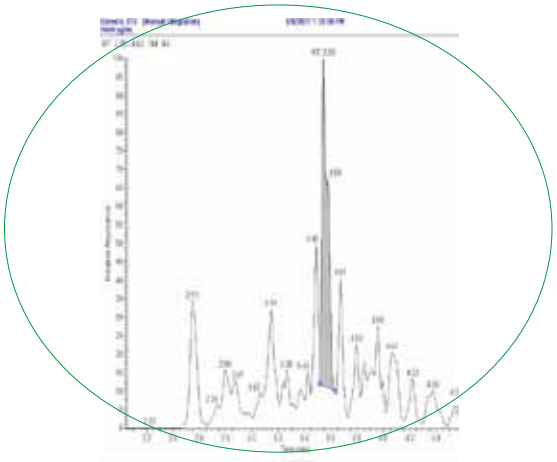


FIGURE 6. Chromatogram of 5 ng.mL^{-1} Exendin-4 (LLOQ).

TABLE 2. Exendin-4 calibration table; dynamic range from 5 ng.mL^{-1} to 10000 ng.mL.$^{-1}$

Nominal Concentration (ng/mL)	Replicate #	Mean Calculated Concentration	Stdev	% CV
5	4	5.143	0.418	8.13
10	4	9.693	0.520	5.36
25	4	24.37	1.22	5.00
50	4	45.46	0.705	1.55
100	4	92.83	13.0	14.0
250	4	261.7	5.31	2.03
500	4	507.3	2.03	0.40
1000	4	966.0	25.2	2.61
2500	4	2675.6	60.7	2.27
5000	4	5205	169	3.24
10000	4	9740	150	1.54

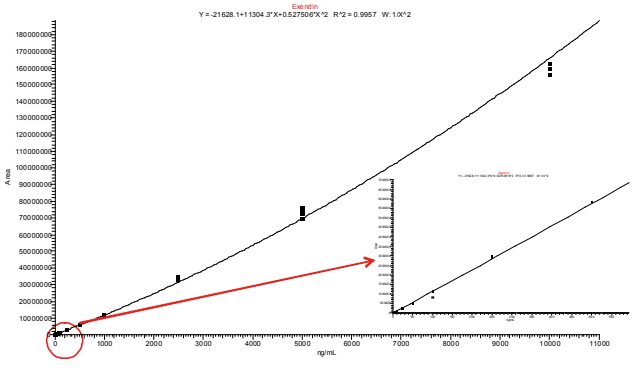


FIGURE 7. Exendin-4 calibration curve, dynamic range from 5 ng.mL^{-1} to 10 µg.mL^{-1}

GLP-1:

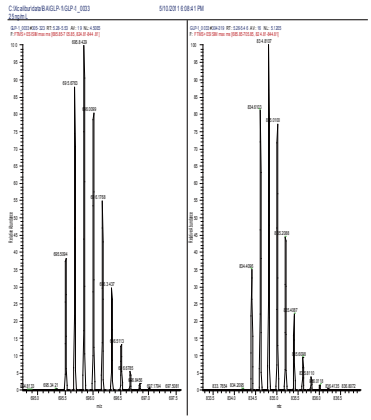


FIGURE 8. Spectra of the acquired mass peaks of GLP-1 at 70,000 resolution.

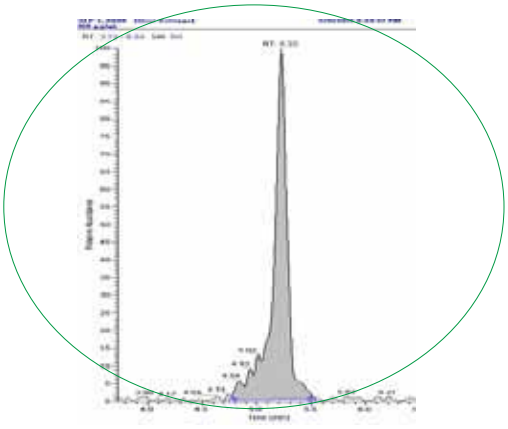


FIGURE 9. Chromatogram of 5 ng.mL^{-1} GLP-1 (LLOQ).

TABLE 3. GLP-1 summary table; dynamic range from 0.1 ng.mL^{-1} to 10000 ng.mL.$^{-1}$

Nominal Concentration (ng/mL)	Replicate #	Mean Calculated Concentration	Stdev	% CV
0.1	4	0.099	0.0098	9.91
0.25	4	0.251	0.0205	8.16
0.5	4	0.534	0.0337	6.31
1	4	0.905	0.0226	2.50
2.5	4	2.69	0.0578	2.15
5	4	5.36	0.126	2.36
10	4	9.99	0.120	1.20
25	4	26.6	0.664	2.50
250	4	50.6	0.562	1.11
100	4	80	0.692	0.86
250	4	243	14.2	5.86
500	4	462	15.5	3.35
1000	4	860	7.90	0.92
5000	4	5383	62.8	1.17
10000	4	9796	164	1.67

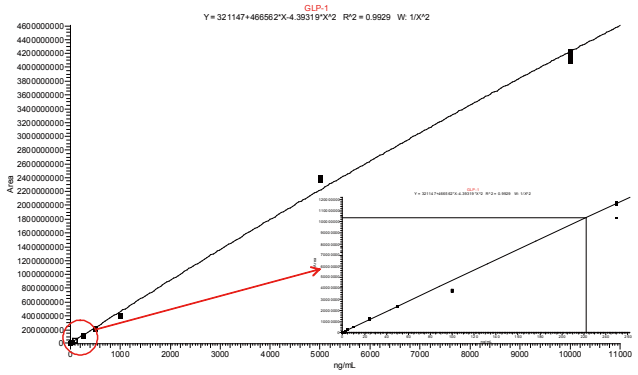
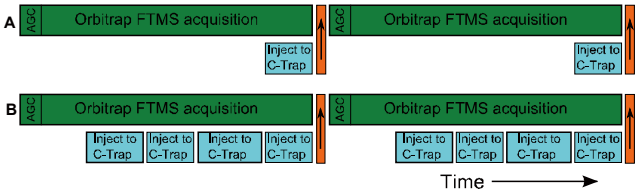


FIGURE 10. GLP-1 calibration curve; dynamic range from 5 ng.mL^{-1} to 10 µg.mL.$^{-1}$

Principle of Multiplexing

Figure 11 compares the standard operation mode with the spectrum multiplexing approach in the scan-to-scan AGC (automatic gain control) mode. In both modes, the instrument is operating in parallel mode. While one scan is acquired, the ions for the next scan are already collected. In the standard operation mode, only one precursor ion species is injected. With spectrum multiplexing, the idle time is used to inject several precursor ion species, resulting in higher throughput.

FIGURE 11. Standard operation mode (A) versus spectrum multiplexing (B).



Conclusion

- The Q Exactive™ high-performance benchtop quadrupole-Orbitrap mass spectrometer is a sensitive quantitation instrument that displays equal or better LOQs than the LOQs reported in the literature for high-end triple quadrupole instruments.[1] The Q Exactive mass spectrometer was found to be the instrument of choice for high-sensitivity quantitation with the following LOQs:
  - Insulin to an LOQ of 250 pg.mL^{-1}
  - Exendin-4 to an LOQ of 5 ng.mL^{-1}
  - GLP-1 to an LOQ of 0.1 ng.mL^{-1}
- The use of multiplexed SIMs is very effective for the quantitation of larger peptides such as insulin, exendin-4 and GLP-1. This technology makes the Q Exactive mass spectrometer highly selective.
- Because the instrument's multiplexing capability is coupled with advanced signal processing, its scan speed is fast enough to capture data across narrow UHPLC peaks. For example, 12 - 15 scans were recorded across the 6 s-wide exendin-4 chromatographic peak.
- These experimental results portend the applicability of the Q Exactive mass spectrometer for the quantitation of peptides, small proteins and oligonucleotides.

References

1. Quantification of Insulin at Micro dosing Levels Using LC-MS/MS, Montreux LCMS conference, Louis Maljers, Jonathan McNally & Rohan Thakur.
2. Application of DBS for quantitative assessment of the peptide Exendin-4; comparison of plasma and DBS method by UHPLC–MS/MS, Jonathan R Kehler, Chester L Bowen, Sharon L Boram & Christopher A Evans, Platform Technology and Science, Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Pharmaceuticals, 709 Swedeland Road, King of Prussia, PA 19406, USA.
3. "Multiple C-Trap Fills as a Tool for Massive Parallelization of Orbitrap Mass Spectrometry- a new Concept for Targeted Mass Analysis." Oliver Lange; Jan- Peter Hauschild; Alexander Makarov; Ulf Fröhlich; Catharina Crone; Yue Xuan; Markus Kellmann; Andreas Wieghaus. Thermo Fisher Scientific, Bremen, Germany; ASMS 2011, Denver, CO, USA. Monday Poster #103.

Developing a Method to Protect the Integrity of Racing Using Targeted SRM: Detection and Quantitation of rhEPO/DPO in Horse Plasma

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Overview

Purpose: To develop a method for the detection and confirmation of rhEPO/DPO in horse plasma using a targeted protein assay and labeled internal standards.

Methods: Combined immunoaffinity separation, enzymatic digestion, and mass spectrometry has been used to confirm the presence of rhEPO in horse plasma.1 The use of an SRM method for targeted protein detection enabled measurements of retention times, ion ratios, and labeled internal standards to confirm and quantify the presence of rhEPO in horse plasma.

Results: Using labeled internal standards, rhEPO was detected, quantified and confirmed in administered horse plasma 72 hours following administration, simulating real world situations.

Introduction

Recombinant human erythropoietin (rhEPO)[2] and Darbepoetin-alpha (DPO)[3] are genetically engineered protein-based drugs used for the treatment of anemia by stimulating red blood cell production. The ability of these agents to stimulate red blood cell production has led to use and abuse by human and equine athletes and, thus, violates the rule of fair competition resulting in their classification as banned substances by the horse racing industry. In addition, continued administration to horses can result in anemia.[3] Despite the negative aspects of rhEPO for horses, a reliable, verifiable, and legally defensible method for identification and confirmation of rhEPO/DPO has been elusive due to the very low concentrations administered. Sample collection is typically acquired only after competition, which could be in excess of 72 hours following administration. Testing of rhEPO/DPO is further confounded by the complexity of the matrices in which the drug is typically found–plasma and urine.

Methods

All experiments were performed using a Thermo Scientific TSQ Quantum Access triple quadrupole mass spectrometer equipped with a Thermo Scientific Surveyor™ MS Pump and MicroAS Autosampler (Thermo Fisher Scientific, San Jose, CA) operated in mSRM mode monitoring six diagnostic peptides that differentiate rhEPO and DPO from equine EPO. (Scheme 1). In addition to the six diagnostic peptides, four stable isotope labeled internal standards for the T_4, T_6, T_{11}, and T_{17} rhEPO proteotypic peptides were used for absolute quantification and additional confirmation of the presence of rhEPO/DPO (Thermo Biopolymers, Thermo Fisher Scientific, Ulm, Germany). Method development was performed using neat rhEPO/DPO protein digests. (Amgen, Inc., Thousand Oaks, CA).

HPLC separations were achieved using a Hypersil Biobasic™ C18 100 × 0.5 mm column and a binary solvent system consisting of A) 0.1% formic acid and B) MeCN (0.1% formic acid). A gradient profile of 2-40% B

in 12 minutes was used at 60 µL/min.

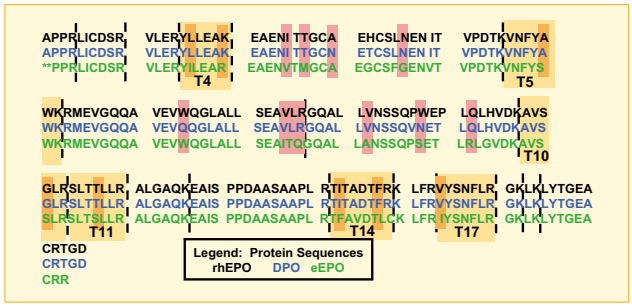
Sample preparation included immunoaffinity separation using rabbit and mouse IgG antibodies linked to magnetic beads. Following separation, the resulting protein was filtered and enzymatically digested with an enzymatic or proteolytic cleavage from which a set of diagnostic peptides representing rhEPO/DPO was chosen as candidate biomarkers for confirmation of the presence of rhEPO/DPO in horse plasma.[1]

Two different sets of samples were prepared and analyzed. The first set was a controlled spiking experiment in which a known quantity of rhEPO was spiked into 1 mL of digested horse plasma to determine detection efficiency. The second sample set was plasma extracted as a function of time following rhEPO administration (iv) of 8000 IU. The time points for extraction ranged from 0 hr to 72 hours. Each of the time point samples was spiked with 10 fmol/µL of the labeled peptide standards.

Results and Discussion

Scheme 1 shows the basis of identification for rhEPO/DPO in equine plasma. The results of enzymatic digestion produced multiple diagnostic markers that can be used to increase the confidence of the presence of the foreign substance in the equine athlete. In addition, the method described enables detection of rhEPO or DPO due to the conserved sequence for each protein over the targeted peptides. Figure 1 shows summed SRM chromatograms for (1A) DPO and (1B) rhEPO using the same SRM transitions. Clearly, the retention times are closely identical for both samples indicating the experimental method is robust for either drug.

Figure 2 shows the summed SRM chromatograms for the four targeted rhEPO peptides and the labeled analogues. The labeled peptide can be used to confirm the correct elution time as well as the ion ratio provided more than one transition was used to monitor each peptide. A level of 500 amol on column was used to test the detection capabilities of the approach used, which would equate to a concentration of ca. 1.7 ng/mL. Note that the responses of T_4, T_{11}, T_{17} markers were greater than 10000 counts, indicating lower levels of detection to be about 10x lower (or 0.2 ng/mL) without requiring nanoliter flow



Scheme 1. Comparison of protein sequences for rhEPO, DPO, and equine EPO. The dashed lines represent sites of enzymatic cleavages and the red boxes highlight non-conserved sequence sites between rhEPO/DPO and equine EPO. The targeted peptides are marked with a gold box.

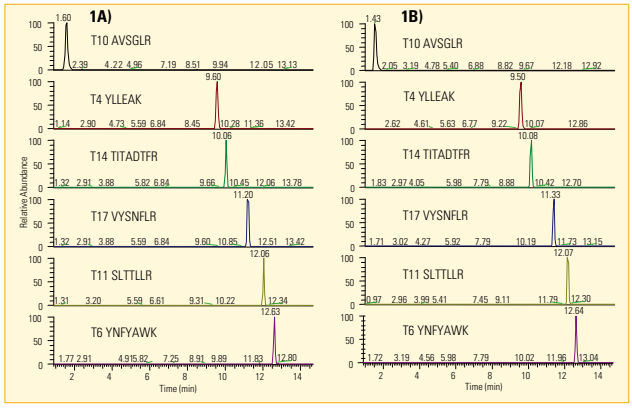


Figure 1. SRM chromatographic traces for each of the targeted peptides for 1A) DPO and 1B) rhEPO enzymatic digest using identical experimental method.

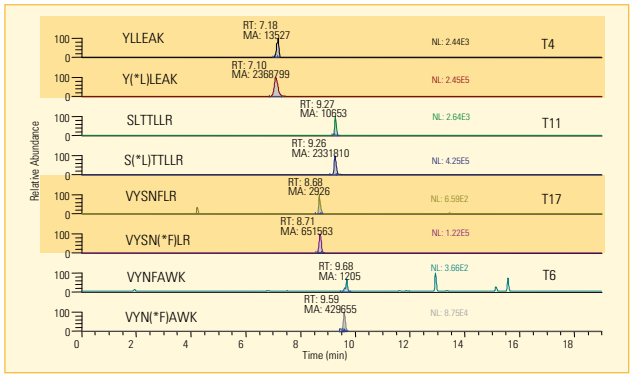


Figure 2. SRM responses for four targeted rhEPO peptides and the corresponding stable isotope labeled peptide. The measured response is for a total of 500 amol on column for the unlabeled rhEPO and 100 fmol for the labeled rhEPO peptides.

rates, which simplifies the experiment and increases the robustness of the method.

In addition to establishing the correct retention times for targeted peptides, the stable-isotope labeled peptides can be used for correct ion ratio determination as an additional means of verification. Figure 3 shows comparative full-scan product ion spectra for the (3A) unlabeled and (3B) labeled T_{11} peptide. Note the y-series detected for each, providing sequencing information and site determination for the stable isotope labeled residue such as the a_2/b_2 fragments as well as the y_6 for the unlabeled peptide. The two product ions used for detecting the T_{11} peptide were the y_4 and y_5 ions. The calculated abundance ratios for the unlabeled and labeled peptides were ca. 25%. The insets to the right of Figure 3 show the measured ion abundance for each SRM transition at 500 amol level. The calculated ratio is within experimental error to be used as an additional means of confirmation for the targeted peptide elution.

Figure 4 shows the quantification curve calculated for the controlled rhEPO spiking of horse plasma. The values show excellent agreement between theoretical and experimentally determined levels based on the integrated peak area ratios between the unlabeled and labeled targeted rhEPO peptides. The %CVs for each was less than 20% at 500 amol level indicating excellent capabilities to quantify the presence of rhEPO in plasma. While a positive confirmation would only require one diagnostic peptide to be present, this method yields four proteotypic peptides that could be used unequivocally to increase the confidence in a positive determination.

The second sample set was used to test the entire workflow. A female horse (500 kg) was administered rhEPO intravenously using 8000 IU (0.08 mg/kg) for four days. Following the injection on the fourth day, blood was withdrawn at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 48, and 72 hour intervals. Samples for each time point were processed using the method outlined previously, reducing complexity of the resulting protein digest mixture. The protocols of most horse racing commissions require the saliva, urine, and/or blood sample to be taken from the winning horse following completion of a race. The 72 hour time window represents a possible maximum duration between the final doping and racing while maintaining a pharmacological effect following administration of rhEPO/DPO. The 8000 IU dose is also an estimate of the dose required to induce the desired biological effects of increasing oxygen carrying capacity for equine athletes. The proposed protocol must enable a reduction of sample loss through the number of sample purification, filtering, reconstitution, and digestion steps prior to mass spectral analysis. Figure 5 shows the summed SRM chromatograms for the four targeted rhEPO peptides with their stable-isotope labeled internal standards. Three of the four peptides showed a positive response with little signal attributed to the T_6 peptide at the 72 hour time point. Although the response for the T_4 peptide does not appear to be measurable, closer inspection shows an integrated peak area over 2000 counts observed to have the same retention time as that for the labeled T_4 peptide at 7.27 minutes.

Comparison of chromatographic retention times for the administered rhEPO study with the spiked rhEPO study (Figure 2) showed excellent chromatographic reproducibility, with retention times that shifted less than 6-10 seconds, enabling an additional means of confirmation for the presence of rhEPO in the extracted horse plasma. Based on the integrated peak area ratios for the three detected rhEPO biomarkers, a total of ca. 0.05 ng/mL was present in the horse plasma following a 72-hour delay between rhEPO administration and sample collection.Comparison of LC-MS/MS results with those measured using ELISA show similar levels (0.04 ng/mL–data not presented) indicating excellent agreement between the two methods.

Using a stable-isotope labeled internal standard provides two clear advantages: identification of the correct retention times, as shown above, and determination of the correct ion ratio for the monitored product ions. Figure 3 demonstrates the consistency of the ion ratios measured following CID for both full scan MS/MS detection as well as SRM analysis for the T_{11} labeled and unlabeled rhEPO peptides. The same measurements can be used to confirm the presence of rhEPO at each time point. Figure 6 shows the measured ion abundance for the y_4 and y_5 fragment ions for the unlabeled and labeled T_4 peptides at the time points of 72, 10, and 0.5 hrs following the final rhEPO administration. The measured ion ratios for the unlabeled

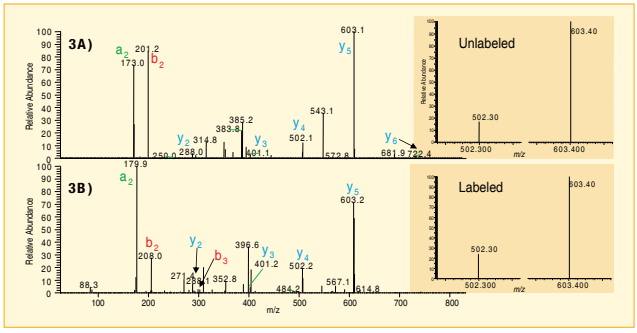


Figure 3. Comparative Quantitation Enhanced Data-Dependent™ MS/MS spectra for 3A) unlabeled and 3B) labeled T_{11} peptides at 50 fmol on column. The inset shows the measured ion intensity for the SRM transitions for the unlabeled and labeled T_{11} peptides at 500 amol on column.

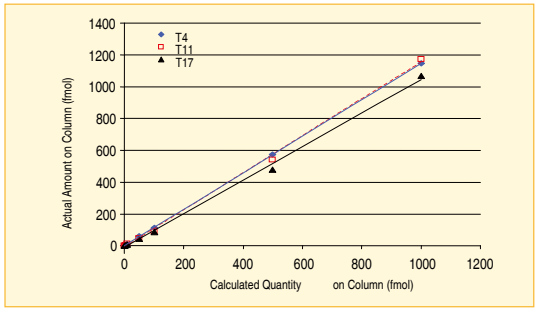


Figure 4. Quantification curve for neat rhEPO analysis. The calculated levels were determined using area ratios of the labeled analogues.

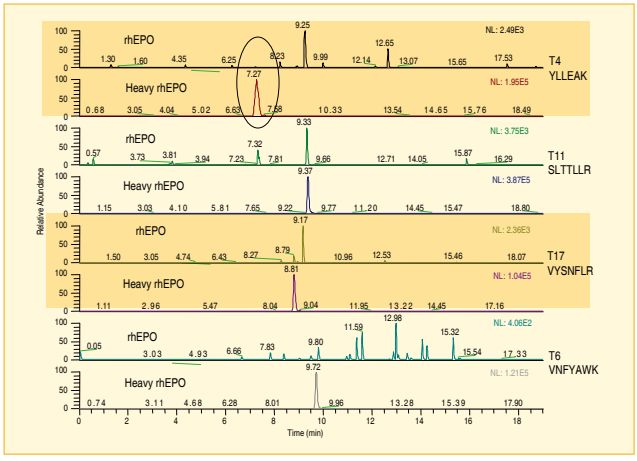


Figure 5. Summed SRM chromatograms for the four targeted rhEPO peptides and their labeled derivatives for the horse plasma extraction sample collected 72 hours following rhEPO administration.

T_4 peptides were consistently between 20 and 25% while the ratio for the labeled T_4 peptide was consistently between 30 and 35%. The slight increase in the ratio for the labeled peptide was observed for the three other pairs of signature peptides (see Figure 3).

Figure 7 shows the calculated rhEPO concentration in the extracted horse plasma samples for T_4 and T_6 peptides. The levels were calculated using the integrated area ratios between the targeted rhEPO peptide and their corresponding labeled internal standards. The calculated concentration for two targeted peptides agree with those obtained using two different labeled standards to monitor the concentration of rhEPO in the test sample. In addition to mass spectral determination, ELISA was also used to A) predict the presence of rhEPO and B) calculate the level of rhEPO in plasma at each time point. The ELISA results nicely corresponded with those calculated using the targeted SRM approach; in fact, the levels estimated at 48 and 72 hours agreed well (0.06 and 0.04 ng/mL, respectively), increasing the confidence in the calculated concentrations.

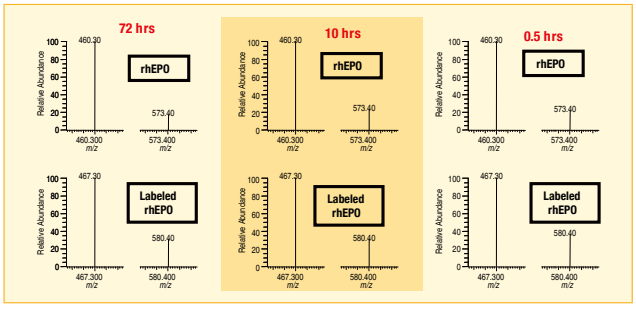


Figure 6. Comparative ion abundance ratios for the T_4 peptide at three different time points for plasma collection. The top row is the measured ion abundance for the unlabeled peptide and the bottom row is the response from the labeled peptide.

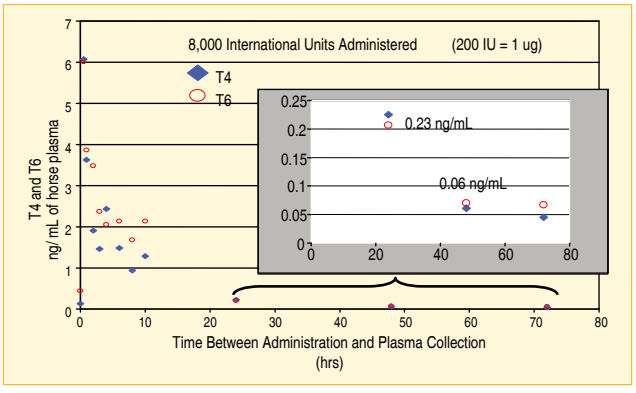


Figure 7. Calculated rhEPO levels in extracted horse plasma as a function of time delay between administration and sample collection. The calculated levels were based on the area ratios of T_4 and T_6 targeted rhEPO peptides and the labeled internal standards.

Conclusions

The approach presented here provides a sensitive and selective method for preparing and analyzing horse plasma for the presence of rhEPO or DPO. The advantages of this method include the ability to use up to six diagnostic peptides to confirm or refute the presence of either illegal protein-based drug.

The use of stable-isotope labeled analogues provides further means of confirming the presence of diagnostic peptides based on chromatographic retention times and ion ratios.

The sensitivity demonstrated enabled detection up to 72 hours following the last administration of rhEPO, increasing the confidence that the described method is useful in the racing industry to maintain a level field of competition.

Of particular interest is the measured sensitivity that was achieved using microspray, increasing the analysis time while simplifying the experimental method and thus, enabling more laboratories the option of employing rhEPO/DPO screening.

References

1 Guan, F., Uboh, C., E., Soma, L. R., Birks, E., Li, X., Chen, Y., Mbuy, G., LC-MS/MS Method in the Confirmation of Recombinant Human Erythropoietin and Darbepoietin-alpha in Equine Plasma, accepted by Analytical Chemistry (see WP 245 for additional applications).

2 Krantz, S. B. Erythorpoietin, *Blood*, **1991**, 77, 419-434.

3 Egrie, J. C., Browne, J. K., Development and Characterization of Novel Erythropoiesis Stimulating Protein (NESP), *British Journal of Cancer*, **2001**, 84, 3-10.

4 Piercy, R. J., Swardson, C. J., Hinchcliff, K. W., Erythroid Hypoplasia and Anemia Following Administration of Recombinant Human Erythropoietin to Two Horses, *Journal of the American Veterinary Medical Association*, **1998**, 212, 244-247.

Acknowledgements

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TSQ Vantage 质谱仪测定两种多肽类药物戈舍瑞林和艾塞那肽

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前言

戈舍瑞林 (Goserelin，图 1) 是一种治疗乳癌和前列腺癌的多肽类药物，艾塞那肽 (Exenatide，图 2) 是首个化学全合成的肠促胰岛素类似物，可降低 2 型糖尿病患者的空腹和餐后血糖。

和传统药物比较，蛋白多肽类药物的体内检测面临相当大的难度，其主要原因是：一，这类药物多为生理活性物质，化学结构特殊，稳定性差，体内处置十分复杂。二，生物体内存在大量干扰物质，如结构相同或相似的内源性蛋白多肽类物质，细胞因子、蛋白类治疗药物降解产物、体液基质、体内结合蛋白以及针对治疗药物的抗体等。故要求检测方法应高度特异。如何特异地将目标蛋白多肽分子与干扰物质区分是一大难题。三，该类药物生理活性强，用药剂量很小，通常为微克级水平，致体液中药物浓度极低，故要求检测方法必须具有极高的灵敏度。

以往蛋白多肽研究主要依赖光谱法、生物检定和免疫分析等方法。其中光谱法的专属性和灵敏度较低，主要适用于分析生物样本中含量较高的药物；生物检定缺乏特异性、灵敏度差且耗时；免疫法的特点是快速、简便和灵敏度高，但该方法建立时须针对每一种药物制备特异性抗体和标记药物，通常需要购买相应的试剂盒，且不适用于多组份复杂药物的分析，所以主要局限于生化药物的样本分析。

LC-MS/MS 集高效液相色谱的高分离性能与质谱的高灵敏度、高专属性的优点于一体，因为其对多数药物结构的通用性、检测的专属性和灵敏度等各方面的优势，已迅速成为药物代谢与药物动力学研究中采用的主要分析方法。利用 LC-MS/MS 从事蛋白质多肽类药物药代动力学研究已成为未来发展的趋势。

TSQ Vantage 质谱仪的高灵敏度和独有的高选择性离子检测模式 (H-SRM, FWHM<0.2 amu）使其适合于多肽类药物药代动力学研究。TSQ Vantage 质谱仪的优越性能来源于它在离子传输方面的技术创新。新型的 S 形离子光学透镜系统使用新型的静电场技术捕获离子，并有效地将其传输进入双曲面四极杆质量分析器。相比以 Skimmer 为基础的离子传输设计，S 形透镜设计具有显著的先进性，它可消除质量歧视，并降低昂贵的分子涡轮泵的气体负荷。这项创新设计可保持更干净离子光学传输路径，同时降低了背景噪音，提高了灵敏度。除此之外，TSQ Vantage 质谱仪采用的四级杆是共轭双曲面四级杆，符合理论上四级杆中的电场分布，因而使其拥有高选择性离子检测模式，可以用于排除共流出物中质荷比相近化合物的污染，显著提高复杂样品中的灵敏度。总之，这套新系统使小分子、生物分子及多肽领域的科学家能够检测超痕量的化合物，并具有极佳的精密度和重现性。

本实验采用 TSQ Vantage 质谱仪，建立了快速、准确、高灵敏度测定两种多肽类药物戈舍瑞林 (Goserelin) 和艾塞那肽 (Exenatide) 的方法。

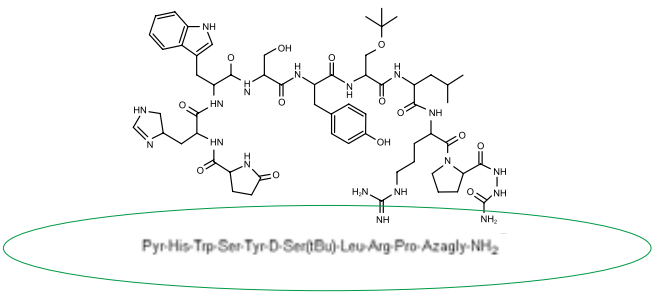

图 1. 戈舍瑞林结构式

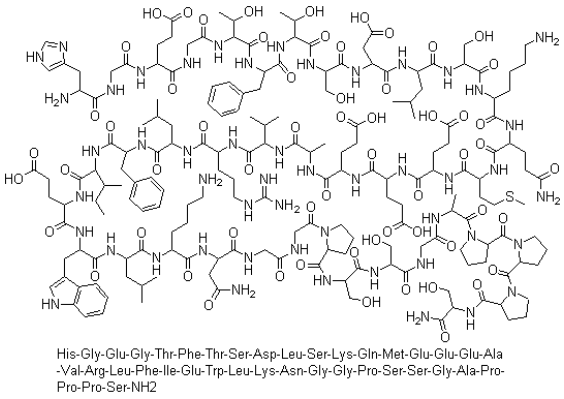

图 2. 艾塞那肽结构式

实验部分

2.1 仪器与试剂

TSQ Vantage 串联质谱仪（赛默飞世尔科技 Thermo Fisher Scientific 公司），配置 Surveyor 液相色谱系统（Thermo Fisher Scientific 公司），Thermo Hypersil Gold C18 柱（100×2.1mm，3 μm）。

标准品：戈舍瑞林 1.0mg，用甲醇 :0.2% 甲酸 (80:20) 配制并稀释成浓度为 1.0 mg/mL 的混合标准溶液；艾塞那肽 1.0mg，用甲醇 :0.2% 甲酸 (80:20) 配制并稀释成浓度为 1.0mg/mL 的混合标准溶液。

甲醇（德国 Merck 公司），甲酸（HPLC 级，美国 Thermo Fisher 公司），水为二次蒸馏水。

2.2 色谱与质谱条件

戈舍瑞林色谱条件：Thermo Hypersil Gold C18 柱（100×2.1mm，3 μm），柱温 35 ℃；流动相 A：0.01% 甲酸水溶液，流动相 B：甲醇，采用梯度洗脱（洗脱程序见表 1）；流速：0.20mL/min；进样体积：10 μL。

表 1. 戈舍瑞林梯度洗脱程序

时间（min）	A（%）	B（%）
0	70	30
1	70	30
2	5	95
4	5	95
4.1	70	30
5	70	30

戈舍瑞林质谱条件： ESI 正离子模式。喷雾电压：4.0 KV; 鞘气：25 arb；辅助气：10 arb；毛细管温度：380 ℃；碰撞气：氩气（1.5 mtorr）。采用选择反应监测（SRM）扫描模式，戈舍瑞林的 SRM 参数见表 3。

艾塞那肽色谱条件：Thermo Hypersil Gold C18 柱（100×2.1mm，3 μm），柱温 35℃；流动相 A: 0.2% 甲酸水溶液，流动相 B：甲醇，采用梯度洗脱（洗脱程序见表 2）；流速：0.20mL/min；进样体积：10 μL。

表 2. 艾塞那肽梯度洗脱程序

时间（min）	A（%）	B（%）
0	80	20
1	80	20
2	5	95
4	5	95
4.1	80	20
5	80	20

艾塞那肽质谱条件： ESI 正离子模式。喷雾电压：4.5 KV; 鞘气：35 arb；辅助气：10 arb；毛细管温度：350 ℃；碰撞气：氩气（1.5 mtorr）。采用选择反应监测（SRM）扫描模式，艾塞那肽的 SRM 参数见表 3。

表 3. 戈舍瑞林和艾塞那肽的 SRM 参数

化合物（Comp.）	分子式（Formula）	母离子（parent ion）	子离子（Product Ion）	碰撞能量（Collision Energy, V）
戈舍瑞林（Goserelin）	$C_4F_9SO_3NA$	635.375（+2 价）	607.217	15
艾塞那肽（Exenatide）	$C_{184}H_{282}N_{50}O_{60}S$	1047.595（+4 价）	395.852	32

结果与讨论

3.1 LC-MS/MS 方法优化及灵敏度考察

经优化流动相和质谱参数，采用 ESI 正离子模式检测，经各质谱参数优化后，两种多肽类药物戈舍瑞林和艾塞那肽均有很高的灵敏度，定量限分别为 2pg/mL 和 20pg/mL。碰撞能量优化结果见图 3（a）和图 3（b）。在该实验条件下所得典型 LC-MS/MS 色谱图见图 4（a）-图 4（d）。

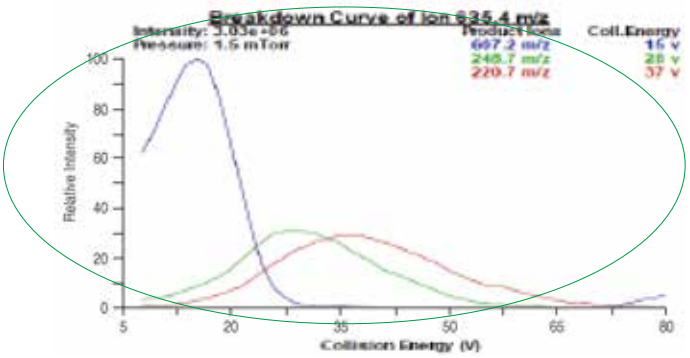

图 3（a）. 戈舍瑞林碰撞能量优化

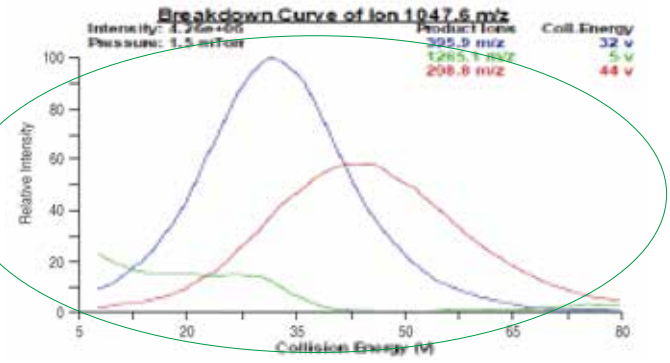

图 3（b）. 艾塞那肽碰撞能量优化

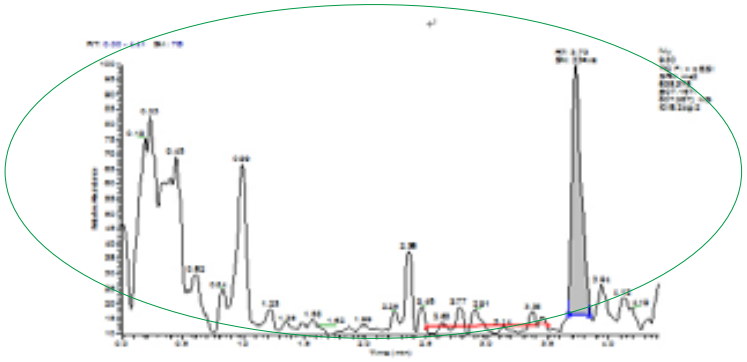

图 4（a）. 戈舍瑞林定量限（LOQ，2pg/mL）

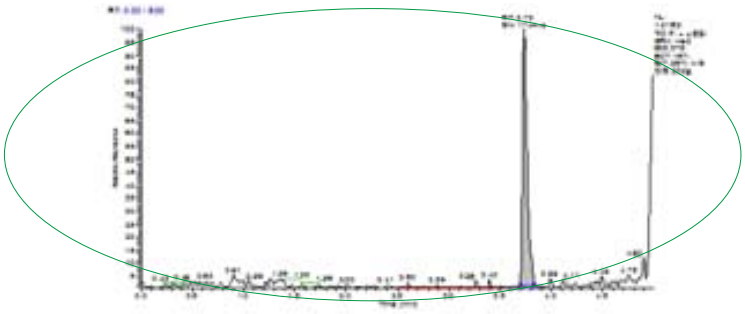

图 4（b）. 戈舍瑞林（20pg/mL）

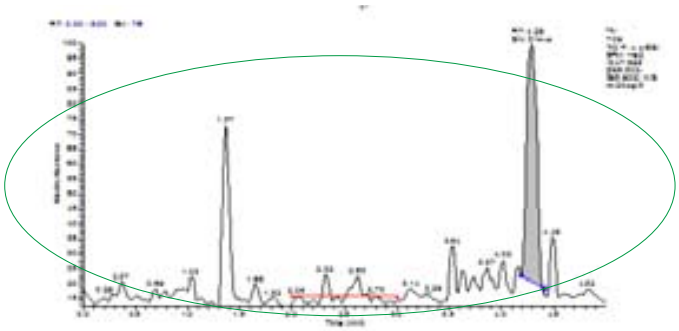

图 4（c）. 艾塞那肽定量限（LOQ，20pg/mL）

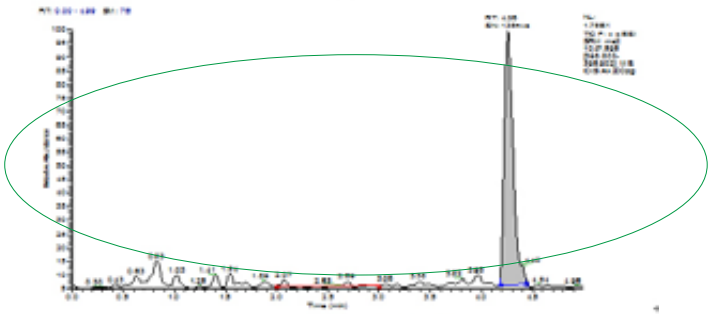

图 4（d）. 艾塞那肽（200pg/mL）

3.2 方法的线性和重复性考察

戈舍瑞林：将浓度为 1mg/mL 的混合标准溶液逐级稀释成 1000，200，100，20，10，5，2 pg/mL 系列标准溶液，分别进样分析。以分析物子离子色谱峰面积对质量浓度作标准曲线，所得线性方程及线性系数见图 5（a）。2 pg/mL 样品连续进样三次，RSD 为 7.36%，重复性满足要求。

艾塞那肽：将浓度为 1mg/mL 的混合标准溶液逐级稀释成 2000，1000，200，100，50，20pg/mL 系列标准溶液，分别进样分析。以分析物子离子色谱峰面积对质量浓度作标准曲线，所得线性方程及线性系数见图 5（b）。2 0pg/mL 样品连续进样三次，RSD 为 9.17%，重复性满足要求。

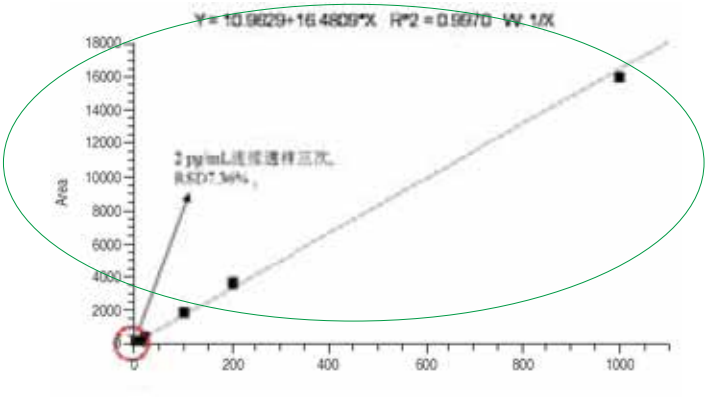

图 5（a）. 戈舍瑞林标准曲线

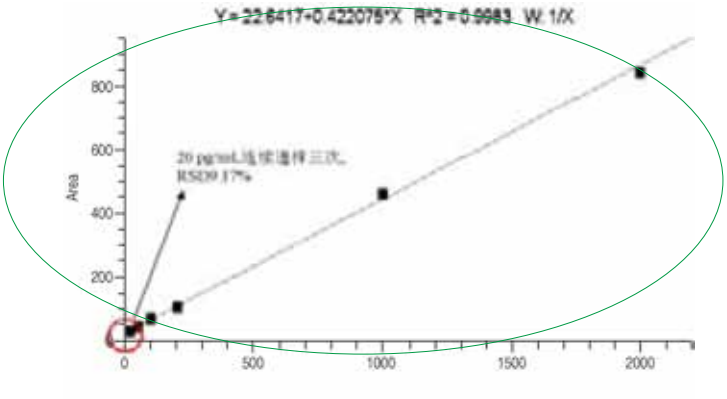

图 5（b）. 艾塞那肽标准曲线

结论

本文通过 TSQ Vantage 质谱仪建立的两种多肽类化合物戈舍瑞林和艾塞那肽的高效液相色谱－电喷雾串联质谱 (LC-ESI-MS/MS) 检测方法。该方法灵敏度高，定量限分别达到了 2pg/mL 和 20pg/mL，标准曲线线性良好，重复性满足要求。

除了考察标准溶液，实验中我们还以犬血浆基质预处理后添加样品 (2ng/mL) 为分析对象，分别考察了分辨率分别为 FMWH=0.7 和 FMWH=0.2 时的性噪比和选择性。结果表明，分析戈舍瑞林时，FMWH=0.2 时样品的性噪比 S/N 提高 2.3 倍，选择性增强；分析艾塞那肽时，FMWH=0.2 时样品的性噪比 S/N 提高了 5 倍，同时在复杂基质中极大地改善了色谱上的分离度。结果表明，TSQ Vantage 具有的高选择性离子检测模式可以极大地改善在复杂基质中样品的选择性和性噪比，尤其在血浆和组织这样复杂的生物样本中优势更为明显。

Absolute Quantitation of Targeted Endogenous Salivary Peptides using Heavy Isotope-labeled Internal Standards and High-Resolution Selective Reaction Monitoring Mass Spectrometry

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Introduction

Salivary gland secretions have significant potential as a noninvasive source of disease biomarkers and recent compilations of the salivary protein repertoire became valuable resources for parallel studies focusing on oral and systemic diseases.[1] One novel finding shows the presence of a complex suite of peptides and protein fragments in the low molecular weight fraction (< 10kDa) of which a subset have biological activity.[2] However, the expression levels and absolute quantitative amounts of these biologically active peptides among different salivary gland secretions have not been yet investigated. Recently, we have demonstrated the utility of high-resolution selected reaction monitoring (H-SRM) method on a triple quadrupole instrument for highly selective and sensitive quantitation of targeted peptides in complex matrices.[3] In this study, a targeted peptide quantitation assay is created for a triple quadrupole mass spectrometer to determine the natural abundance of six peptides in sixteen samples from the salivary gland secretions of eight donors. In order to get absolute quantitative information from the targeted peptides, isotopically labeled peptides were used as internal standards. To increase the detection capabilities of the assay, an H-SRM approach was employed using the Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer in which the mass resolution on the first quadrupole (Q1) was set to 0.4 FWHM.

Goal

To develop a highly sensitive, selective, accurate and precise method for absolute quantitation of endogenous salivary peptides on a triple quadrupole mass spectrometer by using isotopically labeled peptides as internal standards.

Experimental Conditions

Peptide Selection and SRM Method Design

Six endogenous salivary peptides (DSHAKRHHGY, DSHAKRHHGYK, DSHAKRHHGYKR, HEKHHSHRGYR, GRPQGPPQQGGHQQ & AAPDEKVLDSGFR) that showed potential as oral disease biomarkers were selected as the quantitative targets.[2] For each targeted peptide, an isotopically labeled counterpart was synthesized [(DSHA*KRHHGY, DSHA*KRHHGYK, DSHA*KRHHGYKR, HEKHHSHR*GYR, GRPQGP*PQQGGHQQ, AAP*DEKVLDSGFR (Thermo Fisher Scientific, Ulm, Germany)] according to the AQUA peptide quantification strategy.[4] For optimization of the H-SRM assay, each heavy peptide was infused by syringe pump into the mass spectrometer. Most abundant precursor ions were selected and two to four most abundant fragment ions were chosen for each precursor ion, yielding a total of 46 H-SRM transitions for targeting the six peptide pairs (Table 1).

Sample Preparation

Parotid saliva/secretion (PS) and sublingual/submandibular (SMSL) gland secretions were collected from eight human donors, yielding sixteen saliva samples (eight for PS series and eight for SMSL series). The low-molecular-weight fraction was prepared by ultrafiltration (nominal molecular weight limit 10,000) and used without further clean-up. The six isotopically labeled peptides were mixed together to make a 50 fmol/µL AQUA peptide stock mixture. 2.5 µL of the stock AQUA mixture and 2.5 µL of TFA were spiked into a 20 µL aliquot of prepared low-molecularweight fraction from each salivary gland secretion of eight donors respectively. The final concentration of spiked AQUA peptides was 5 fmol/µL in each saliva sample and contained 10% TFA. Each saliva sample was diluted from 20 µL to 25 µL.

Table 1. SRM transition list used for targeting the six native peptide/isotopically labeled peptide pairs

Protein	Peptide	Precursor *m/z* (Q1)	Charge	Product *m/z* (Q3)	Ion Type	Charge
Peptide 1	DSHAKRHHGY	403.20	3	335.80	y_8	3
		403.20	3	364.90	y_9	3
		403.20	3	399.20	y_6	2
		403.20	3	434.70	y_7	2
Heavy Peptide 1	DSHA*KRHHGY	404.53	3	337.27	y_8	3
		404.53	3	366.17	y_9	3
		404.53	3	399.20	y_6	2
		404.53	3	436.67	y_7	2
Peptide 2	DSHAKRHHGYK	334.70	4	252.63	M-18	4
		334.70	4	330.36	y_9	3
		334.70	4	378.54	y_4	2
Heavy Peptide 2	DSHA*KRHHGYK	335.70	4	252.60	M-18	4
		335.70	4	331.36	y_9	3
		335.70	4	379.90	y_4	2
Peptide 3	DSHAKRHHGYKR	373.75	4	344.94	y_{11}	4
		373.75	4	369.19	M-18	4
		373.75	4	430.57	y_{10}	3
Heavy Peptide 3	DSHA*KRHHGYKR	374.75	4	345.90	y_{11}	4
		374.75	4	370.18	M-18	4
		374.75	4	431.90	y_{10}	3
Peptide 4	HEKHHSHRGYR	361.68	4	304.82	y_7	3
		361.68	4	357.18	M-18	4
		361.68	4	388.20	y_6	2
		361.68	4	456.73	y_7	2
Heavy Peptide 4	HEKHHSHR*GYR	364.30	4	308.17	y_7	3
		364.30	4	359.68	M-18	4
		364.30	4	393.20	y_6	2
		364.30	4	461.75	y_7	2

Protein	Peptide	Precursor *m/z* (Q1)	Charge	Product *m/z* (Q3)	Ion Type	Charge
Peptide 5	GRPQGPPQQGGHQQ	491.65	3	409.72	b_8	2
		491.65	3	496.26	b_5	1
		491.65	3	526.23	y_5	1
		491.65	3	879.41	y_8	1
Heavy Peptide 5	GRPQGP*PQQGGHQQ	493.65	3	412.70	b_8	2
		493.65	3	496.26	b_5	1
		493.65	3	526.23	y_5	1
		493.65	3	879.41	y_8	1
Peptide 6	AAPDEKVLDSGFR	468.95	3	421.60	y_{11}	3
		468.95	3	631.80	y_{11}	2
		702.86	2	466.24	y_4	1
		702.86	2	631.82	y_{11}	2
		702.86	2	793.42	y_7	1
Heavy Peptide 6	AAP*DEKVLDSGFR	470.95	3	423.60	y_{11}	3
		470.95	3	634.80	y11	2
		705.86	2	466.24	y4	1
		705.86	2	634.80	y11	2
		705.86	2	793.42	y7	1

LC/MS

Nano-HPLC

Pump:	Thermo Scientific Surveyor MS pump equipped with MicroAS autosampler
Column:	PicoFrit™ C18 column from New Objective (75 µm × 100 mm)
Post-split Flow Rate:	300 nl/min
Buffer A:	0.1% Formic acid/H_2O
Buffer B:	0.1% Formic acid/100% ACN
Gradient:	0% B to 95% B in 30 min
Injection Volume:	2 µL

MS

Mass Spectrometer:	TSQ Vantage with a standard Ion Max source equipped with a column adapter for nanoflow (New Objective)
H-SRM Set Up:	Q1, 0.4 FWHM; Q3, 0.7 FWHM; Q2, 1.5 mTorr (Ar), Dwell time, 20 ms
CE:	Optimized by using each AQUA peptide

Note: Each sample was run in triplicate

Results

Optimal Q1 Resolution

The main advantage of high resolution isolation is its ability to selectively detect low abundance peptide peaks within a complex background. Nearly all commercial instruments are used with unit resolution (0.7 FWHM) for precursor ion selection and would otherwise suffer significant signal loses when using higher resolution (0.4 FWHM or 0.2 FWHM) isolation. The Thermo Scientific TSQ Series are well known for higher resolution for precursor ion selection while maintaining high transmission efficiency.[5] To determine if the H-SRM improves quantitative precision for targeted peptides, pooled saliva sample was spiked with all six internal standards, analyzed at three different Q1 resolutions (0.7 FWHM, 0.4 FWHM & 0.2 FWHM). Figure 1 shows an example of SRM trace for heavy peptide GRPQGP*PQQGGHQQ (10 fmol on column) at three different Q1 resolutions. The interference peak observed while using unit resolution (0.7 FWHM) was eliminated when using higher resolution (0.4 FWHM

and 0.2 FWHM). The signal-to-noise ratio was greatly improved while using higher selectivity for Q1 precursor ion. After evaluating all the target peaks, 0.4 FWHM resolution at Q1 was found to be selective enough to reduce chemical interferences from the saliva background.

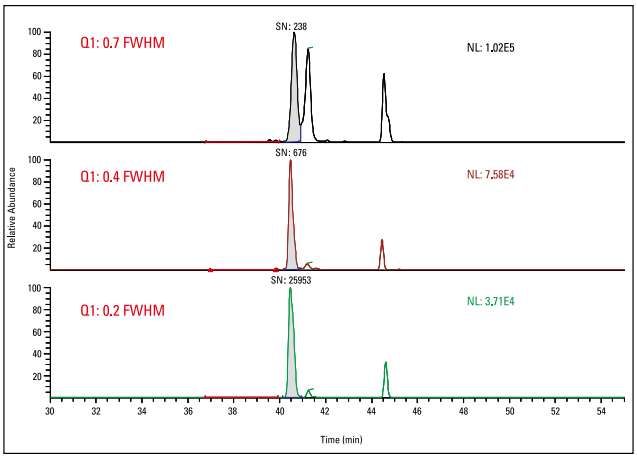

Figure 1. Comparison of the chromatographic traces from the analysis of the heavy peptide GRPQGP*PQQGGHQQ at three different Q1 resolutions

Detection Limit and Linear Dynamic Range for Targeted Endogenous Peptides

With the AQUA strategy, the absolute quantitation of the native peptide is determined by comparing the abundance of the known AQUA internal standard peptide to the abundance of the native peptide.[4] To determine the detection limit and quantitative linearity of targeted native saliva peptides, the six heavy AQUA peptides were mixed and spiked into the pooled saliva sample over a concentration range of four orders of magnitude (0.05 fmol, 0.5 fmol, 5 fmol, 50 fmol and 100 fmol per µL of pooled saliva sample). By using the new RF-only ion ring technology (S-lens) for maximum ion transmission, the TSQ Vantage can deliver highly sensitive and reproducible quantitative results for very low-level analytes.[6] In our experiment, both excellent sensitivity and wide linear dynamic range were achieved. Figure 2a shows the summed SRM chromatographic traces of HEKHHSHR*GYR/ HEKHHSHRGYR peptide pair with 50 attomoles of HEKHHSHR*GYR loaded on column. Figure 2b shows the heavy peptide peak clearly detected by the retention time (RT) alignment of monitored multiple fragment ions from the heavy labeled peptide. Figure 3 shows the calibration curve of AQUAlabeled peptide HEKHHSHR*GYR across the concentration range of 0.05 fmol to 100 fmol on column by using the native peptide in the pooled saliva sample as the internal standard. Excellent linearity was obtained over four orders of magnitude (R^2=0.9962).

Targeted Native Peptide Confirmation and Absolute Quantitation by AQUA

The targeted peptide peak from each sample was confirmed by using both the retention time and multiple fragment ion ratios from the co-eluting isotopically labeled internal standard (Figure 4). Five targeted peptides were detected in all sixteen saliva samples. The H-SRM assay was performed in triplicate for each saliva sample. The

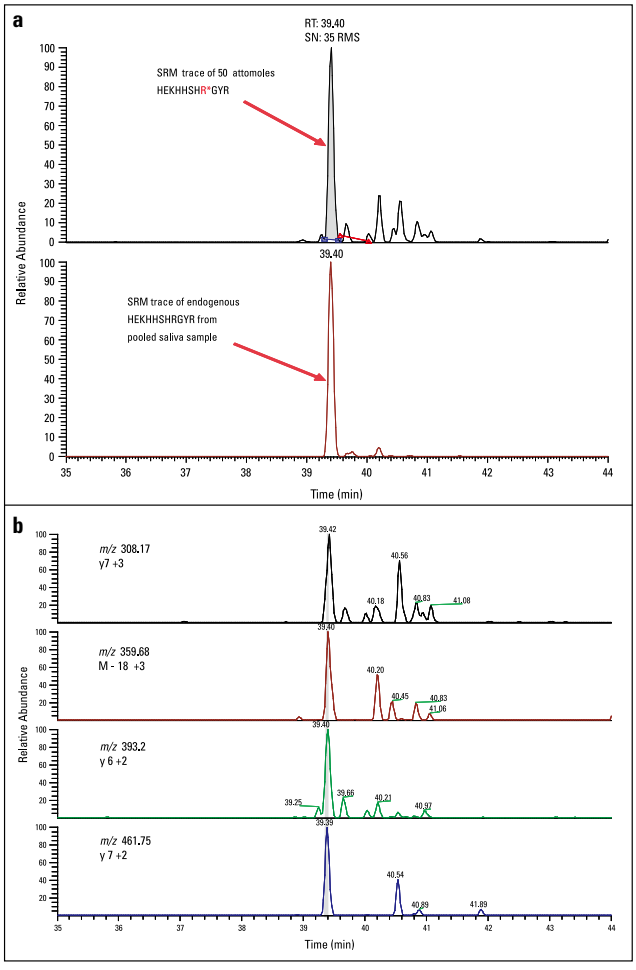

Figure 2. a) SRM chromatograms of the HEKHHSHR*GYR/ HEKHHSHRGYR peptide pair (50 attomoles of HEKHHSHR*GYR on column). b) Individually monitored fragment ion chromatograms from the heavy peptide.

sixth targeted peptide (AAPDEKVLDSGFR) was detected only in Y PS and Y SMSL saliva samples. Extracted ion chromatograms were generated and the analyte peaks integrated. The quantities of endogenous peptides were calculated using the mean-peak-area ratios (n=3) of endogenous peptide/isotopically labeled peptide pairs multiplied by the absolute amount of internal standard (5 fmol/µL) and 1.25X dilution factor. Figure 5 shows an example of how to calculate the absolute amount of peptide AAPDEKVLDSGFR using this strategy. Although this native peptide existed at a very low concentration level (calculated 60 attomoles on column), the peak was clearly detected with a signal-to-noise ratio of 20. Table 2 shows an example of the absolute quantitation results of six targeted peptides from the saliva sample of Y SMSL. The analytical precision across the three replicate runs was good. The relative standard deviation (RSD) of five peptides was less than 12% (average 8.5%). The RSD of low level peptide AAPDEKVLDSGFR was also within acceptable range (25%).

The summary for all the calculated results of each native peptide in the eight PS series samples and the eight Y SMSL series samples are shown in Tables 3 and 4, respectively. Abundances of peptides varied significantly between donors and glandular sources. The peptide AAPDEKVLDSGFR was unique to the gland secretions of one donor. Another peptide HEKHHSHRGYR was consistently more abundant in sublingual/submandibular than in parotid saliva. The clinical significance for these abundance variations are under further investigation.

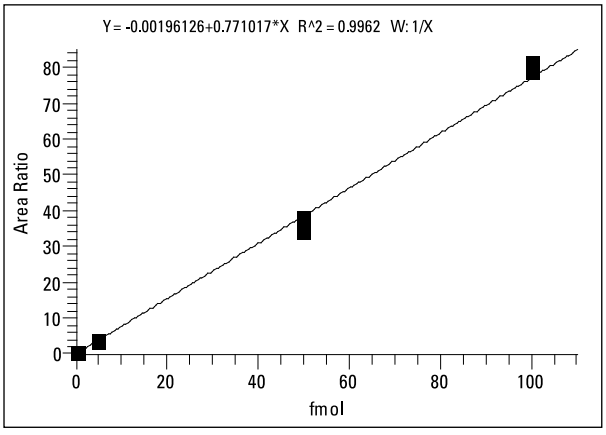

Figure 3. Linear calibration curve for the heavy peptide HEKHHSHR*GYR (50 amol to 100 fmol on column), n=3

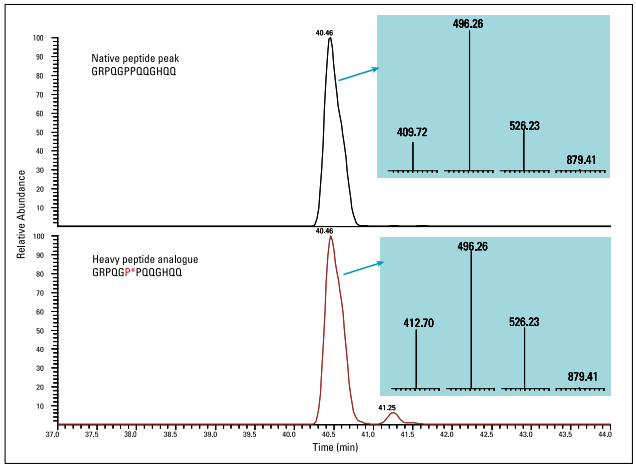

Figure 4. Confirmation of targeted native peptide peaks using both RT and multiple fragment ion ratios of the co-eluting heavy isotopic-labeled peptide analogue

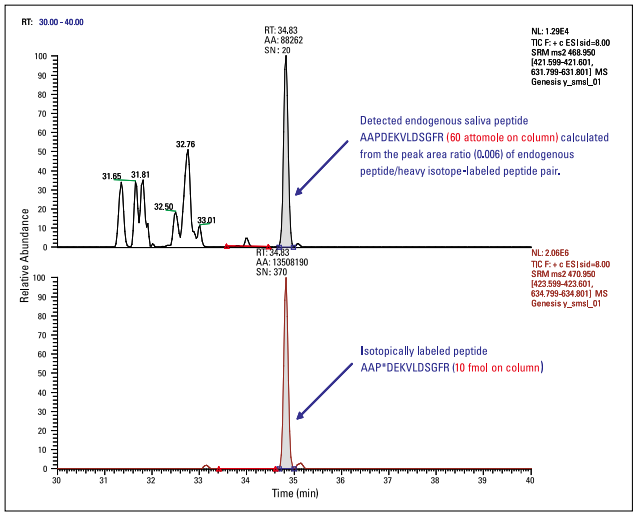

Figure 5. Absolute quantitation of targeted native peptide AAPDEKVLDSGFR by using the isotopically labeled peptide as an internal standard

Table 2. Absolute quantitative results of six targeted peptides from the saliva sample of Y SMSL using the AQUA strategy

Peptide	Sequence	Ratio (Native/Labeled) n=1	Ratio (Native/Labeled) n=2	Ratio (Native/Labeled) n=3	Mean Ratio (Native/Labeled) Mean	Calculated Native Amount* fmol/μL	RSD %
Peptide 1	DSHAKRHHGY	10.866	11.591	11.308	11.255	70.3	3.25
Peptide 2	DSHAKRHHGYK	11.094	12.754	13.050	12.299	76.9	8.57
Peptide 3	DSHAKRHHGYKR	4.269	3.408	3.681	3.786	23.7	11.62
Peptide 4	HEKHHSHRGYR	0.063	0.079	0.079	0.074	0.5	12.48
Peptide 5	GRPQGPPQQGGHQQ	3.423	3.284	2.998	3.235	20.2	6.70
Peptide 6	AAPDEKVLDSGFR	0.006	0.008	0.005	0.006	0.04	25.46

* Calculated native peptide amount = mean ratio of (Native/Labeled) x 5 fmol/μL x 1.25

Table 3. Quantitative summary for the parotid saliva samples of eight human donors

Peptide	Sequence	Y PS fmol/μL	E PS fmol/μL	EL PS fmol/μL	KR PS fmol/μL	DO PS fmol/μL	KS PS fmol/μL	LN PS fmol/μL	S PS fmol/μL
Peptide	Sequence	fmol/μL	fmol/μL	fmol/μL	fmol/μL	fmol/μL	fmol/μL	fmol/μL	fmol/μL
Peptide 1	DSHAKRHHGY	13.2	1.4	33.2	20.6	104.1	6.1	50.4	11.9
Peptide 2	DSHAKRHHGYK	44.9	13.1	56.3	47.4	71.5	24.5	66.7	35.7
Peptide 3	DSHAKRHHGYKR	25.9	3.0	66.7	22.8	98.5	6.9	51.2	24.6
Peptide 4	HEKHHSHRGYR	2.5	0.2	5.7	3.7	7.6	0.7	3.1	2.1
Peptide 5	GRPQGPPQQGGHQQ	62.9	36.2	124.5	48.1	30.8	37.7	48.8	46.1
Peptide 6	AAPDEKVLDSGFR	0.0006							

Table 4. Quantitative summary for the sublingual/submandibular saliva samples of eight human donors

Peptide	Sequence	Y SMSL fmol/μL	E SMSL fmol/μL	EL SMSL fmol/μL	KR SMSL fmol/μL	DO SMSL fmol/μL	KS SMSL fmol/μL	LN SMSL fmol/μL	S SMSL fmol/μL
Peptide 1	DSHAKRHHGY	70.3	0.6	9.1	52.6	75.7	33.6	2.2	35.3
Peptide 2	DSHAKRHHGYK	73.1	3.5	42.4	59.2	112.2	58.5	10.3	59.0
Peptide 3	DSHAKRHHGYKR	23.7	0.6	17.7	4.2	58.8	38.1	2.3	41.5
Peptide 4	HEKHHSHRGYR	0.500	0.003	0.200	0.034	0.700	0.400	0.090	1.430
Peptide 5	GRPQGPPQQGGHQQ	20.2	4	74.0	183.8	34.0	353.5	16.8	85.9
Peptide 6	AAPDEKVLDSGFR	0.04							

Conclusions

1. A 46-transitions H-SRM assay on a new TSQ Vantage triple quadrupole mass spectrometer was used to successfully determine the absolute amounts of six targeted endogenous peptides from parotid and sublingual/ submandibular gland secretions of eight human donors using the AQUA strategy without any prior peptide enrichment or sample clean up.
2. The H-SRM assay was highly sensitive and enabled to detect the native salivary peptides at less than 10 attomole/μL level.
3. The analytical precision of the H-SRM assay was good. The RSDs of five peptides were less than 12% (average 8.5%). The RSD of the low-level peptide AAPDEKVLDSGFR was also within acceptable range (25%).
4. Significant abundance variations for each targeted endogenous peptide were observed between donors and between glandular sources. The biological meaning for these variations is a subject of further investigation.

References

1. Paul Denny *et al.*, The Proteomes of Human Parotid and Submandibular/ Sublingual Gland Salivas Collected as the Ductal Secretions; J. *Proteome Res*. **2008** 7: 1994-2006.
2. Markus Hardt, H. Ewa Witkowska, Sally Webb, Lindsay R. Thomas, Scott E. Dixon, Steven C. Hall, and Susan J. Fisher, Assessing the Effects of Diurnal Variation on the Composition of Human Parotid Saliva: Quantitative Analysis of Native Peptides using ITRAQ Reagents, *Anal. Chem.* **2005** 77: 4947-4954.
3. Reiko Kiyonami, Scott Peterman, Rosa Viner, Amol Prakash, and Vlad Zabrouskov, A new Methodology for targeted peptide quantitation in complex mixtures using a high resolution triple quadrupole mass spectrometer. Application note of Thermo Fisher Scientific, #412
4. Scott A. Gerber, John Rush, Olaf Stemman, Marc W. Kirschner, and Steven P. Gygi, Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc. Natl. Acad. Sci. U.S.A. (2003) 100: 6940-6945.
5. Kevin J. McHale and Nick Duczak, Jr., Applying Highly-Selective Reaction Monitoring (H-SRM) for the Assay of Midazolar and 1-Hydromidazolam in Plasma on the TSQ Quantum Ultra. 2005. Application note of Thermo Fisher Scientific, #347.
6. Eloy R. Wouters, Maurizio Splendore, Michael W. Senko, John E. P. Syka, Jean-Jacques Dunyach, Design of a progressively spaced stacked ring ion guide for improved ion transmission at high pressure, ASMS 2008 poster #Th006.

The Importance of Linear Dynamic Range for Small Molecule and Targeted Peptide LC-MS/MS Quantitation

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Introduction

Mass spectrometry (MS) can be divided into ion generation, ion separation, and ion detection. While the techniques of ionization and mass analysis receive much attention, ion detection is less frequently reviewed. The impact of ion detection on quantitative analysis, however, is significant, especially for applications requiring wide dynamic range and high sensitivity such as small molecule quantitation during drug research, and protein quantitation. The demand for simpler methods and quick turn-around of results is high in today's competitive research environments. Typical MS detectors have to perform at very high count rates (>10^6 counts/s) with minimum refresh times for a wide dynamic range of responses and have low noise characteristics for very sensitive limits of detection.[1]

Continuous-dynode electron multipliers (CDEM) are typically used in a triple stage quadrupole because they are compact, rugged, and customizable. The CDEM technique can either be used as "analog" counting mode or "pulse" counting mode. In the pulse counting mode, individual electron pulses from each incident ion are counted as discrete events, in an attempt to buffer against noise created by thermal events or electron impact ionization (EI) of background gases. The limitation of the pulse counting technique is a fall off in linearity due to pulse "pile-up" effects where multiple ion events at high incident rates cannot be distinguished.[1]

In the analog mode, the incident ion creates a cascade of electrons which generates a current that is then converted to a voltage, followed by digitization. Advances in electronics and better vacuum have virtually eliminated common detector noise such as Johnson, Shot, Flicker, & EI of background gases. The key advantage is that a single incident ion can be amplified by 10^6x or more giving high sensitivity and wide dynamic ranges.[1] This is the key reason the CDEM in analog mode with advanced electronics is used in the Thermo Scientific TSQ Vantage mass spectrometer.

The benefit of having a wide linear dynamic range is especially important for small molecule quantitation during drug research, and for targeted peptide quantitation. In small molecule quantitation during drug research, the dosage form can cause an order of magnitude difference in quantitative results. In proteomics, the dynamic range of proteins in real samples can be in excess of 10 orders of magnitude with further complications arising from varying ionization efficiencies for the representative enzymatic peptide fragments. Thus having a wide dynamic range makes experimentation simpler by avoiding re-assays and dilution issues.

Goal

Demonstrate linear dynamic range in excess of four orders of magnitude using a simple fit for small molecule and biomolecule quantitative applications.

Experimental Conditions

Chromatographic Conditions, Small Molecule

Small Molecule:	Paroxetine
HPLC Pump:	Thermo Scientific Accela pump
Autosampler:	CTC™ PAL (CTC Analytics, Basel, Switzerland)
Column:	Thermo Scientific Hypersil GOLD C18 50 mm × 2.1 mm (3 µm) column

HPLC Method, Small Molecule

A linear gradient of 10% Solvent B (acetonitrile containing 0.1% formic acid) to 95% B over five minutes was used to chromatograph paroxetine. Solvent A was water containing 0.1% formic acid. The flow rate was 1 mL/min. Injection volumes of 10 µL were used.

Chromatographic Conditions, Biomolecule

Biomolecule:	Horse Heart Myoglobin Tryptic digest
HPLC Pump:	Thermo Scientific Surveyor MS pump
Autosampler:	MicroAS autosampler (Spark Holland, Netherlands)
Column:	PicoFrit® C18 column from New Objective (75 µm × 100 mm)

HPLC Method, Biomolecule

A linear gradient from 2% B (0.1% FA/100% ACN) to 50% B in 45 min was used to chromatograph horse heart tryptic digest fragments in *E. coli* protein matrix. Buffer A was 0.1% FA/2% ACN/98% H_2O. Post-split flow rate: 300 nL/min. Sample loading: Directly loaded on column. Injection amount: 1 µL.

Mass Spectrometer Conditions, Small Molecule

Mass Spectrometer:	TSQ Vantage™
Ionization Mode:	HESI-II in positive ion mode
Ion Sweep Gas:	5 au
Ion Transfer Tube Temp:	300 ℃
Sheath Gas:	60 au
Aux Gas:	30 au
Resolution:	0.7 Da (FWHM) on Q1 and Q3
Scan Time:	0.2 s
Scan Width:	0.002 Da
Chrom Filter:	5 s
Selected Reaction Monitoring:	Paroxetine 330.20 > 192.1 Da Alprazolam 309.1 > 281.0 Da (ISTD)
Collision Energy:	22 V
Collision Gas Pressure:	1.5 mTorr

Mass Spectrometer Conditions, Biomolecule

Mass Spectrometer:	TSQ Vantage
Ionization Method:	Ion Max source equipped with a column adapter for nanoflow (New Objective, Woburn, MA) in positive ion mode
Ion Sweep Gas:	0
Ion Transfer Tube Temp:	180 ℃
Sheath Gas:	0
Aux Gas:	0
Resolution:	0.7 Da (FWHM) on Q1 and Q3
Scan Time:	0.02 s
Scan Width:	0.002 Da
Chrom Filter:	15 s

Multiple Selected Reaction Monitoring

Peptide ALELFR Representing Myoglobin:	374.72 > 435.3 Da 374.72 > 564.3 Da 374.72 > 677.5.3 Da
Collision Energy:	374.72 > 435.3 Da 10 V 374.72 > 564.3 Da 10 V 374.72 > 677.5.3 Da 12 V
Collision Gas Pressure:	1.5 mTorr

Results and Discussion

Figures 1 and 2 show over four orders of linear dynamic range for the quantitative analysis of Paroxetine and Myoglobin tryptic fragment (ALELFR) on the TSQ Vantage. Another notable factor is that Paroxetine was quantified using an internal standard (alprazolam) while the Myoglobin tryptic fragment (ALELFR) was quantified using absolute intensity, or, without an internal standard. Both assays demonstrated linearity as indicated by correlation coefficients of 0.9998 (Paroxetine) and 0.9999 (Myoglobin tryptic fragment ALELFR). Having a triple stage quadrupole with a wide linear dynamic range offers substantial advantages: time saved due to lack of re-assays and avoiding complications that are usually associated with dilution (partition, settling, solubility).

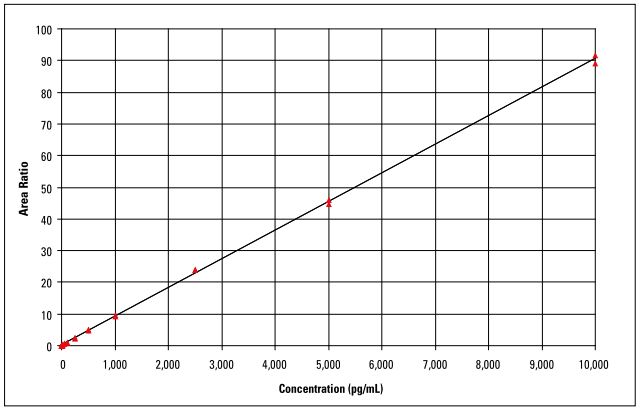

Figure 1. Heated ESI-II based LC-MS/MS analysis of Paroxetine showing four orders of linear dynamic range (1-10,000 pg/mL). A linear gradient of 10% Solvent B (acetonitrile containing 0.1% formic acid) to 95% B over five minutes was used. Solvent A was water containing 0.1% formic acid. The column used was Hypersil GOLD™ C18 50 mm × 2.1 mm (3 µm) and the flow rate was 1 mL/min. Injection volumes of 10 µL were used.

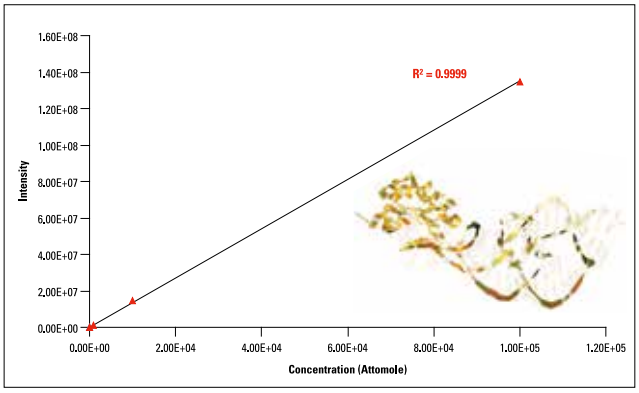

Figure 2. Linear dynamic range of four orders of magnitude for the peptide quantitation experiment. Horse heart myoglobin (0.01 fmol - 100 fmol) was spiked into *E. coli* tryptic digest. Excellent linear calibration curve was generated from 0.01 fmol to 100 fmol for peptide ALELFR without using internal standard.

Conclusions

A linear dynamic range in excess of four orders of magnitude using a simple fit for a small molecule and a biomolecule can be achieved on the TSQ Vantage triple stage quadrupole instrument. This allows for faster results and simpler LC-MS/MS methods for small molecule quantitation during drug research, and for targeted peptide quantitative analysis.

References

1. Koppenaal, D.W., et al. Anal. Chem. p419a. 2005.

Identification and Quantification of Low-Abundance Proteins in Biotherapeutics by a Sensitive and Universal LC High-Resolution MS-based Assay

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Overview

Purpose: To develop a simple, sensitive and universal platform for identification and quantification of trace-level impurities in biotherapeutics.

Methods: IgG mAb samples with different concentrations of low-abundance proteins were denatured, reduced and alkylated, then digested using trypsin. Samples were analyzed by online nano-flow LC and high-resolution MS. Identification and quantification of the impurity proteins were performed using software designed for qualitative and quantitative proteomics.

Results: Due to the difference in ionization efficiency of peptides, three out of four trace-level protein impurities were identified at 5ppm using database searching in through the software. The observed dynamic range was 5 orders of magnitude. Good linearity was observed over the range of 10-1000 ppm for Lysozyme and carbonic anhydrase in the presence of IgG, with correlation coefficients (R^2) greater than 0.99. Lower Limits of quantitation (LLOQs) were detected at 10 ppm in 1.0mg/mL IgG.

Introduction

Host cell proteins (HCPs) accompanied with recombinant biotherapeutics can significantly affect drug efficacy and cause immunogenicity. Detection and quantification of residual HCPs as potential process-related impurities is critical for biopharmaceutical companies in accordance with regulatory agency guidelines. Current analytical methods suffer from long method development times, require prior knowledge of contaminant proteins, and lack the ability to detect a wide range of protein concentrations. LC-MS has been used for HCPs analysis, but qualitative and quantitative studies are traditionally performed using two different MS platforms due to the sensitivity limitations of high-resolution MS. Here, a sensitive and universal assay employing nano-flow LC coupled with a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer is evaluated for simultaneous identification and quantification of low abundant proteins in biotherapeutic products.

Methods

Sample Preparation

Four protein standards, lysozyme (14k Da), carbonic anhydrase (29k Da), cytochrome C (12k Da) and bovine serum albumin (67k Da) were spiked in 1mg/mL of IgG mAb to final relative concentrations of 5-1000 ppm. The mixture was denatured, reduced and alkylated, then equal amount of stable isotope labeled peptides were spiked in each level. After trypsin digestion (1/20=protein/enzyme, 37 C overnight), samples were desalted by Thermo Scientific™ Pierce™ C18 Spin Tips.

Liquid Chromatography

Trypsin digested mAb samples were analyzed on Thermo Scientific™ EASY-nLC 1000™ Liquid chromatograph coupled with a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. Peptides were trapped using a Thermo

Scientific™ Acclaim™ PepMap™ C18 column (75µm × 2cm) and separated on a Thermo Scientific™ Easy-Spray™ PepMap column (75µm × 50cm) with integrated emitter heated at 40 C at a flow rate of 250 nL/min with solvent A (0.1% formic acid in H_2O) and solvent B (0.1% formic acid in acetonitrile).

Injection volume: 4 µL; Loading volume: 12 µL; Max. pressure: 45 bar with Solvent A

Time [min]	Flow [nL/min]	Mixture [%B]
0	250	2
5	250	5
130	250	25
160	250	40
165	250	95
180	250	95

Mass Spectrometry

The Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Figure 1) was equipped with a Thermo Scientific™ EASY-Spray™ source was employed for MS analysis.

Methods were set with full scan (140,000 resolution) and top 10 data dependant MS/MS (17,500 resolution) in positive mode. The MS parameters are shown in Table 1.

TABLE 1. MS parameters

=== Easy-Spray Source: ===	
• Spray Voltage (+)	1800 V
• Capillary Temperature (+)	250 °C
• Sheath Gas (+)	0
• Aux Gas (+)	0
• Sweep Gas (+)	0
• Heater Temperature (+)	0 °C
• Isolation Window(+)	2 m/z
• NCE(HCD)	27
• S-lens	50
• Positive MS Scan: 1 microscan; Full Scan R=140,000; AGC=3e6; IT=120 ms; Scan range: *m/z* 400-1800; Lock Mass: off	
Top 10 data-dependant MS/MS: R=17,500; AGC=1e5; IT=250ms; First mass: 130 *m/z*	

Data Analysis

The identification of protein impurities was performed using Thermo Scientific™ Proteome Discoverer™ Software revision 1.4. The quantitative analysis of those proteins was performed using Thermo Scientific™ Pinpoint™ Software revision 1.3.

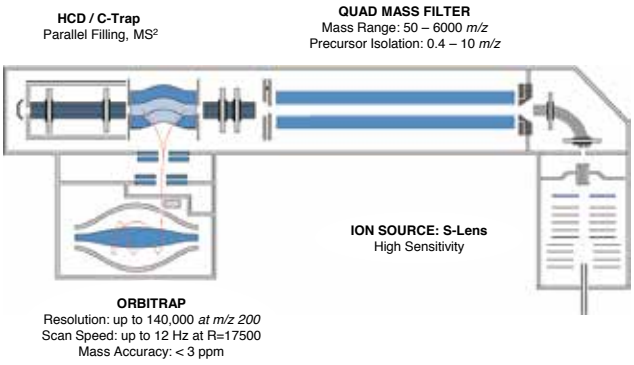

FIGURE 1. Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer

Results

Identification of Low Abundance Protein Impurities

Four proteins with molecular weights from 10k to 70k Da were spiked in 1 mg/mL of mAb (150k Da) to demonstrate the sensitivity and dynamic range of the nano LC-MS based assay. In the presence of large amount of monoclonal antibody and high background noise, selectivity was greatly increased by using high resolving power at 140,000 (FWHM) in full-scan and data-dependant MS/MS at 17,500 (FWHM).

Data was searched using the SEQUEST® algorithm in Proteome Discover Software with Percolator peptide validation (1% FDR) against a customized database with targeted mAb and possible protein impurities.

Searching parameters were set as: Precursor mass tolerance: 15 ppm; Fragment mass tolerance: 20mmu; Enzyme: Trypsin; Missed cleavage: 2; Dynamic modification: Methionine oxidation; N-terminal: Gln to Pyro-Glu; Static modification: Carbamidomethylation on Cysteine. The searching results were filtered with high peptide confidence and peptide rank #1.

Lysozyme, bovine serum albumin and carbonic anhydrase were identified at 10 ppm with the sequence coverage of 34.92%,18.8% and 5.1% due to the ionization efficiency difference of the enzymatically cleaved peptides. Figure 2 shows the sequence coverage of Lysozyme. The concentration is 10 ppm relative to 1 mg/mL IgG. Amino acids highlighted in green are the identified peptides based

on fragment ions matched with theoretical ones. The modified amino acids (AA) were also indicated above the AA in Figure 2, i.e. Cysteine 24 was carbamidomethylated, and Methionine 30 was oxidized.

FIGURE 2. Sequence coverage of spiked 10 ppm of Lysozyme relative to 1 mg/mL IgG.

Among four identified peptides for lysozyme, NTDGSTDYGILQINSR eluted at 92 min. Figure 3 shows the isotope distribution of the peptide at charge 2+, and the three major isotopes are indicated with a red star. The sequence was confirmed by a very good match of the experimental MS/MS spectrum from the precursor ion 877.4216 *m/z* to the theoretical one (Figure 4).

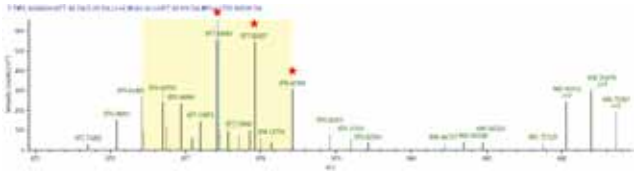

FIGURE 3. Mass spectrum of the isotope distribution of $[M+2H]^{2+}$ peptide NTDGSTDYGILQINSR for Lysozyme.

Quantification of Protein Impurities

Targeted protein quantification on the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer can be performed by (1) Full Scan, (2) Selected Ion Monitoring (SIM), and (3) Targeted MS/MS or Parallel Reaction Monitoring (PRM). The second and third methods provide better sensitivity and selectivity compared to first. However, the first method is ideal for simultaneous quan/qual applications in discovery research.

With a full scan method, the targeted peptide is quantified using the integrated area of the extracted ion chromatogram of the monoisotopic mass or sum of the different isotopes. The post translation modifications (PTMs) of peptides can also be identified.

Proteome Discoverer Software search results (msf files) were imported to Pinpoint Software to automatically generate the accurate mass of isotopes or MS/MS fragment ions based on the identified peptide sequence. The

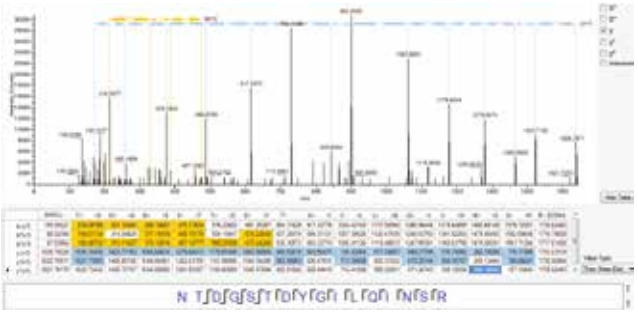

FIGURE 4. Annotated MS/MS spectrum of peptide NTDGSTDYGILQINSR from $[M+2H]^{2+}$ precursor ion 877.4216 m/z for Lysozyme.

signature peptides from different proteins were selected for the quantitation.

Quantification of Lysozyme and Carbonic Anhydrase in Human IgG Sample

Lysozyme was quantified using the signature peptide NTDGSTDYGILQINSR. The signals of three most abundant isotopes at charge 2^+, 877.4212, 877.9226 and 878.4240 *m/z* (in green, purple and blue respectively in Figure 5b) were summed for quantification. Integrated peak area in Figure 6 shows the concentration dependent increase. The calibration curve was generated with duplicate sample injections. Good linearity was observed from 10 ppm (10 pg/µL in IgG sample) to 1000 ppm with R^2=0.991 (Figure 5a).

SEQUEST search results demonstrated the sequence coverage of carbonic anhydrase was 42.31% in the IgG spiked at 1000 ppm. Three isotopes at charge 2^+ of peptide QSPVDIDTK , 501.7588 (green in Figure 7b), 502.2602 (purple) and 502.761 5(blue) *m/z* were integrated to generate calibration curve. Figure 7a shows good linearity with R^2=0.996, 1/X, dynamic range: 10-1000ppm (pg/µL).

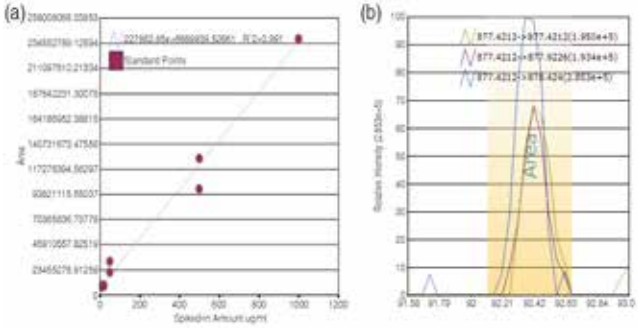

FIGURE 5. Calibration curve (a) of peptide NTDGSTDYGILQINSR for Lysozyme and XICs of three most abundant isotopes (b).

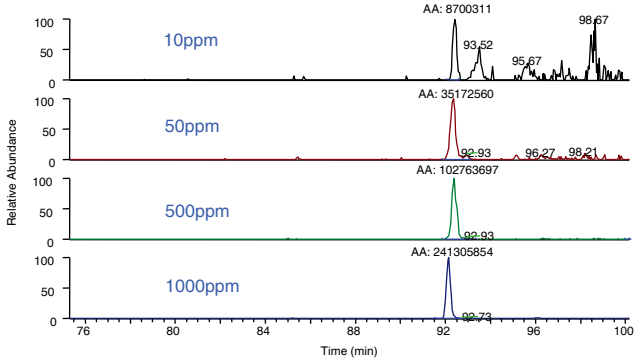

FIGURE 6. XICs of peptide NTDGSTDYGILQINSR with isotopes 877.4212, 877.9226 and 878.4240 *m/z* within 5 ppm mass tolerance window.

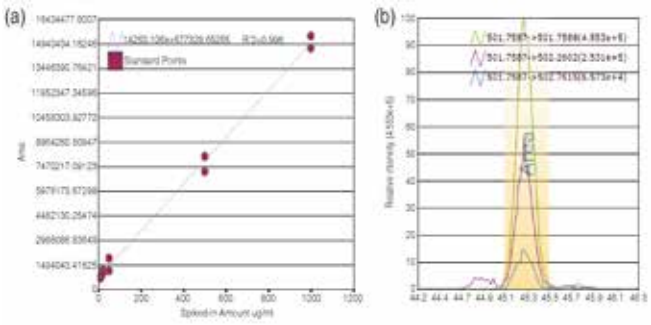

FIGURE 7. Calibration curve (a) of peptide QSPVDIDTK for Carbonic Anhydrase and XICs of the three most abundant isotopes (b).

Conclusion

A simple, sensitive and universal platform was developed for identification and quantification of trace-level protein impurities in biotherapeutics. IgG monoclonal antibody spiked with low abundant proteins was used as the model to test the assay sensitivity and dynamic range.

- The observed dynamic range was 5 orders of magnitude. Lysozyme, carbonic anhydrase and bovine serum albumin were detected at 5 ppm level relative to 1 mg/mL of IgG antibody.
- Good linearity was observed over the range of 10-1000 ppm for Lysozyme and carbonic anhydrase in the presence of IgG, with correlation coefficients (R^2) of 0.991 and 0.996 (n=2), respectively.
- The simultaneous qualitative and quantitative analysis of low abundance proteins was achieved with single injection on the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. This LC-MS based approach can be applied for host cell proteins generated during drug process development by recombinant DNA technology.
- The combination of the EASY-Spray source and EASY-Spray column with integrated emitter and column makes trace-level protein impurity nano-flow analysis more close to routine.

HR/AM Targeted Peptide Quantification on the Q Exactive™: A Unique Combination of High Selectivity, High Sensitivity and High Throughput

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Overview

Purpose: The performance of a novel hybrid quadrupole –orbitrap mass spectrometer was evaluated in targeted peptide quantification.

Methods: Two high resolution/accurate mass (HR/AM) targeted quantification methods were used to analyze 14 peptide standards spiked into a complex E. coli digest background. The data were automatically processed in Pinpoint.

Results: A detection limit of 10 amol and a linear dynamic range of 4 orders were obtained with msx tSIM methods in moderately complex background. A detection limit of 10-50 amol and a linear dynamic range of 3-4 orders were obtained with tHCD methods in strong complex background.

Introduction

Quantitative proteomics enables the identification of large number of protein candidates, which display biologically interesting dynamics on a global scale in the early discovery phase. Targeted MS approach, in particular selected reaction monitoring (SRM), has become the standard technique for quantitatively analyzing tens to hundreds of peptide candidates across large number of samples, either for understanding of signaling regulation or for verification and selection of potential clinical biomarkers. However, the low resolution of triple quadrupole MS limits its ability to achieve high selectivity on the targets from complex backgrounds. It is also difficult to develop SRM method for peptides with high charge states or high mass or for modified peptides.

In this study, the Thermo Scientific™ Q Exactive™ hybrid quadrupole –orbitrap mass spectrometer, a true high resolution and accurate mass (HR/AM) mass spectrometer, was evaluated for targeted protein quantification. The dynamic range and LOD/LOQ of two HR/AM methods, multiplexed targeted selected ion monitoring (msx tSIM) and targeted HCD (tHCD), were investigated.

Methods

Sample Preparation: Thermo Scientific heavy-isotope labeled peptide retention standards were spiked into either 5 ng/µL or 250 ng/µL E coli. whole cell tryptic digest to reach final concentrations of 0, 5 amol/µL, 25 amol/µL, 50 amol/µL, 500 amol/µL, 5 fmol/µL, and 50 fmol/µL.

Liquid Chromatography: Peptides from 2 µL of each sample were separated on a Michrom Magic™ (Bruker-Michrom) C18 nano LC column (75 µm × 15 cm, 3 µm particle) with a 1hr gradient at a flow rate of 350 nL/min. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). LC gradient was 0-2 min, 3%B; 2-47 min, 3-30%B; 47-52 min, 30-95%B; 52-54 min, 95%B; 54-55 min, 95-5% B; 55-60 min, 5%B.

Mass Spectrometry: The samples with 5 ng/µL E coli digest background were analyzed in triplicate with a Full-

msx tSIM method on the Q Exactive MS. The resolution was set at 140K for both scan types. The AGC target was 1E6 for full scan and 2E5 for SIM scan. The maximum ion injection time was 120 ms for full scan and 500 ms for SIM scan. The isolation width for SIM scan was 4 amu. The peptide retention standards were monitored over a 3 min window. Multiplexing level was set at 4, which allowed isolation and accumulation of up to four peptide targets in the c-trap before they were transferred to Orbitrap for detection (Figure 2)

The samples with 250 ng/µL E coli digest background were analyzed in triplicate with a tHCD method. The resolution was set at 17.5K.The AGC target was 5E5. The maximum ion injection time was 500 ms. The isolation width was 2.0 amu. The normalized collision energy (NCE) was 27%.The peptide retention standards were monitored over a 3 min window.

Data Analysis: The data were automatically processed using Pinpoint. Extract ion chromatogram for peptide retention standards were obtained with 5ppm mass tolerance. Peak areas were calculated with 7-point smoothed complete chromatograms. Linear fitting was performed with weighing scheme of 1/x.

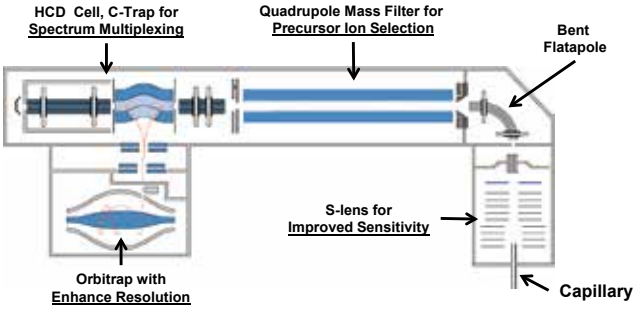


FIGURE 1. Schematic of the Q Exactive MS

Key Features of The Q Exactive MS

- The incorporation of S-lens at the source enhances its sensitivity by up to 5 fold.
- Quadrupole mass filter enables precursor selection for data-dependent MS^2 and selected ion monitoring (SIM).
- Advanced signal processing increases resolution by two folds, which results in a maximum resolution of 140K at *m/z* 200 and a maximum scan speed of 12Hz at a resolution of 17.5K.
- Spectrum multiplexing (msx) and parallel ion injection/ detection significantly improves duty cycle.

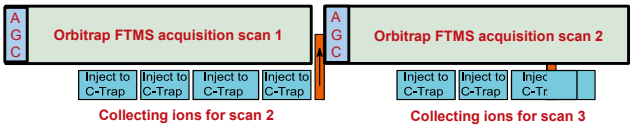


FIGURE 2. Spectrum Multiplexing and Parallel Ion Injection/ Detection Provides High Throughput Analyses

Up to 10 target ions can be isolated by the quadrupoles sequentially, stored in the C-trap, then transferred to the Orbitrap mass analyzer and detected with high resolution simultaneously. The isolation and trapping of the target ions is also concurrent with the Orbitrap mass analyzer detection of the previous ion packet.

Results

1. Targeted Quantification with msx tSIM

Full or SIM spectra were acquired at three different resolutions: 35K, 70K and 140K. Baseline separation of two ions that are only 30 ppm apart is only achievable at the resolution of 140K. The high mass accuracy (< 5 ppm) and high resolution ensures accurate identification of target peptides from complex background with msx tSIM.

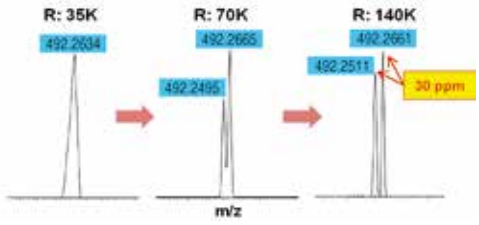


FIGURE 3. High Resolution Ensures Accurate Target Selection

Target peptides in 10 ng of E coli digest were analyzed with alternating full scans and tSIM scans (Figure 4). The peptide GLILVGGYGTR* (558.3259, 2+) is barely visible in the full scan with a 600 amu isolation window (Figure 4A), but is accurately identified with a S/N of 65 and mass deviation of ~0.2 ppm in the SIM scan with a 4 amu isolation window (Figure 4B). The +1 isotope peak is also accurately identified with a S/N of 39 and mass deviation of ~1.4 ppm in the SIM scan.

Extracted ion chromatograms (XIC) of target peptides in SIM scans were obtained with 5 ppm mass tolerance. The XIC of GISNEGQNASIK* (613.3167, 2+) at 100 fmol,

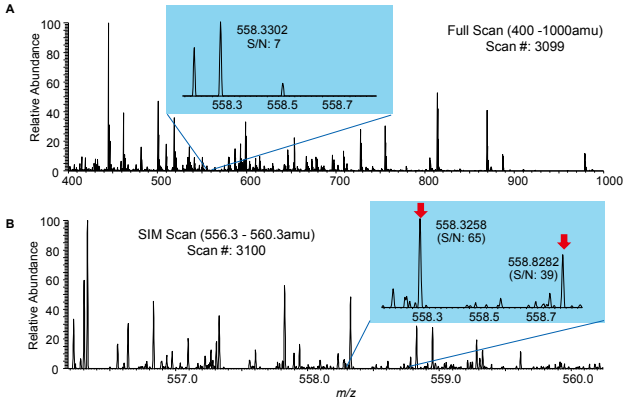


FIGURE 4. High Sensitivity with Quadrupole Based SIM Scan

1 fmol and 10 amol is displayed in Figure 5. Although GISNEGQNASIK* was monitored with two other peptides in the same time range, more than 10 SIM scans with a resolution of 140K were obtained across a 12 sec LC peak, even at a sample amount of 10 amol.

As shown in Figure 6, a 4-order linear dynamic range was obtained for most peptides with the msx tSIM method. Table 1 lists the LOD and LOQ of target peptides for the method. The CV% from triplicate analyses is also included for the LOQ.

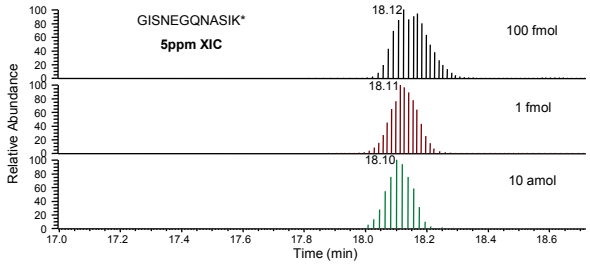


FIGURE 5. Multiplexing Improves Throughput and Results in Accurate Quantification

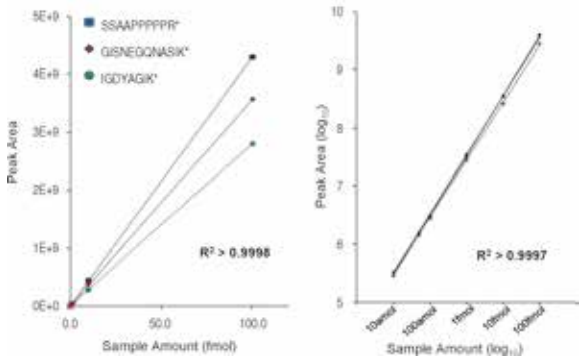


FIGURE 6. Four Order Linear Dynamic Range with msx tSIM Method.

TABLE 1. The LOD and LOQ of Target Peptides are Listed for msx tSIM Method. The CV% is also included for LOQ.

	LOD (amol)	LOQ (amol)	CV% (LOQ)
IGDYAGIK*	10	50	3
SSAAPPPPPR*	10	50	3
HVLTSIGEK*	10	50	3
LTILEELR*	<10	10	4
GLILVGGYGTR*	10	50	7
NGFILDGFPR*	<10	10	14
SAAGAFGPELSR*	<10	10	4
GISNEGQNASIK*	<10	10	10
ELASGLSFPVGFK*	<10	10	10
TASEFDSAIAQDK*	10	50	1
SFANQPLEVVYSK*	<10	10	8
ELGQSGVDTYLQTK*	10	50	7
LSSEAPALFQFDLK*	<50	50	3
GILFVGSGVSGGEEGAR*	50	100	5

2. Targeted Quantification with HCD

With tHCD, high resolution and high mass accuracy allow simultaneous accurate identification of multiple fragment ions from complex mixed MS2 spectra (Figure 7). As shown in Figure 8, 3-4 orders of linear dynamic range were obtained for most peptides with the tHCD method. Table 2 lists the LOD and LOQ of target peptides for the tHCD method. The CV% from triplicate analyses is also included for the LOQ.

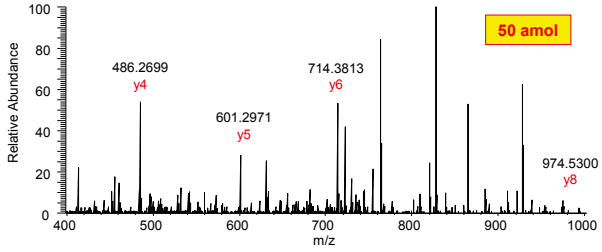


FIGURE 7. HCD Spectrum of Peptide NGFILDGFPR* (573.305, 2+) at 50 amol in 500 ng E coli Tryptic Digest Background.

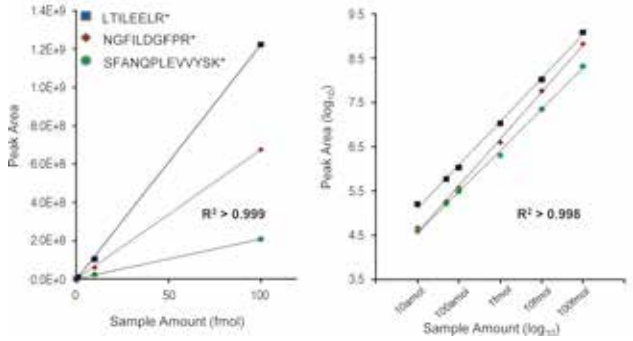


FIGURE 8. Three - Four Order Linear Dynamic Range with tHCD Method.

TABLE 2. The LOD and LOQ of Target Peptides are Listed for tHCD Method. The CV% is also included for LOQ.

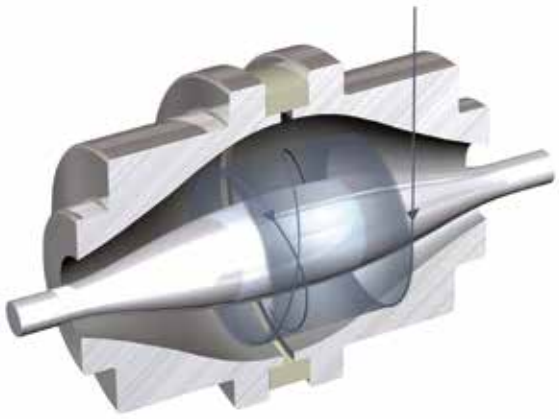
	LOD (amol)	LOQ (amol)	CV% (LOQ)
IGDYAGIK*	>10	50	6
SSAAPPPPPR*	10	50	11
HVLTSIGEK*	50	100	11
LTILEELR*	10	50	3
GLILVGGYGTR*	>10	50	8
NGFILDGFPR*	>10	50	6
SAAGAFGPELSR*	10	50	4
GISNEGQNASIK*	10	50	5
ELASGLSFPVGFK*	>10	50	2
TASEFDSAIAQDK*	10	50	1
SFANQPLEVVYSK*	<10	10	12
ELGQSGVDTYLQTK*	10	50	6
LSSEAPALFQFDLK*	10	50	6
GILFVGSGVSGGEEGAR*	>10	50	8

Conclusion

The Q Exactive MS is well suited for targeted quantification providing two powerful HR/AM approaches, msx tSIM and tHCD. Both approaches demonstrate high selectivity, high sensitivity and high throughput due to several hardware and software innovations.

- For the msx tSIM approach, high mass accuracy (< 5 ppm) and high resolution (140K) ensures accurate identification of target ions in the presence of complex background, while spectrum multiplexing dramatically increases throughput without sacrificing resolution.
- A LOD of 10 amol and 4-order linear dynamic range was obtained for most peptides with msx tSIM method in the presence of medium complex background.
- Different from triple-quadrupole-based SRM, all fragment ions are simultaneously detected with high mass accuracy and high resolution using the tHCD approach. The HR/AM of fragment ions provides unmatched selectivity. The simultaneous detection of all fragment ions allows more efficient sampling of target peptides and results in improved sensitivity. A scan speed of 12 Hz at a resolution of 17,500 ensures high throughput.
- A LOD of 50 amol and 3-4 orders linear dynamic range was obtained for most peptides with tHCD method in the presence of strong complex background.

精确　稳定



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