Journal of Visualized Experiments

N-glycan Profiling of Glycoproteins by Hydrophilic Interaction Liquid Chromatography-with Fluorescence and Mass Spectrometric Detection

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE62751R2
Full Title:	N-glycan Profiling of Glycoproteins by Hydrophilic Interaction Liquid Chromatography-with Fluorescence and Mass Spectrometric Detection
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TITLE:

N-glycan Profiling of Glycoproteins by Hydrophilic Interaction Liquid Chromatography with Fluorescence and Mass Spectrometric Detection

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SUMMARY:

N-glycan profiling of glycoproteins is essential for discovering novel biomarkers and understanding glycan functions in cellular events. Additionally, *N*-glycan analysis of protein biopharmaceuticals is very important for human use. In this current article, a high-throughput strategy for identifying and quantifying *N*-glycan structures was presented using the HILIC-FLD-MS/MS technique.

ABSTRACT:

Glycosylation is a vital modification found in proteins. *N*-glycan profiling of glycoproteins is required to detect novel biomarker candidates and determine glycan alterations in diseases. Most commercially available biopharmaceutical proteins are glycoproteins. The efficacy of these drugs is affected by glycosylation patterns. Therefore, an in-depth characterization method for the *N*-glycans is necessary. Here, we present a comprehensive approach for qualitative and quantitative analysis of *N*-glycans using hydrophilic interaction liquid chromatography equipped with fluorescence detection and tandem mass spectrometry (HILIC-FLD-MS/MS). *N*-glycans were released from glycoproteins with a facile method and labeled by a procainamide fluorophore tag in the strategy. Subsequently, the procainamide labeled *N*-glycans were analyzed by a HILIC-FLD-MS/MS technique. In this approach, *N*-glycan structures were confirmed by the tandem mass spectrometric analysis, whereas fluorescence detection was used for the quantitative analysis. An application for data analysis of the detected *N*-glycan peaks is described in the study. This protocol can be applied to any glycoprotein extracted from various species.

INTRODUCTION:

Glycosylation is a vital post-translational modification observed in proteins[1]. Multiple enzymatical processes regulate glycosylation modification in cellular organisms. Glycans are attached to the proteins by these enzymatical processes, and the proteins subjected to this modification are called glycoproteins[1]. Two glycosylation types are commonly observed in proteins. *O*-glycosylation is the attachment of *O*-glycans to the side chain of serine or threonine amino acid residues. *N*-glycosylation is the attachment of *N*-glycans to the side chain of asparagine amino acid residue in a protein.

The structure, stability, and folding of the proteins are affected by glycan attachments[2]. The glycosylation process dramatically influences the functions of the proteins, and glycoproteins regulate many cellular functions in organisms[3,4]. For example, heavily glycosylated proteins protect their glycoproteins from proteolytic degradation[5]. Another example is glycans of thyroid gland proteins that regulate Tg transport and hormone synthesis[6,7]. To explain their roles in cellular events, an in-depth characterization of glycoproteins is required[8].

N-glycan profiles of the glycoproteins change in disease situations[9-12]. Profiling *N*-glycans derived from crucial glycoproteins or body fluids is required to discover novel biomarkers and understand the enzymatic activity changes in disease cases. On the other hand, most protein biopharmaceuticals are glycoproteins, and their glycan profiles influence drug efficacy[13]. Therefore, an acceptable method of *N*-glycan profiling must be performed in developing proper protein biopharmaceuticals for human use[14].

Glycomics is an emerging discipline used to identify and quantify glycan structures of glycosylated molecules[15,16]. Many methods have been utilized for profiling the glycans of glycosylated species, including NMR[17] and MS[18]. Hydrophilic Interaction Liquid Chromatography-with Fluorescence Detection (HPLC-HILIC-FLD) is the gold standard method for profiling *N*-glycans derived from glycoproteins[19]. When this strategy is combined with mass spectrometric detection, identifying *N*-glycan structures could be easier and more reliable. Most fluorescence tags used in *N*-glycan analysis with mass spectrometry have low ionization efficiencies. In contrast, procainamide increases the ionization efficiencies of *N*-glycans, which is used to obtain efficient tandem mass spectra of *N*-glycan structures[20,21]. Specific fragments can be obtained from this strategy by tandem mass spectrometry for the structural identification of *N*-glycans such as core fucosylated[22] (proc-HexNAc1Fuc1) and bisecting types[23] (proc-Hex1HexNAc3, proc-Hex1HexNAc3Fuc1).

This study demonstrates a facile protocol for the *N*-glycan profiling of glycoproteins with HILIC-FLD-MS/MS. The presented method includes four steps: (1) releasing of *N*-glycans from glycoproteins (2) labeling of *N*-glycans by a procainamide tag (3) purification of the procainamide labeled *N*-glycans, and (4) data analysis.

PROTOCOL:

NOTE: The human plasma used is commercially available (**Table of Materials**). No further biological samples obtained from humans were used.

1. Glycan release

1.1. Denaturation of (glyco-)proteins

1.1.1. Prepare the glycoprotein standards (e.g., IgG, a monoclonal antibody) at a concentration of a 10 $\mu g \cdot \mu L^{-1}$ in deionized H_2O. For human plasma, the concentration used is 70 $\mu g \cdot \mu L^{-1}$.

NOTE: The samples should be vortexed until all solid proteins are dissolved. Human plasma (lyophilized) was used for the preparation of the plasma samples.

1.1.2. Take 20 µL of glycoprotein samples (200 µg) and lyophilized human plasma (1.4 mg).

1.1.3. Add 40 µL of 2% SDS (sodium dodecyl sulphate) (w/v).

1.1.4. Incubate the samples at 60 °C for 10 min to denature the (glyco-)proteins.

NOTE: The samples could be mixed at 500 rpm by a thermomixer during the incubation. A shaking water bath could be used alternatively.

1.2. Glycan release

1.2.1. Add 20 µL of 4% Igepal-CA630 to the samples from step 1.1.4.

1.2.2. Add 20 µL of 5x PBS (phosphate buffer saline).

1.2.3. Prepare PNGase F enzyme at the concentration of 1 $U \cdot \mu L^{-1}$ in deionized water.

1.2.4. Add 1 U of enzyme for glycoprotein standards and 2 U of enzyme for human plasma samples.

1.2.5. Incubate the samples at 37 °C for 16 h.

1.3. Procainamide labeling

1.3.1. Prepare labeling solution using procainamide hydrochloric acid (110 $mg \cdot mL^{-1}$ in DMSO/AA (dimethyl sulfoxide /glacial acetic acid) 7/3, v/v).

1.3.2. Prepare reductive amination solution using sodium cyanoborohydride (65 $mg \cdot mL^{-1}$ in DMSO/AA, 7/3, v/v).

CAUTION: Sodium cyanoborohydride is very toxic and flammable. Wear eye shields. 2-picoline borane complex (107 $mg \cdot mL^{-1}$ in DMSO) could be used alternatively.

1.3.3. Mix these solutions in a ratio of 1:1, v/v to prepare the labeling mixture.

1.3.4. Add 100 µL of this labeling mixture to the glycan released sample from step 1.2.5.

1.3.5. Incubate the samples at 65 °C for 2 h.

2. Purification of Procainamide Labeled *N*-glycans by Solid-phase Extraction

(SPE) Cartridge

2.1. Prepare a solution of microcrystalline cellulose (100 $mg \cdot mL^{-1}$) in deionized water.

2.2. Take 300 µL of microcrystalline cellulose and insert it into the microcentrifuge tubes.

2.3. Wash the microcrystalline cellulose with 1 mL of deionized H_2O.

2.4. Wash the microcrystalline cellulose with 1 mL of ACN/MQ (acetonitrile/dH_2O), 85/15, v/v for conditioning.

NOTE: The microcentrifuge should be used for 1 min to discard washing solutions carefully.

2.5. Take 120 µL of glycan release solution and mix with 680 µL of ACN to obtain proper loading conditions (85/15, v/v, ACN/sample).

2.6. Mix this mixture with microcrystalline cellulose in the microcentrifuge tubes.

2.7. Incubate it at room temperature in a thermomixer by shaking at 500 rpm for 15 min.

2.8. Transfer the slurry to an SPE cartridge (1 mL volume capacity).

NOTE: Do not allow air to enter cartridge packing.

2.9. Discard the loading solutions by passing slowly (1 drop/second).

NOTE: Ensure that the SPE system is connected to the vacuum pump. Solvents may flow with gravity. When necessary, apply vacuum via a vacuum pump. The applied vacuum pressure should be appropriately adjusted to transfer liquids down slowly.

2.10. Wash the sample by passing 1 mL of ACN/MQ/TFA (acetonitrile/dH_2O/trifluoroacetic acid) solution (85/14/1, v/v/v) twice.

2.11. Wash the sample by passing 1 mL of ACN/MQ mixture (85/15, v/v) twice.

2.12. Elute the procainamide labeled *N*-glycans with 0.75 mL of water.

2.13. Dry the elution solution with a concentrator overnight.

NOTE: Use a concentrator temperature of 45 °C for drying.

2.14. Dissolve the dried samples in a mixture of 100 µL of ACN/MQ (75/25, v/v) and transfer this solution to the vials, including an insert.

NOTE: The redissolved samples should be vortexed for 20 second.

3. HILIC-FLD-MS/MS Analysis

3.1. Insert Tee (T) adaptors to the HILIC column to separate the flow into the two equal volumes. Connect one of both to the FLD detector, the other to the MS detector.

NOTE: The flow lines must be the same lengths.

3.2. Adjust a gradient program for HILIC separations of procainamide labeled *N*-glycans as indicated in **Supplementary Figure 1**. Use Mobile Phase A: 50 mM ammonium formate (pH: 4.4 and Mobile Phase B: 100 % ACN).

NOTE: The gradient program could be further optimized depending on the glycan heterogeneity of samples.

3.3. Click the **Sampler** button and set the injection volume to 10 µL.

3.4. Click the **Column Comp** and adjust the column temperature to 60 °C.

3.5. Click **FLD** and adjust the FLD excitation and emission wavelengths to 310 nm and 370 nm, respectively.

3.6. Adjust the MS source, tune, and MS/MS parameters as displayed in **Supplementary Figure 2**.

NOTE: The MS and MS/MS parameters could be changed depending on the mass spectrometry used in the analysis.

4. Data Analysis

4.1. Identification of procainamide labeled *N*-glycans

4.1.1. Export MS/MS data by a proper format for database searches (e.g., .mgf, .xml).

4.1.2. Insert them into a database search tool for the identification of *N*-glycans.

4.1.3. Select the sample name listed in the software and click the **Glycan Search** button for the identification of procainamide labeled *N*-glycans by using the Carbbank database with given parameters (**Supplementary Figure 3**).

4.1.4. Open the edit chromatograms button in the data analysis software for raw data and select extracted ion chromatogram type by filtering in all MSn.

4.1.5. Add specific masses for core-fucosylated fragment proc-N1F1 (m/z 587.3^{+}) and bisected fragments proc-H1N3 (m/z 1009.5^{+}) and proc-H1N3F1 (m/z 1155.5^{+}).

4.1.6. Determine the precursors containing core-fucosylated and bisected *N*-glycan structure fragments.

4.1.7. Export the identified *N*-glycan structures by the software (**Supplementary Table 1-3**).

4.1.8. Check the precursors detected manually for determination of core-fucosylated and bisected *N*-glycan structures.

4.2. Quantification of procainamide labeled *N*-glycans by an open-source software[24]

4.2.1. Select FLD chromatogram in the chromatograms and click the method in the software. Open a method for exporting chromatograms as .xy file format.

4.2.2. Convert the .xy file format of exported chromatograms to .txt files.

4.2.3. Open the quantitative analysis software.

NOTE: The software used in the study can be downloaded from a Github source (https://github.com/Tarskin/HappyTools).

4.2.4. Follow the instructions and tutorials of the program presented by the developer.

NOTE: It is recommended to use a batch process to export relative areas.

4.2.5. Adjust the setting parameters (**Supplementary Figure 4**). Define four minimum peaks and 27 signal/noise ratios for calibrations.

4.2.6. Open the results file with proper software such as Microsoft Excel for further evaluation.

REPRESENTATIVE RESULTS:

In this presented approach, the *N*-glycans were first released, labeled by the procainamide tag and purified by cellulose-containing SPE cartridges. Then, *N*-glycan analysis of IgG, trastuzumab, and human plasma were performed by an HPLC-HILIC-FLD-MS/MS system. The MS (base peak) and FLD chromatograms of the determined *N*-glycan structures obtained from IgG and trastuzumab are shown in **Figure 1**, respectively. The MS/MS data obtained from these analyses were imported to the software and searched against a glycan database. Example MS/MS annotations of glycan structures from MS/MS spectra are given in **Supplementary Figure 5.** The lists of the detected *N*-glycan structures are provided in **Supplementary Information Table S1 and S2** for IgG and trastuzumab, respectively. In addition, core fucosylated and bisected *N*-glycan types were detected by the analysis of fragment ions (proc-N1F1 (m/z 587.3^{+}), proc-H1N3 (m/z 1009.5^{+}) and proc-H1N3F1 (m/z 1155.5^{+}) obtained from tandem MS analysis.

[place Figure 1 here].

This strategy was also followed by the analysis of human plasma glycoproteome (**Figure 2**). The list of the *N*-glycans were listed in **Supplementary Table S3** including the core fucosylation and bisecting *N*-glycan information. In the strategy, a python-based open-source tool was used to quantify *N*-glycan structures by using FLD chromatograms. The *N*-glycan profile of IgG *N*-glycans were exemplified and displayed in **Figure 3**. Thus, *N*-glycan profiling of glycoproteins were achieved by HILIC-FLD-MS/MS analysis.

[Place Figure 2 here]

[Place Figure 3 here]

FIGURE AND TABLE LEGENDS:

Figure 1: HILIC-FLD-MS/MS analysis of IgG and trastuzumab. Base peak and fluorescent chromatograms of procainamide-labeled *N*-glycans of (A, B) IgG and (**C, D)** trastuzumab, respectively.

Figure 2: HILIC-FLD-MS/MS analysis of human plasma glycome. (A) Base peak chromatogram (**B)** fluorescent chromatogram.

Figure 3: Relative abundances of IgG *N*-glycans.

Supplementary Figure 1: The gradient program applied in the study is illustrated.

Supplementary Figure 2. Mass spectrometric parameters applied in the study.

Supplementary Figure 3: The parameters for searching glycan structures used in the study.

Supplementary Figure 4: Parameters applied for the extraction of peak areas.

Supplementary Figure 5. Annotated MS/MS spectra of IgG *N*-glycans. **(A)** H3N5F1 **(B)** H4N4F1.

Supplementary Table 1: The list of *N*-glycans belonging to IgG obtained from HILIC-FLD-MS/MS analysis.

Supplementary Table 2: The list of *N*-glycans belonging to trastuzumab obtained from HILIC-FLD-MS/MS analysis.

Supplementary Table 3: The list of *N*-glycans belonging to human plasma glycoproteome obtained from HILIC-FLD-MS/MS analysis.

DISCUSSION:

N-glycan profiling of glycoproteins includes challenging steps. Although there are many different methodologies for this purpose, a suitable approach should be selected for both identification and quantification of *N*-glycan structures[14]. HILIC-FLD is the gold standard approach for the quantification of *N*-glycans. However, identification of all *N*-glycan types by FLD detection is not achieved. Therefore, tandem MS analysis is needed for confirming *N*-glycan structures derived from glycoproteins. By the combination of FLD and MS detection in the same system, analysis of the *N*-glycans is more efficient[23]. Due to these reasons, both identification and quantification of *N*-glycans are performed by HILIC-FLD-MS/MS for standard glycoproteins as well as complex samples such as human plasma.

N-glycans are labeled by a fluorescence tag from their reducing ends to detect and quantify them by FLD[25]. *N*-glycans have low ionization efficiencies in mass spectrometric analysis. Procainamide is a fluorescence tag that improves the ionization efficiencies compared with commonly applied tags such as 2-AB (2-aminobenzamide)[20,21]. Thus, we selected this tag for achieving tandem MS analysis of *N*-glycans efficiently. This tag also allows confirmation of core fucosylated and bisected *N*-glycan types by monitoring specific fragments obtained from tandem MS analysis[22,23] as stated in the presented protocol.

Collision-induced dissociation (CID) or higher-energy C-trap dissociation (HCD) is commonly applied to interpret the *N*-glycan spectra[8]. The MS/MS spectra can allow detection of the branching points and elongation of the *N*-glycan structure[26]. In addition, the linkage position of *N*-glycans can be assigned by monitoring cross-ring fragmentation in CID and HCD[27]. However, data analysis software is necessary to identify *N*-glycan structures together with instrumentation and bioanalytical methods[28]. Several commercially or freely available tools from literature can be used with the presented approach for interpreting *N*-glycan structures.

Purification of labeled *N*-glycans is usually applied before HILIC-FLD-MS/MS analysis because sample preparation steps contain various chemicals that have interfered with the analysis. Several commercially available sorbents based on HILIC interaction can be used for the purification of *N*-glycans[29]. Cellulose is one of the cheapest alternatives for the purification of *N*-glycans. It is employed not only for batch-mode experiments but also for solid-phase extraction applications. In addition, purifications can be achieved in 96 well plate platforms using cellulose. The presented method with the purification step may be optimized depending on the sample size. On the other hand, alternative materials such as porous graphitized carbon (PGC) can be inserted into the protocol to purify procainamide labeled *N*-glycans. Furthermore, PGC-based purification can be used with cellulose-based HILIC purification to increase purification efficiency[30].

Data analysis for profiling *N*-glycans is a time-consuming process. Recently, a python-based open-source tool to quantify peaks of *N*-glycans has been demonstrated[24]. This tool allows automated data analysis workflow for peak selection and retention time calibration. In addition, a large amount of data belonging to *N*-glycan samples can be analyzed by a batch-mode application. This tool makes the data analysis faster and easier. Additionally, other software can be inserted into

the current strategy to evaluate the obtained data. In conclusion, a facile method was demonstrated here to profile *N*-glycans using HILIC-FLD-MS/MS. This approach can apply to any glycoproteins such as protein biopharmaceuticals as well as complex samples to profile *N*-glycans.

ACKNOWLEDGMENTS:

This work was partly supported by the Ministry of Development-Republic of Turkey with project number: 2016 K121230. Bekir Salih gratefully acknowledges the Turkish Academy of Science (TUBA) for the partial financial support.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

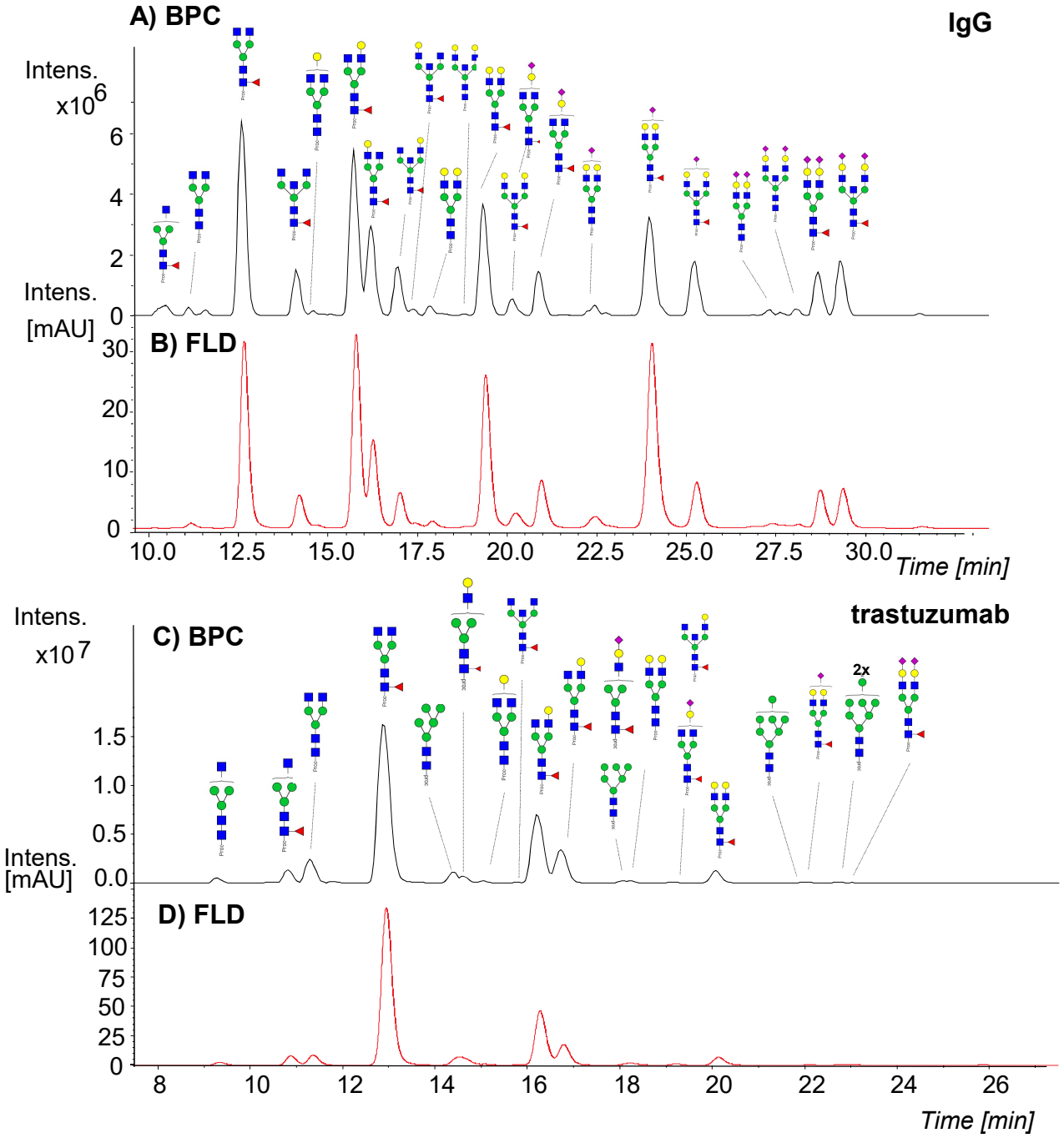

Figure 2

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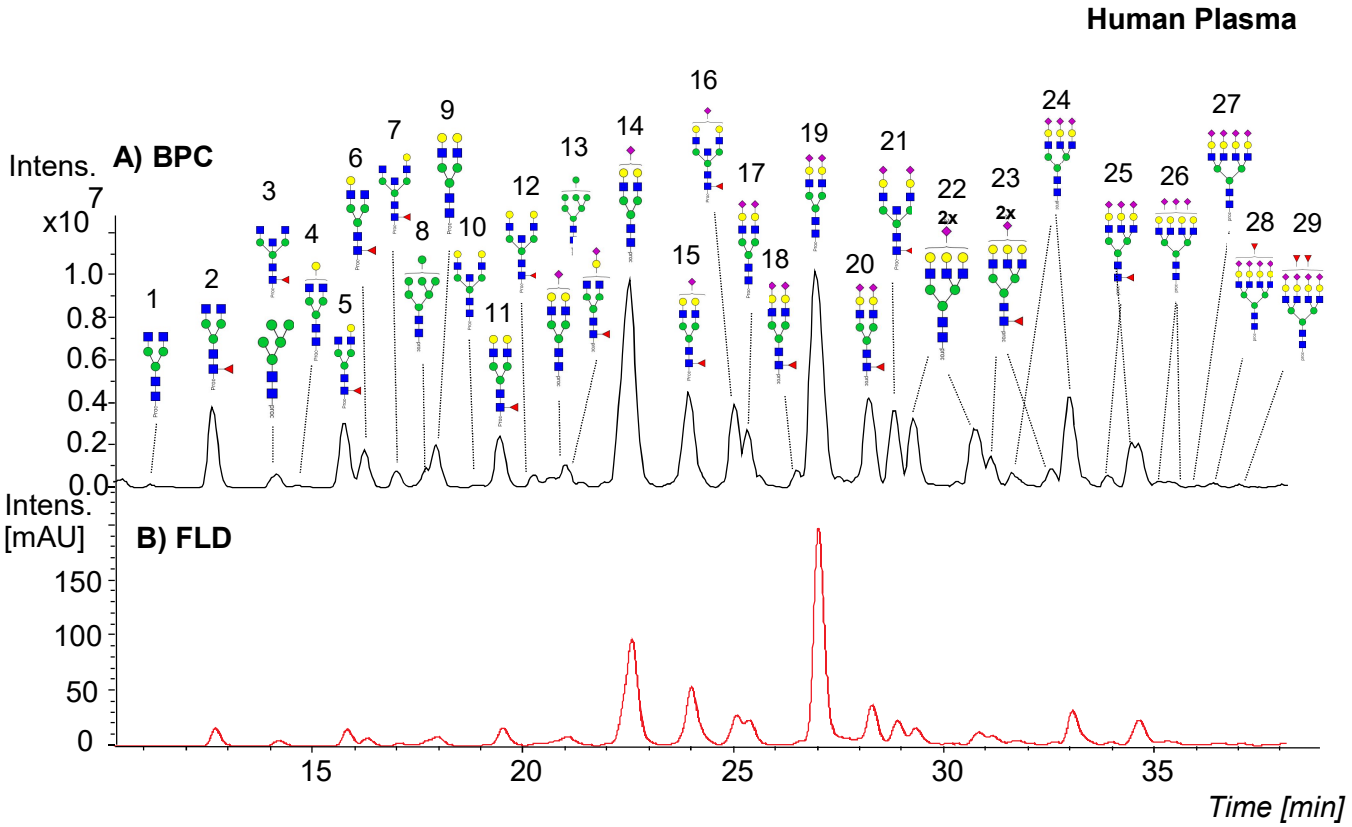

Figure 3

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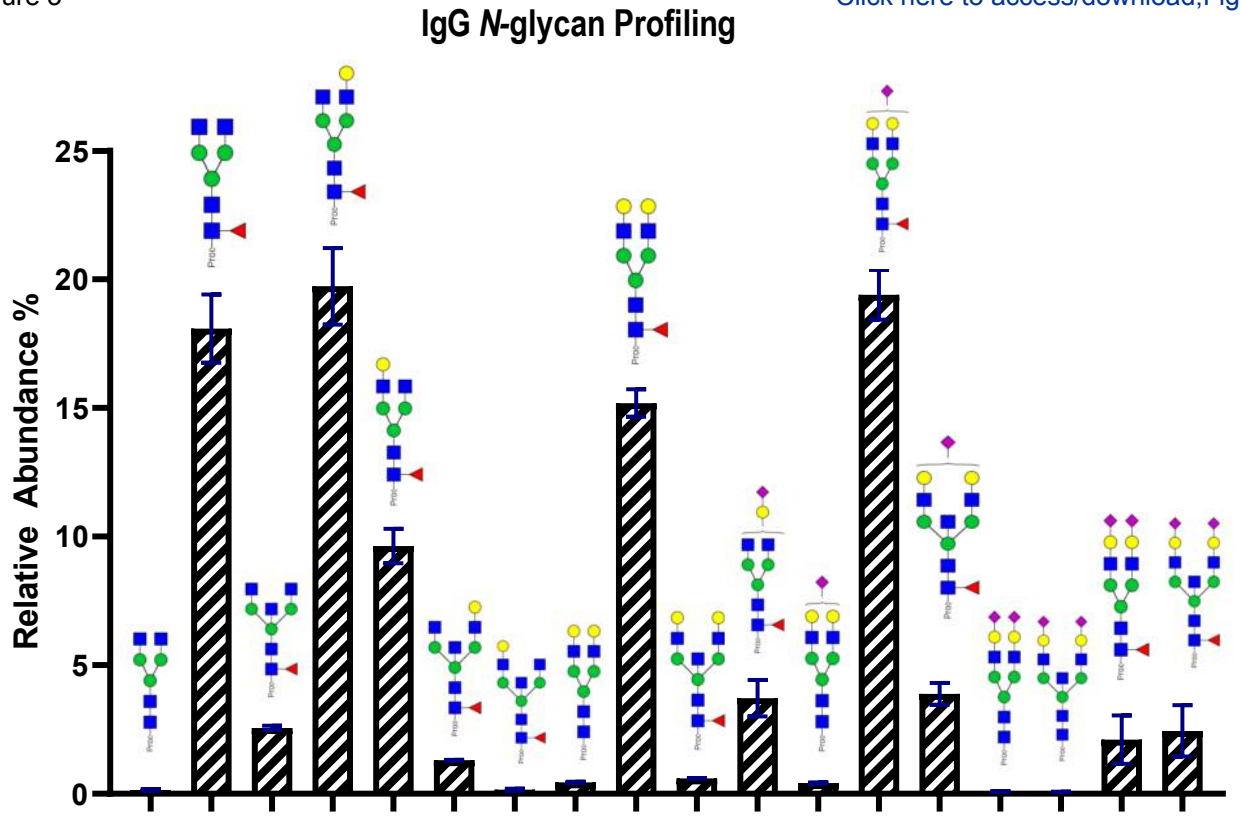

We appreciate the time and effort you and the reviewers dedicated to providing feedback on our manuscript. We have incorporated most of the suggestions made by the reviewers. Those changes are highlighted within the manuscript.

Please see below, in red, our detailed response to comments.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Haci Mehmet Kayili and Bekir Salih provide a well-engineered protocol for N-glycan profiling of glycoproteins by HILIC-FLD with parallel HILIC-MS2, after enzymatic digest and labelling of the N-glycans with procainamide. This method allows quantification by FLD with orthogonal in-depth characterization by MS2. The protocol is good structured and very detailed, allowing reproducibility of the described method. Furthermore, the authors include an exemplary open-source software for data analysis, increasing the educational character of the manuscript. I strongly recommend publication of their work in JoVE with some minor concerns stated below.

Major Concerns:

-

Minor Concerns:

-General: I would recommend some grammatical rephrasing, but this may be done during the copy-editing process (e.g. line 44: "Multiple enzymatical processes are regulating the glycosylation modification in cellular organism".

As you highlighted, grammar errors will be checked during the copy-editing process. We also reread the manuscript to clarify sentences.

-Abstract: In line 30 it is said: "Here, we present a comprehensive approach for qualitative and quantitative analysis of N-glycans using hydrophilic interaction liquid chromatography equipped with a fluorescence detector and a tandem mass spectrometry (HILIC-FLD-MS/MS)." I would recommend rephrasing to "fluorescence detection and tandem mass spectrometry", as the rest of the sentence describes techniques, while "fluorescence detector" is an instrument.

The sentence was changed in the manuscript, as the reviewer stated.

-Introduction: Considering the educational character of JoVE I recommend to go a bit deeper into details in the introduction (e.g. line 46 and following: "The glycosylation process dramatically influences the functions of the proteins2. Glycoproteins regulate many cellular functions in organisms3." Maybe name some examples, like 3D-structure or protein-protein interaction. Also the other parts of the introduction could be provided with more detais (depending of course, if there is a page limit or not).

We added a little bit more detail about glycosylation into the introduction part, as the reviewer stated.

-Protocol: In one incubation step the thermomixer is mentioned. If it is also used int the other incubations steps, I would add it. Although different labs have different mass spectrometers, also the settings for the ionization and mass spectrometry would be of interest in terms of reproducibility

The other incubation tool such as a shaking water bath was added to the related part of the protocol.

Reviewer #2:
Manuscript Summary:
Well prepared

Major Concerns:
The presented combination of Split after HILIC with fluorescence and MS is interesting and allows the proper characterization of carbohydrates in biosimilars and purified glycoproteins. My only concern is with regard to the MS part. It is basically focused on MS analysis. Following the abstract, it should be convenient to extend the discussion to MS/MS methods.

A part was added to the discussion part for evaluating MS methodology in the presented strategy.

Minor Concerns:
In the introduction for glycoprofiling, the authors could also mention other techniques. For instance, recent advances in the application of NMR methods: Unione et al. ACS Central Sci 2019, 5(9):1554-1561

The NMR technique was cited as stated by the reviewer as a glycoprofiling method.

Reviewer #3:
This manuscript describes a protocol for N-glycan profiling of glycoproteins, and an example is given on an IgG (not defined), trastuzumab, and human plasma glycoproteins. The topic is appropriate for JoVE and is of interest to researchers working in this field.

IgG from human serum was listed in the Materials list.

Major concerns:
- English is poor. Major revision is needed to make the manuscript easily understandable.

A proof-reading system of jove will check English of the manuscript prior to publication. We also reread the manuscript carefully.

- You do not explain with enough details what is the advantage of procainamide compared to other derivatizing agents such as 2-AB, 2-AA, RapiFluor-MS, ...

A part regarding the advantage of the procainamide tag was inserted into the discussion part.

- The HPLC column that is used is not described in the protocol!!!

HPLC column used in the study was inserted in the Materials List.

- MS parameters are not provided in the protocol. Even if it has to be adapted from one MS equipment to another, it should be given as an example.

MS parameters used in the study were added into the Supplementary Information. (Figure s2).

- I don't understand why HappyTools is used. All the chromatography software allow the integration and quantification of peaks. Using third-party software is strongly discouraged in pharma environments.

It was aimed to introduce and quantify *N*-glycans using open-source software. We have thought that this would be good for the users who don't reach appropriate software. Furthermore, batch processes can be quickly done by this open software when compared with commercial alternatives. On the other hand, users can utilize their software for the quantification of procainamide labeled *N*-glycans. Happytools could be the only open-source option.

- Discussion does not give many information that is not already included in the introduction. It should be revised.

Discussion were revised.

- Examples of MS and MS/MS spectra should be presented.

Two MS/MS spectra were given in Supplementary Information Figure 5.

Minor concerns:
- In the introduction, you mention that glycomics is an emerging field. The publication you cite is from ... 2010! This is not what I consider as being "emerging".

The citation was updated.

- With an overnight evaporation in a SpeedVac, don't you observe partial desialylation?

No. We use this process at a relatively low temperature.

- What is the temperature for the evaporation?

It was 45 ^{o}C which was inserted into the protocol section.

- In the protocol, you should explain what is the role of Igepam-CA630. This is not a common reagent for this type of sample preparation.

"IGEPAL® CA-630 is a nonionic, non-denaturing detergent suitable for solubilization, isolation and purification of membrane protein complexes." It is the alternative nonionic reagent of NP-40. Sigma-Aldrich has replaced Nonidet P-40 with Igepal CA-630. It increases the solubilization efficiency of proteins.

- Abbreviations should be defined (eg MQ or AA)

Abbreviations were explained in the text.

- Mobile phases A and B in the text are reversed compared to what is shown in Figure S1

Yes. It was written reverse. Mobil phases were written correctly.

- No capital letters should be used for "trastuzumab" (written "trastuzumAb" in the protocol)

All were rewritten without capital letters.

- In Table S1, what do "FragCov %" stand for? Percentages higher than 100% are sometimes reported.

Frag. Cov % represents the percentage ratio of total detected fragments of *N*-glycans to the theoretically generated fragments from in-silico digestion of the related glycans using a database. Additional multiple charged fragments can be detected in the MS/MS spectra in addition to single charged fragments. This may increase the Frag. Cov. % for calculating this feature.

Editorial and production comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Done.

3. Please provide an email address for each author.

Done.

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Done.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Sep Pack, Protein Scape Software, GlycoQuest, HappyTools, etc.

All of them were removed.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Done.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Done.

8. Please describe the actions in complete steps.

Done.

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Done. All steps were reread for giving more detail. Some parts were added.

10. Please ensure the representative results show the effectiveness of your technique backed up with data. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

All necessary results were inserted.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

No need. All figures used were obtained for this study.

12. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

Done.

13. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Some parts were added to explain some detail regarding to protocol.

14. Please sort the materials table in alphabetical order.

Done.

15. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

Done.

Table

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemantary Table 1.xlsx

Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]
1	Hex3HexNAc3dHex1-proc	740.3234	2	740.3266	1478.6386	-0.0064
2	Hex3HexNAc4-proc	768.8346	2	768.8373	1535.6601	-0.0055
3	Hex3HexNAc4dHex1-proc	841.8635	2	841.8663	1681.718	-0.0055
3	Hex3HexNAc5dHex1-proc	943.4019	2	943.406	1884.7974	-0.008
4	Hex4HexNAc4-proc	849.8603	2	849.8637	1697.7129	-0.0068
5	Hex4HexNAc4dHex1-a-proc	615.5961	3	615.5975	1843.7708	-0.0043
6	Hex4HexNAc4dHex1-b-proc	922.8895	2	922.8927	1843.7708	-0.0064
7	Hex4HexNAc5dHex1-a-proc	1024.4288	2	1024.4324	2046.8502	-0.0071
8	Hex4HexNAc5dHex1-b-proc	1024.4289	2	1024.4324	2046.8502	-0.007
9	Hex5HexNAc4-proc	620.9269	3	620.9292	1859.7657	-0.007
10	Hex5HexNAc5-proc	688.6205	3	688.6223	2062.8451	-0.0054
11	Hex5HexNAc4dHex1-proc	669.6136	3	669.6152	2005.8236	-0.0046
12	Hex5HexNAc5dHex1-proc	737.3065	3	737.3083	2208.903	-0.0054
13	Hex4HexNAc4NeuAc1dHex1-proc	712.6274	3	712.6294	2134.8662	-0.0058
14	Hex4HexNAc4NeuAc1dHex1-proc	1068.4367	2	1068.4404	2134.8662	-0.0075
15	Hex5HexNAc4NeuAc1-proc	717.9597	3	717.961	2150.8611	-0.0039
16	Hex5HexNAc4NeuAc1dHex1-proc	766.645	3	766.647	2296.919	-0.0058
17	Hex5HexNAc5NeuAc1dHex1-proc	834.3381	3	834.3401	2499.9984	-0.0058
18	Hex5HexNAc4NeuAc2-proc	814.9904	3	814.9928	2441.9566	-0.0071
19	Hex5HexNAc5NeuAc2-proc	882.6839	3	882.6859	2646.0446	-0.0109
20	Hex5HexNAc4NeuAc2dHex1-proc	863.6771	3	863.6788	2588.0145	-0.0049
21	Hex5HexNAc5NeuAc2dHex1-proc	931.3698	3	931.3719	2791.0938	-0.0064

Rt [min]	Score	FragCov. [%]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ m/z [ppm]	Core Fucosyla	Bisected
10.7	61.5	40	1479.6395	1479.6459	-0.0032	-4.3	+	
11.14	90.5	82	1536.6618	1536.6673	-0.0028	-3.58		
12.53	89.9	83	1682.7198	1682.7253	-0.0027	-3.24	+	
14.18	110.2	126	1885.7966	1885.8046	-0.004	-4.27	+	+
14.45	93	88	1698.7134	1698.7202	-0.0034	-3.98		
15.65	84.8	76	1844.7738	1844.7781	-0.0014	-2.33	+	
16.03	78.9	64	1844.7717	1844.7781	-0.0032	-3.46	+	
16.99	99.8	102	2047.8503	2047.8575	-0.0036	-3.47	+	+
17.1	83	72	2047.8505	2047.8575	-0.0035	-3.41	+	+
17.78	119.3	150	1860.766	1860.773	-0.0023	-3.74		
18.69	79.4	71	2063.847	2063.8524	-0.0018	-2.61		+
19.22	96.7	97	2006.8263	2006.8309	-0.0015	-2.29	+	
20.01	76.1	61	2209.9049	2209.9103	-0.0018	-2.42	+	+
20.57	74.6	60	2135.8677	2135.8735	-0.0019	-2.72	+	
20.82	66.7	46	2135.866	2135.8735	-0.0037	-3.5	+	
22.29	78.6	68	2151.8645	2151.8684	-0.0013	-1.79		
24.22	77	64	2297.9205	2297.9263	-0.0019	-2.52	+	
25.18	68.6	49	2500.9999	2501.0057	-0.0019	-2.33	+	+
27.15	70.5	67	2442.9567	2442.9638	-0.0024	-2.91		
27.91	79.4	69	2647.041	2647.0519	-0.0036	-4.1		+
28.73	74.4	61	2589.0168	2589.0217	-0.0016	-1.89	+	
29.3	77.9	66	2792.0947	2792.1011	-0.0021	-2.3	+	+

Table

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Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]	Rt [min]
1	Hex3HexNAc3-proc	667.2955	2	667.2976	1332.5807	-0.0043	9.25
2	Hex3HexNAc3dHex1-proc	740.3239	2	740.3266	1478.6386	-0.0053	10.62
3	Hex3HexNAc4-proc	768.8343	2	768.8373	1535.6601	-0.006	11.22
4	Hex3HexNAc4dHex1-proc	841.8635	2	841.8663	1681.718	-0.0055	12.78
5	Hex5HexNAc2-proc	727.8089	2	727.8108	1453.607	-0.0038	14.3
6	Hex4HexNAc4-proc	849.8606	2	849.8637	1697.7129	-0.0063	14.47
7	Hex4HexNAc3dHex1-proc	821.3504	2	821.353	1640.6914	-0.0052	14.55
8	Hex3HexNAc5dHex1-proc	943.4033	2	943.406	1884.7974	-0.0054	15.79
9	Hex4HexNAc4dHex1-proc	922.8892	2	922.8927	1843.7708	-0.0069	16.06
10	Hex4HexNAc4dHex1-proc	922.8886	2	922.8927	1843.7708	-0.0081	16.66
11	Hex4HexNAc3NeuAc1dHex1-proc	966.8977	2	966.9007	1931.7868	-0.0061	18
12	Hex6HexNAc2-proc	808.8352	2	808.8372	1615.6598	-0.0039	18.13
13	Hex5HexNAc4-proc	930.8873	2	930.8901	1859.7657	-0.0057	18.53
14	Hex4HexNAc4NeuAc1dHex1-proc	1068.4368	2	1068.4404	2134.8662	-0.0072	19.07
15	Hex4HexNAc5dHex1-proc	1024.4289	2	1024.4324	2046.8502	-0.0069	19.12
16	Hex5HexNAc4dHex1-proc	1003.9159	2	1003.9191	2005.8236	-0.0065	20
17	Hex7HexNAc2-proc	889.8603	2	889.8636	1777.7126	-0.0065	21.93
18	Hex5HexNAc4NeuAc1dHex1-proc	766.6451	3	766.647	2296.919	-0.0056	22.65
19	Hex8HexNAc2-proc	970.8868	2	970.89	1939.7654	-0.0064	25.17
20	Hex5HexNAc4NeuAc2dHex1-proc	863.6766	3	863.6788	2588.0145	-0.0066	25.85

Score	FragCov. [%]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ m/z [ppm]	Core Fucosyla	Bisected
92	94	1333.5837	1333.588	-0.0021	-3.2		
73	60	1479.6406	1479.6459	-0.0027	-3.58	+	
97.8	109	1536.6614	1536.6673	-0.003	-3.88		
78.7	70	1682.7197	1682.7253	-0.0028	-3.28	+	
87.3	85	1454.6105	1454.6143	-0.0019	-2.6		
101.2	114	1698.7139	1698.7202	-0.0032	-3.71		
83.1	75	1641.6935	1641.6987	-0.0026	-3.16	+	
82.5	88	1885.7992	1885.8046	-0.0027	-2.86	+	
74.8	61	1844.7712	1844.7781	-0.0034	-3.74	+	
79.8	70	1844.77	1844.7781	-0.004	-4.38	+	
69.1	51	1932.788	1932.7941	-0.0031	-3.16	+	
99.4	109	1616.6632	1616.6671	-0.002	-2.42		
84.3	85	1860.7673	1860.773	-0.0028	-3.06		
67.8	51	2135.8663	2135.8735	-0.0036	-3.36	+	
71.9	61	2047.8505	2047.8575	-0.0035	-3.37	+	
89.7	89	2006.8244	2006.8309	-0.0032	-3.23	+	
113.7	141	1778.7134	1778.7199	-0.0033	-3.66		
73.6	57	2297.9207	2297.9263	-0.0019	-2.44	+	
111.5	137	1940.7663	1940.7727	-0.0032	-3.32		
69.3	51	2589.0152	2589.0217	-0.0022	-2.54	+	

Table

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Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]
1	Hex3HexNAc4-proc	768.8349	2	768.8373	1535.6601	-0.0048
2	Hex3HexNAc4dHex1-proc	841.863	2	841.8663	1681.718	-0.0065
3	Hex5HexNAc2-proc	727.8083	2	727.8108	1453.607	-0.005
3	Hex3HexNAc5dHex1-proc	943.4023	2	943.406	1884.7974	-0.0073
4	Hex4HexNAc4-proc	849.8603	2	849.8637	1697.7129	-0.0068
5	Hex4HexNAc4dHex1-proc	922.8894	2	922.8927	1843.7708	-0.0065
6	Hex4HexNAc4dHex1-proc	922.8899	2	922.8927	1843.7708	-0.0055
7	Hex4HexNAc5dHex1-proc	1024.4285	2	1024.4324	2046.8502	-0.0077
8	Hex6HexNAc2-proc	808.8343	2	808.8372	1615.6598	-0.0058
9	Hex5HexNAc4-proc	930.887	2	930.8901	1859.7657	-0.0064
10	Hex5HexNAc5-proc	1032.4259	2	1032.4298	2062.8451	-0.0079
11	Hex5HexNAc4dHex1-proc	1003.9151	2	1003.9191	2005.8236	-0.008
12	Hex5HexNAc5dHex1-proc	1105.4551	2	1105.4588	2208.903	-0.0073
13	Hex5HexNAc4NeuAc1-proc	717.9599	3	717.961	2150.8611	-0.0032
13	Hex4HexNAc4NeuAc1dHex1-proc	1068.4371	2	1068.4404	2134.8662	-0.0066
13	Hex7HexNAc2-proc	889.8608	2	889.8636	1777.7126	-0.0055
14	Hex5HexNAc4NeuAc1-proc	1076.4338	2	1076.4378	2150.8611	-0.008
15	Hex5HexNAc4NeuAc1dHex1-proc	1149.463	2	1149.4668	2296.919	-0.0076
16	Hex5HexNAc5NeuAc1dHex1-proc	834.3376	3	834.3401	2499.9984	-0.0074
17	Hex5HexNAc4NeuAc2-proc	814.9904	3	814.9928	2441.9566	-0.0071
18	Hex5HexNAc4NeuAc2dHex1-proc	863.6766	3	863.6788	2588.0145	-0.0064
19	Hex5HexNAc4NeuAc2-proc	814.9908	3	814.9928	2441.9566	-0.006
20	Hex5HexNAc4NeuAc2dHex1-proc	863.6765	3	863.6788	2588.0145	-0.0069
21	Hex5HexNAc5NeuAc2dHex1-proc	931.3692	3	931.3719	2791.0938	-0.0081
22	Hex6HexNAc5NeuAc2-proc	937.035	3	937.0397	2808.0974	-0.0143
23	Hex6HexNAc5NeuAc2dHex1-proc	985.3868	3	985.3895	2953.1467	-0.0081
24	Hex6HexNAc5NeuAc3-proc	1034.0671	3	1034.0716	3099.1928	-0.0133
25	Hex6HexNAc5NeuAc3dHex1-proc	1082.7531	3	1082.7575	3245.2507	-0.0132
26	Hex7HexNAc6NeuAc3proc	1155.4425	3	1155.4461	3463.3164	-0.0036
27	Hex7HexNAc6NeuAc4-proc	1252.4744	3	1252.4779	3754.4118	-0.0035
28	Hex7HexNAc6NeuAc4dHex1-proc	1301.1608	3	1301.1638	3900.4697	-0.003
29	Hex7HexNAc6NeuAc4dHex2-proc	1349.8461	3	1349.8498	4046.5276	-0.0037

Rt [min]	Score	FragCov. [%]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ m/z [ppm]	Core Fucosyla	Bisected
11.19	90.3	94	1536.6625	1536.6673	-0.0024	-3.14		
12.84	75.4	65	1682.7188	1682.7253	-0.0032	-3.86	+	
14.02	86.5	83	1454.6093	1454.6143	-0.0025	-3.43		
14.11	88.3	91	1885.7974	1885.8046	-0.0036	-3.85	+	+
14.38	98.9	110	1698.7133	1698.7202	-0.0034	-4.02		
15.71	79	68	1844.7716	1844.7781	-0.0033	-3.53	+	
16.45	79.3	69	1844.7726	1844.7781	-0.0028	-2.99	+	
16.9	76.4	65	2047.8498	2047.8575	-0.0038	-3.74	+	+
17.31	95	100	1616.6613	1616.6671	-0.0029	-3.58		+
17.88	112.8	141	1860.7666	1860.773	-0.0032	-3.41		
18.84	82.4	77	2063.8445	2063.8524	-0.0039	-3.82		
19.44	86.2	82	2006.8229	2006.8309	-0.004	-3.98	+	
20.23	79.9	71	2209.9029	2209.9103	-0.0037	-3.32	+	+
20.62	97.7	102	2151.8652	2151.8684	-0.0011	-1.49		
21.04	63	44	2135.8669	2135.8735	-0.0033	-3.09	+	
21.28	108.6	125	1778.7144	1778.7199	-0.0027	-3.09		
22.41	85	80	2151.8604	2151.8684	-0.004	-3.74		
23.81	63	44	2297.9187	2297.9263	-0.0038	-3.3	+	
25	69	49	2500.9982	2501.0057	-0.0025	-2.98	+	+
25.2	98.1	101	2442.9568	2442.9638	-0.0024	-2.89		
26.5	70.2	51	2589.0154	2589.0217	-0.0021	-2.46	+	
26.87	94.4	93	2442.9578	2442.9638	-0.002	-2.47		
28.11	70.8	53	2589.0148	2589.0217	-0.0023	-2.68		
28.74	70.3	51	2792.093	2792.1011	-0.0027	-2.89	+	+
29.25	76.1	60	2809.0904	2809.1047	-0.0048	-5.08		
31.04	67.6	48	2954.1459	2954.1539	-0.0027	-2.73	+	
31.43	66.6	50	3100.1868	3100.2001	-0.0044	-4.3		
33.23	49.6	35	3246.2448	3246.258	-0.0044	-4.06		
35.3	nd	nd	3464.3129	3464.3236	-0.0107	-3.09		
37	nd	nd	3755.4089	3755.4191	-0.0102	-2.72		
39.5	nd	nd	3901.4678	3901.477	-0.0092	-2.36		
40.3	nd	nd	4047.5237	4047.5349	-0.0112	-2.77		

S1

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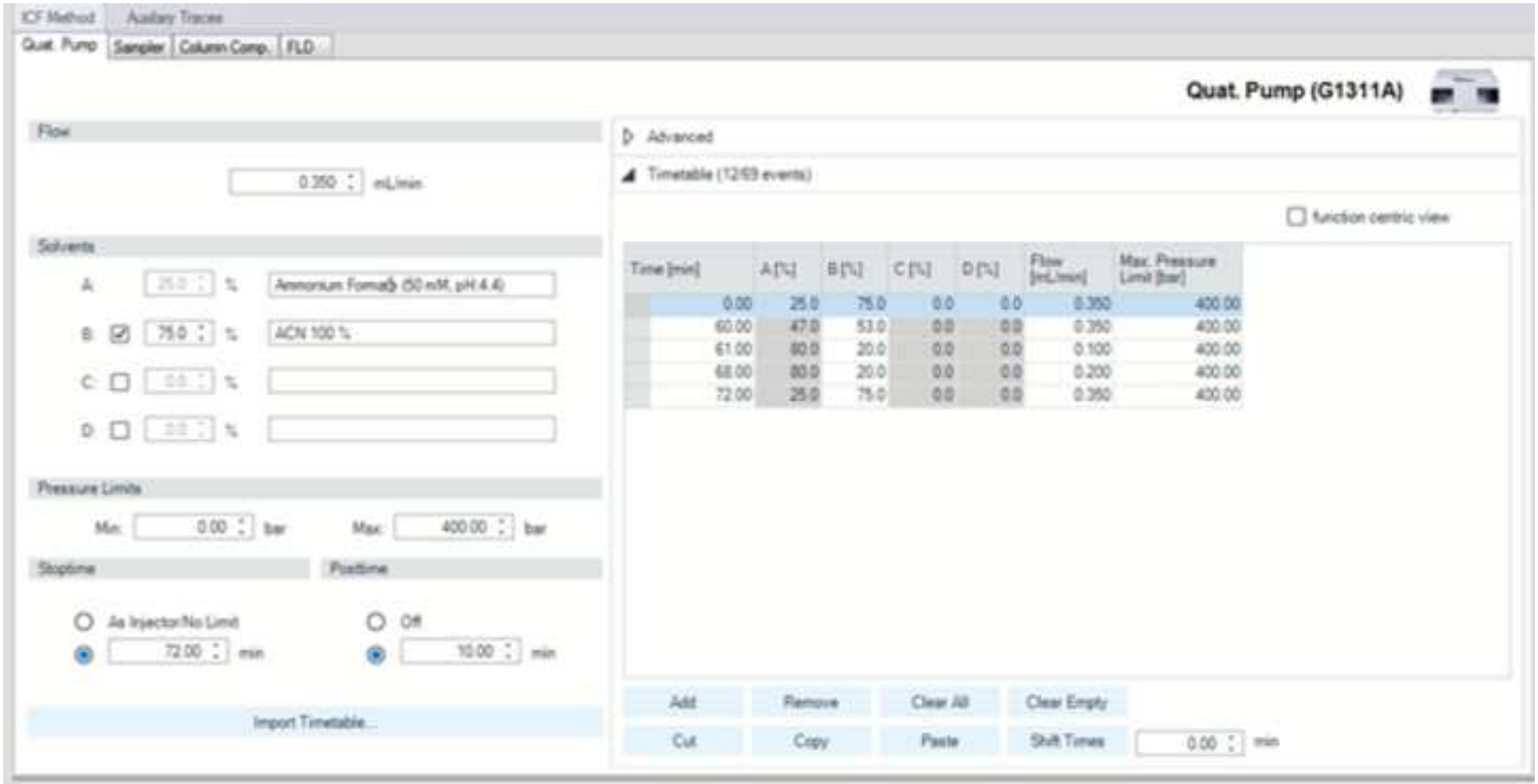

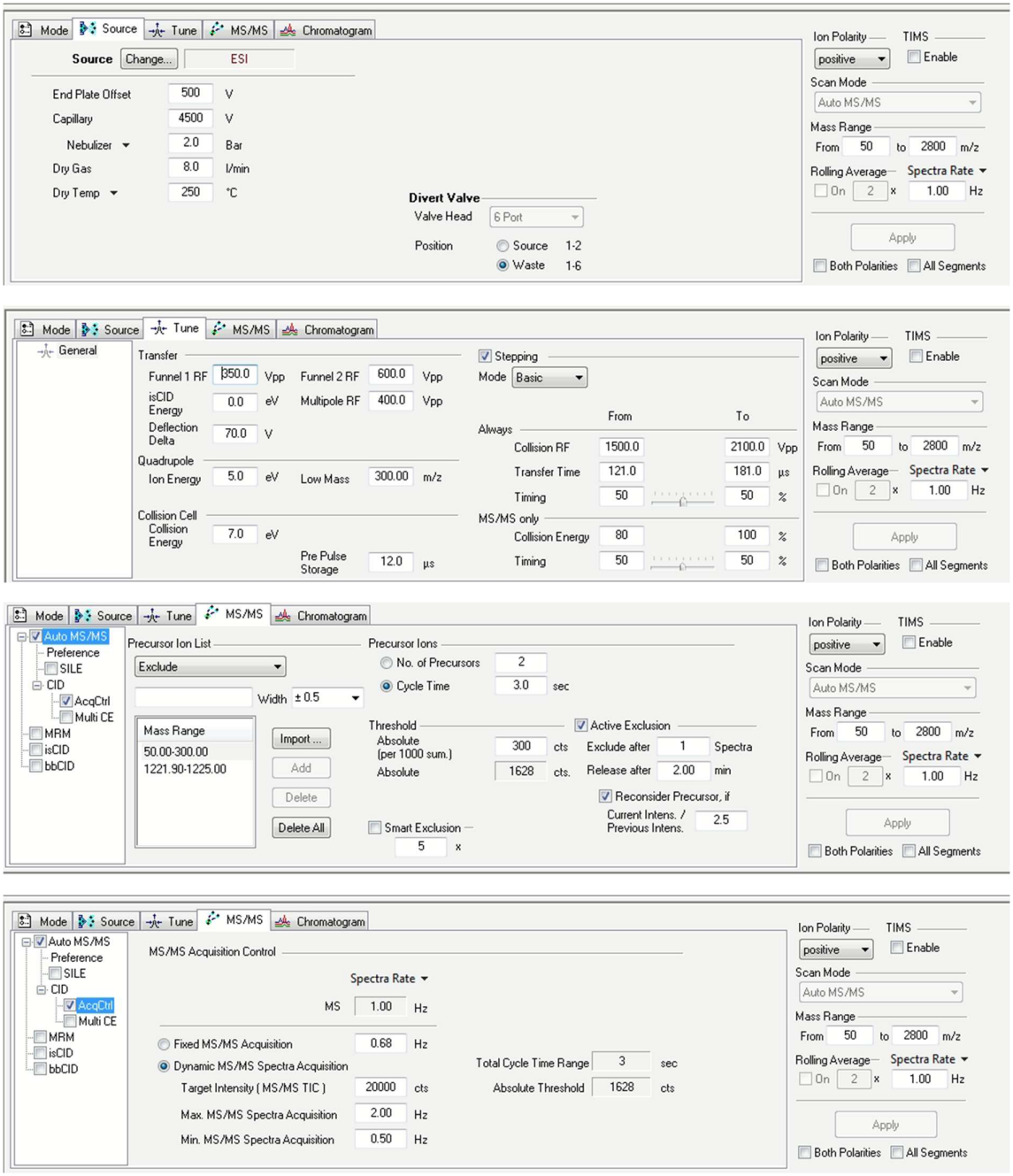

Supplementary Figure 2. Mass spectrometric parameters applied in the study.

S3

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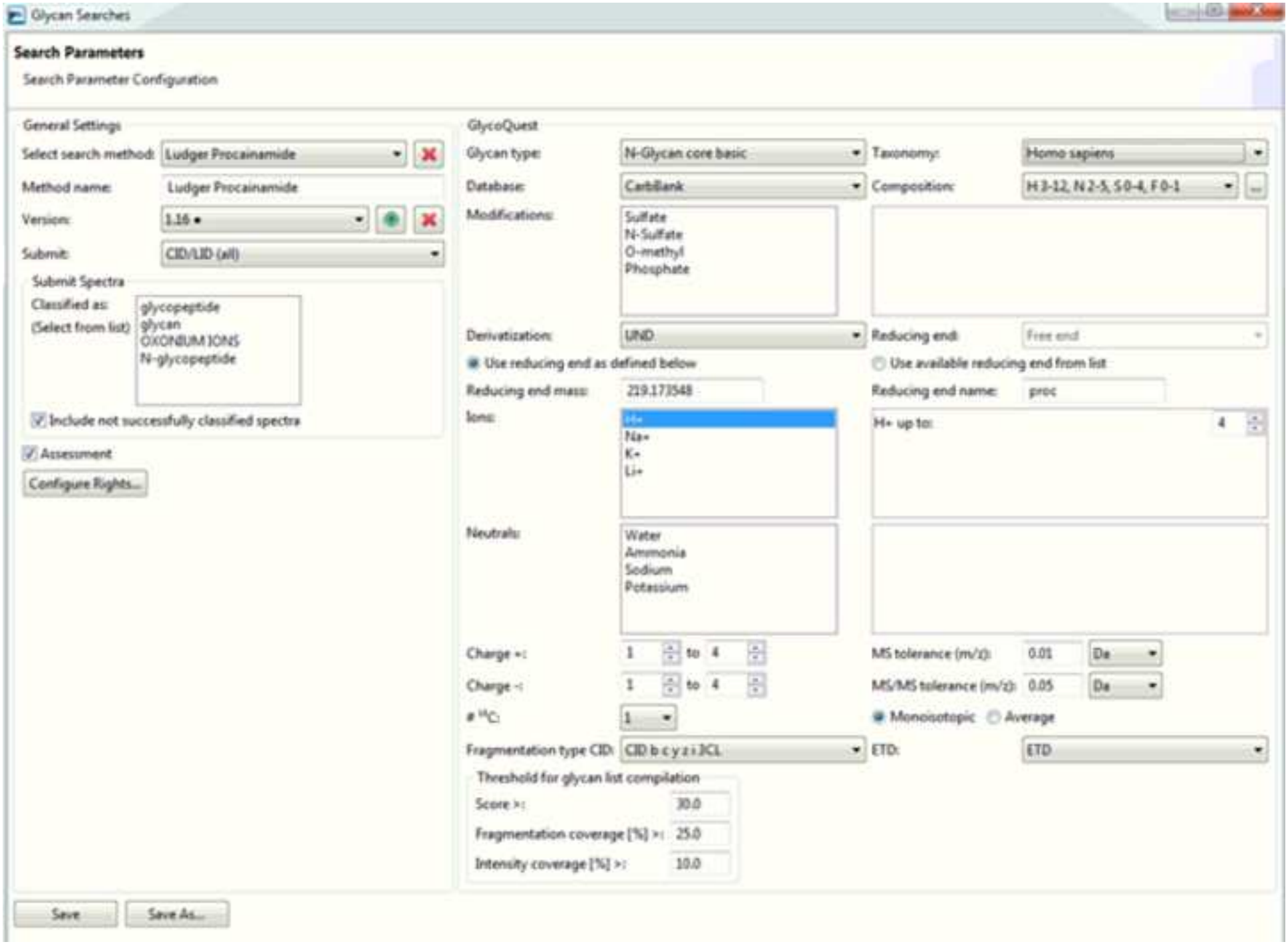

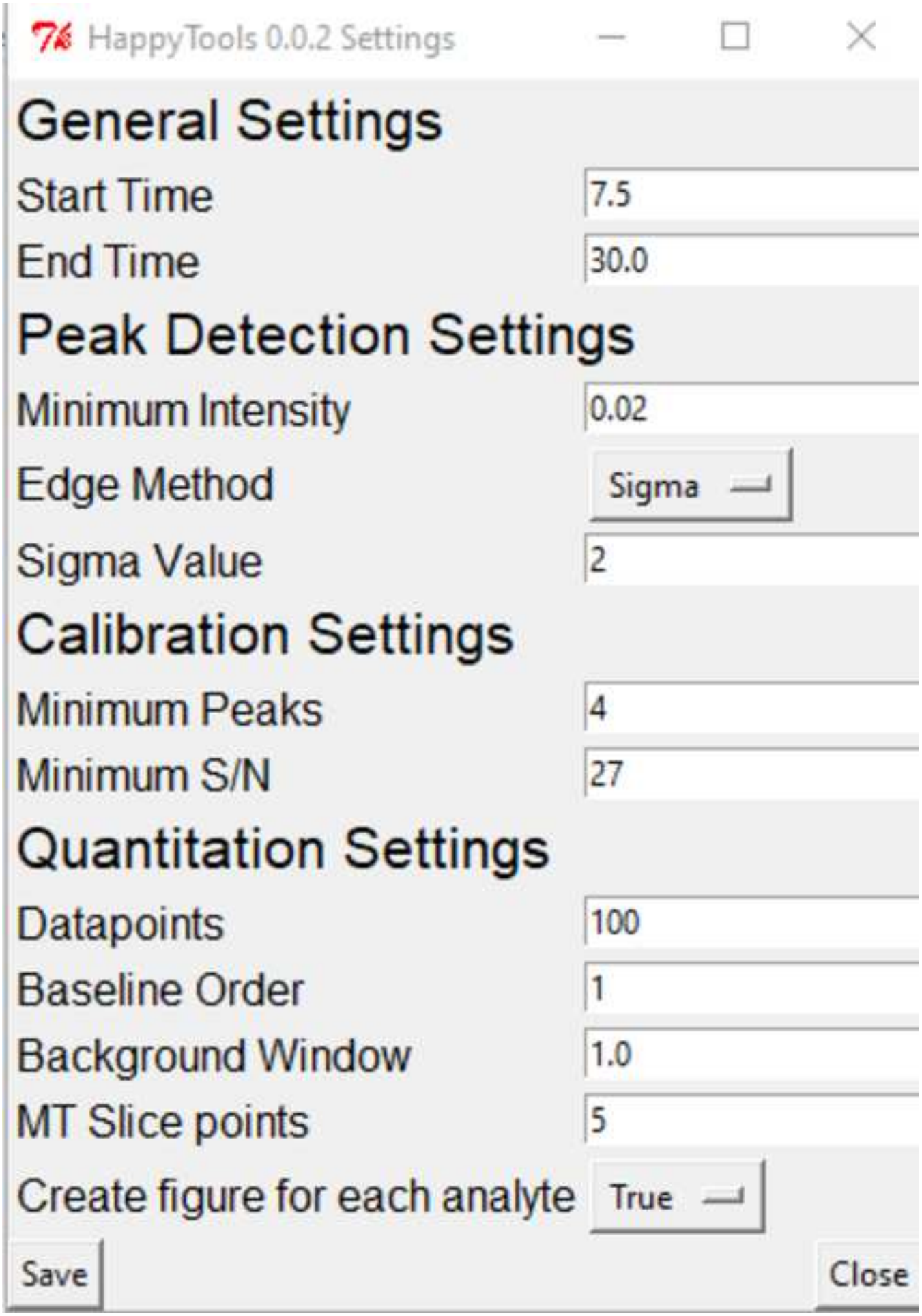

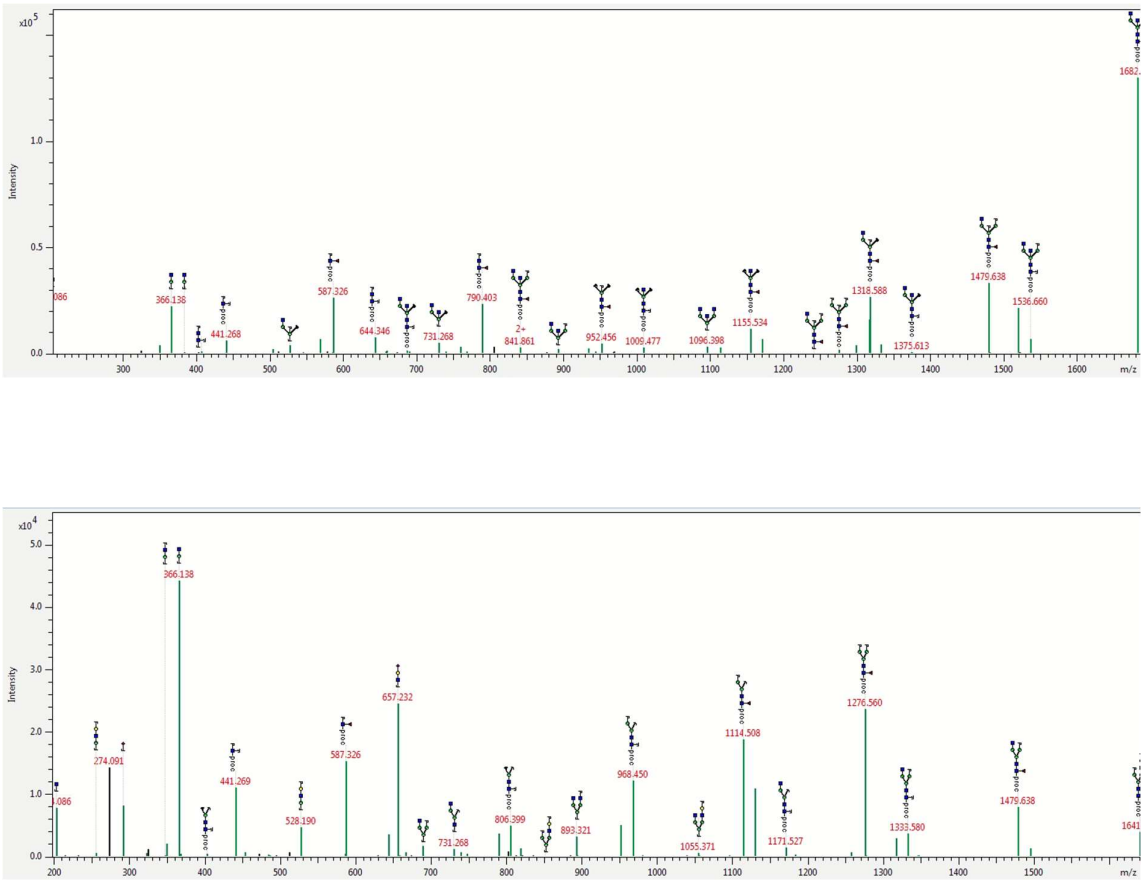

Supplementary Figure 5. Annotated MS/MS spectra of IgG *N*-glycans. **(A)** H3N5F1 **(B)** H4N4F1.