Comprehensive N-glycan Profiling, Characterization and Detailed Structure Analysis

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1 ABSTRACT

We describe a strategy for uniting quantitative profiling with comprehensive N-glycan characterization where detailed structural understanding of biopharmaceutical products is a fundamental requirement. Fluorescence profiling by high performance liquid chromatography is carried out in combination with accurate mass analysis and online MS/MS integrated with spectral-library database matching. These protocols enable the rapid and confident assignment of known structures and aid de novo interpretation of unknowns to provide an exacting characterization of N-glycans encountered in glycol-engineered products, non-traditional protein expression systems and/or glycosidase treated samples. Standard exoglycosidase treatments may be augmented with permethylated MSn allowing for resolution of linkage and branching complexities in greater detail, and providing for orthogonal evaluation of enzymatic activities. Terminal glycan epitopes transparent to accurate mass analysis are approached using positive analytical data. The evaluation of potentially bioactive motifs by mass spectrometry augments or supplants the need for biological inference and structural designations using bracketed cartoon compositions. Key aspects of optimal sample preparation, including fluorescent labeling and sample purification prior to LC-MS to remove background components, HPLC column selection, and preferred mobile phases for best MS/MS spectral quality are discussed based on the needed sensitivity and minimization of signal-diluting adducts. An important consideration is the reproducibility of high vs. low energy collisions on Q-TOF vs. Ion Trap (IT) instruments. The use of MS/MS and IT-MSn spectra for spectrum-matching is treated systematically with novel consideration given to ion m/z vs. intensity plots. The overall strategy presented is designed to approach fundamental structural challenges involved in the characterization of glycol-engineered products and other potentially complex N-glycan pools.

2 CONCLUSIONS

An overall strategy for comprehensive N-glycan profiling, including characterization and MS-based detailed structural analysis, is presented. Critical elements include sample preparation, HPLC column and mobile phase selection, and mass spectrometer settings. Florescence profiling coupled with online accurate MS, and MS/MS spectral matching, enables rapid and confident assignment of known structures. Permethylated MSn analysis provides information on glycan linkages and branching to facilitate de novo interpretation of any unknowns. The overall strategy presented is suitable for the characterization of N-glycans from biopharmaceutical products including diverse, and highly complex N-glycan pools.

3 REFERENCES

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2 Ashline DJ, Hanneman AJ, Zhang H, Reinhold VN; J Am Soc Mass Spectrom. 2014, 25(3):444-453
3 Wuhrer M, Koeleman CA, Hokke CH, Deelder AM; Rapid Commun Mass Spectrom. 2006, 20(11): 1747-54

4 RESULTS AND DISCUSSION

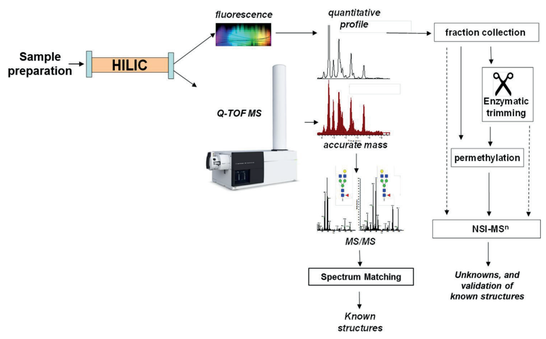


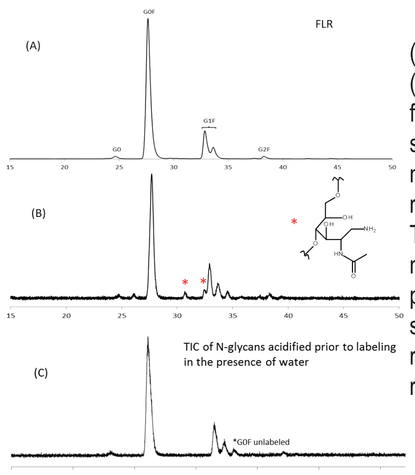
Figure 1. Workflow for combining HPLC-fluorescence quantification with accurate mass, MS/MS and MSn analysis.

Sample Preparation Considerations

Factors important for minimizing artefactual unknowns in profiles:

- Choice of enzyme for N-glycan release: purified PNGase F vs. recombinant N-glycanase (Endo F-Free)
- Labeling in the presence of water is quick and convenient but may result in some unlabeled species (Figure 2B).
  - Sample acidification prior to fluorescent labeling removes reducing terminal amine to lower the level of unlabeled species (Figure 2C).
- Sample clean-up prior to fluorescent labeling and use of non-aqueous conditions can further minimize unlabeled species.

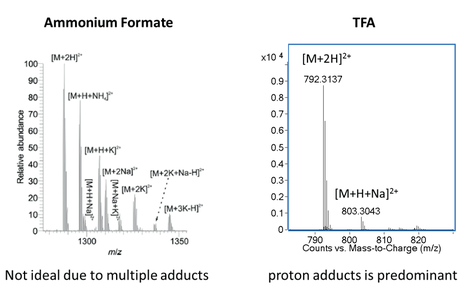
Figure 2. Comparison of total ion chromatograms (TIC) of N-glycans from direct fluorescence labeling of released N-glycans vs. acidification before fluorescence labeling.



(A) Fluorescence chromatogram. (B) TIC obtained from direct fluorescence labeling. The species at 30.7 and 32.4 minutes match unlabeled glycans with the reducing end in amine form. These unlabeled species were minimized in a second sample preparation by acidifying the sample briefly after N-glycan release prior to 2-AB labeling, the resulting TIC is shown in (C).

HILIC HPLC Considerations

- Choice of column1: Waters ACQUITY UPLC BEH Glycan Column, 130Å, 1.7 µm
- Choice of mobile phase additive1: TFA vs. ammonium formate (Figure 3)



Not ideal due to multiple adducts

proton adducts is predominant

Figure 3. Comparison of MS spectra of N-glycans obtained with ammonium formate vs.TFA as mobile phase additive. Multiple adducts of a single N-glycan were observed with ammonium formate as the additive, while a predominant proton adduct was observed with TFA as the additive, resulting in increased sensitivity and easy selection of precursor ions for MS/MS analysis.

Mass Spectrometry Analysis Considerations

MS source optimization to reduce in-source fragmentation
QTOF ion optics tuning to reduce MS1 fragmentation of fragile ions

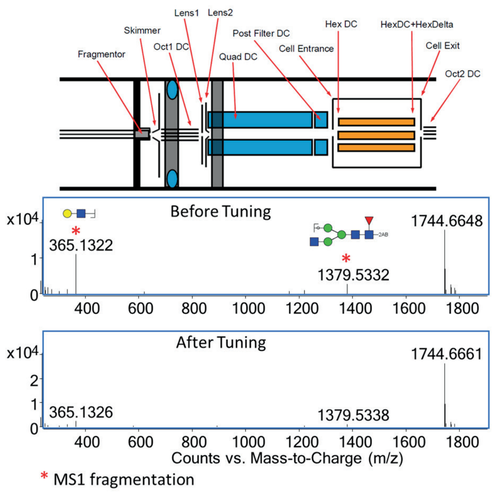


Figure 4. Tuning ion optics elements for reduced MS1 fragmentation

The voltage difference between Oct1DC and HexDC, as well as all of the elements in between, were optimized to reduce ion acceleration before the ions reach the collision cell. As a result, MS1 fragmentation was reduced (as shown for G1F) indicated by the decrease of 365 and 1379 Da peaks.

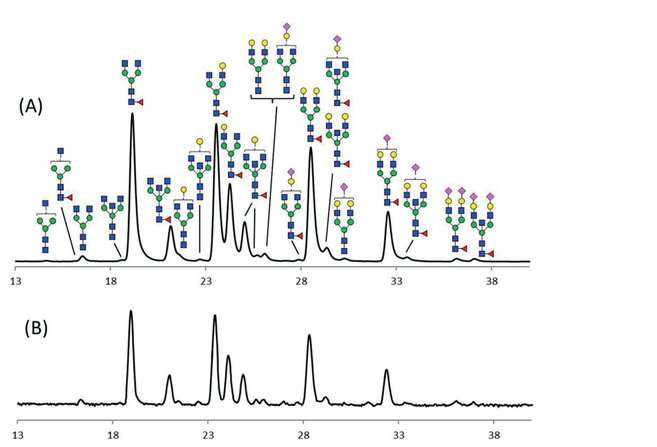


Figure 5. HILIC HPLC profiles of 2-AB labeled N-linked glycans from human IgG: (A) Fluorescence chromatogram (B) Total ion current chromatogram. N-Glycans are assigned based on accurate masses. 1:1 correspondence between FLD and TIC is key to linking quantification with characterization.

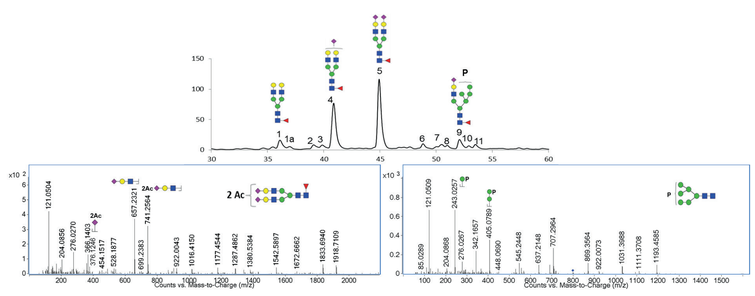


Figure 6. HILIC HPLC profile of 2-AB labeled N-glycans released from a viral glycoprotein. (A) Fluorescence chromatogram, (B) MS/MS spectrum of peak 1a, (C) MS/MS spectrum of peak 3. Accurate mass coupled with online MS/MS analysis provide confident assignment of N-glycan compositions.

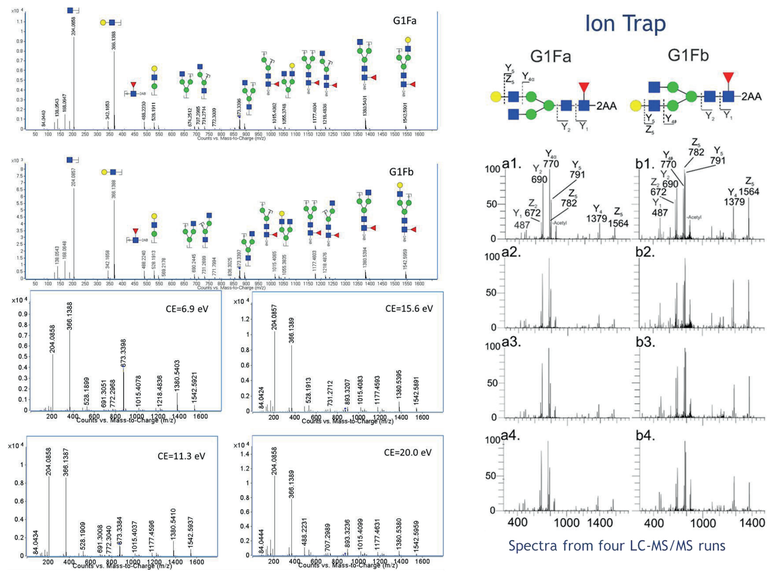


Figure 7. MS/MS spectra of N-glycan isomers, G1Fa and G1Fb, obtained on a Q-TOF (left panel) and an ion trap (right panel) mass spectrometer.
Under the same collision energy, G1Fa and G1Fb fragmentation intensity patterns are similar on a Q-TOF, but differ significantly on an ion trap.2
Under different collision energies, the fragmentation intensity patterns differ on a Q-TOF, but not on an ion trap.2
MS/MS spectra obtained on different trap mass analyzers are nearly identical and are ideal for building spectrum library for matching.2

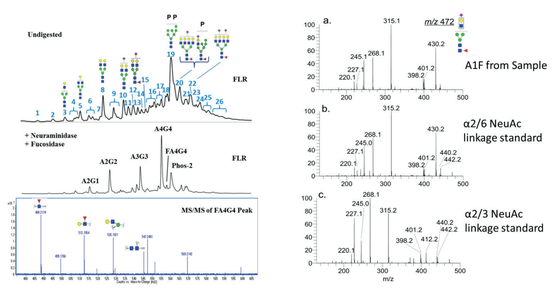


Figure 8. Comparison of MS/MS and MSn for structural analysis of N-glycans.
Left panel: To assist in structure analysis of a complex pool of N-glycans released from a glycoprotein, the 2-AB labeled N-glycans were treated with exoglycosidase α2-3,6,8 neuraminidase and α1-2,4,6 fucosidase. After treatment, a fucosylated glycan was still observed. MS/MS analysis of the fucosylated species gives ambiguous data about the location of fucose due to possibility of fucose rearrangement of protonated ions3.
Right panel: Permethylated Na+ ions are not prone to rearrangement, and are best for detailed structure analysis. Permethylated MSn analysis and spectrum matching vs. standards to provide conclusive assignment of α(2/6) NeuAc linkage in a biopharmaceutical sample.

5 ACKNOWLEDGMENTS

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