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State-of-the-art Glycopeptide Identification Using an Orbitrap Astral Mass Spectrometer

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Abstract

Purpose: Demonstrate the performance of the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer for glycopeptide analysis.

Methods: 1) Chromatographic separation using a Thermo Scientific™ Vanquish™ Neo UHPLC system, operated in a direct injection configuration, 2) Data-dependent acquisition (DDA) analysis on the Orbitrap Astral MS using stepped collision energies.

Results: more than 3000 glycopeptides were identified with higher scores using stepped collision energies.

Introduction

Post-translational modifications (PTMs), such as phosphorylation or glycosylation of proteins, are essential in defining and regulating protein functions. Hence, their identification and site localization is crucial in understanding biological functions and disease mechanisms. While improvements in instrumentation, computational tools, and sample preparation methodologies have made fields such as glycoproteomics realizable for many labs, consistently detecting glycopeptides in clinical samples such as human plasma remains a challenging endeavor due to the complexity and the huge dynamic range posed by these types of samples. From a sample preparation perspective, glycopeptide enrichments have been shown to improve the identification of glycopeptides. From an instrumentation perspective, besides the conventional collision-induced dissociation (CID) or higher collision energy (HCD), glycopeptide identification and site localization are improved by the use of electron-transfer dissociation (ETD) or a combination of the ETD and higher collision energy (EThCD) for fragmentation. Recent reports [1] have suggested that using two different serialized collision energies (CEs) in the same analyzed spectrum improves glycopeptide detection. Therefore, in this work, we sought to demonstrate that the Orbitrap Astral mass spectrometer [2] is a suitable platform for glycoproteomics analysis.

Materials and methods

Sample preparation

For these evaluations we used Human Plasma K2 EDTA Gender Pooled Normal (BioIVT)**.** 400 uL of plasma was depleted using HSA/Immunoglobulin Depletion Resin (Thermo Scientific). The depleted sample was processed using a Thermo Scientific™ EasyPep™ Maxi MS Sample Prep Kit. Glycopeptide enrichment was done using Oasis MAX SPE (Waters) columns in HILIC mode. Peptide yields were assessed using a Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay.

LC-MS

The peptides were separated on an Aurora Ultimate TS 25×75 C18 UHPLC column using a Vanquish Neo UHPLC system coupled online to an Orbitrap Astral mass spectrometer. The LC mobile phases used were water with 0.1% formic acid (solvent A) and 80% acetonitrile in water with 0.1% formic acid (solvent B) (both UPLC Grade). The Orbitrap Astral MS was operated in a positive ion mode and configured to collect DDA MS/MS using one, two, and three CEs per scan. For single CE experiments, the fragmentation was performed in the ion processor, and for multiple collision energies, “stepped collision energy”, the fragmentation was done in the IRM (highlighted in red, shown Figure 1). A summary of the MS, LC and MS parameters are shown in Tables 1 and 2. All ions from each stepped collision energy injection are accumulated in the IRM and then passed to the Astral analyzer simultaneously, for analysis and detection. The schematic in Figure 2 shows how the stepped collision energy was implemented.

Data Analysis

Raw data files were searched using PMI-Byonic (v.5.3.44, Protein Metrics) against the UniProt Human FASTA (UP000005640_9606, downloaded 17 May 2024) and a built-in library of 132 human *N*-glycans, 57 human plasma N-glycan and 9 common O-glycan all set as ‘rare1’. Carbamidomethylation (+57.0214) was set as a fixed modification and oxidation (+15.9949) as ‘common1’. Tryptic digest was selected (RK, ‘C-terminal cutter’, fully-specific, max. 2 missed cleavage). In addition, we used the following parameters: precursor tolerance: 10 ppm, fragment tolerance (HCD): 20 ppm, and protein false-discovery rate (FDR): 1%. Identified glycopeptide information (‘Spectra’ tab of each Byonic output file) was imported into R and PSMs were further filtered with the following thresholds: presence of glycan in ‘Glycans NHFAGNa’ column, Byonic score > 200.

Master Scan	
Detector	Orbitrap
Orbitrap Resolution	180000
scan Range	380-2000
Normalized AGC Target (%)	500
Injection Time (ms)	50
RF Lens(%)	40
MIPS Filter	
MIPS Mode	Peptide
Charge State	
Charge State	2-5
Dynamic Exclusion Filter	
Exclude after n times	1
Exclusion duration	20
mass tolerance high\low	10
ddMSnScan	
Detector Type	Astral
isolation width	2
HCD collision Energy (%)	See Figure 4
Scan Range (m/z)	120-2000
Injectiom Time (ms)	10
AGC Target (%)	100
Cycle Time (sec)	0.5

Table 1. MS parameters used for the evaluation.

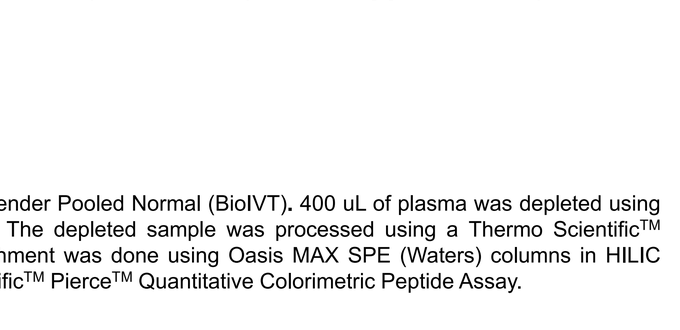

Figure 1. Instrument layout of the Orbitrap Astral mass spectrometer. The parallel operation scheme of Orbitrap and Astral analyzers is shown on the top left.

Duration	Flow rate [ul\min]	% B
0.00	0.30	4
0.4	0.30	6
0.5	0.30	8
30.9	0.30	28
6	0.30	45
1.2	0.30	95
3	0.30	95

Table 2. LC gradients used for the high-throughput evaluation.

Results

One reason that the Orbitrap Astral MS is able to rapidly analyze samples is by taking advantage of scan parallelization, thus attaining a scanning frequency of up to 200 Hz. However, it has been demonstrated that the use of multiple collision energies is essential to enhance the structural characterization of posttranslational modifications such as phosphorylation and glycosylation. The schematic implementation of stepped collision energy on the Orbitrap Astral MS is shown in Figure 2. In this example, three injections are performed in series, with the ions accumulated in the IRM (see red shaded area of Figure 1.). Following the accumulation, all fragmented ions are released into the Astral analyzer, and new injections can begin in parallel to the onward flight of the ions into the analyzer. It can therefore be seen that the number of serialized injections and the individual injection time are the main determining factors in the repetition rate of this type of experiment.

The implementation of stepped collision energy on the Orbitrap Astral MS was quickly evaluated using Flexmix ions and compared to that of the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer using the same acquisition parameters. Figure 3. shows the repetition rate achieved for stepped collision energy experiments with two and three NCEs (normalized collision energies) implemented in an Orbitrap Exploris 480 MS and an Orbitrap Astral MS. For a typical 10 ms DDA scan, it is shown that the Orbitrap Astral MS is almost 2x faster than the Orbitrap Exploris 480 MS. For shorter injection times, the Orbitrap Astral MS achieved up to 3x the repetition rate of the Orbitrap Exploris 480 MS, demonstrating the potential for world-leading analytical speeds for this type of measurement.

Next, we separated 250 ng glycopeptides enriched from human plasma, and the eluted peptides were then measured on the Orbitrap Astral MS. For the fragmentations, different collision energies were used to evaluate the effect of collision energies compared to the newly implemented stepped collision energy. The results for all the collision energies used in the evaluation are shown in Figure 5. The use of stepped collision energy increases the number of identified glycol peptides by approximately 33%. In addition, the peptide scores are much higher compared to those identified with single NCE (see heat map in Figure 6), hence resulting in an overall enhancement in glycan backbone determination.

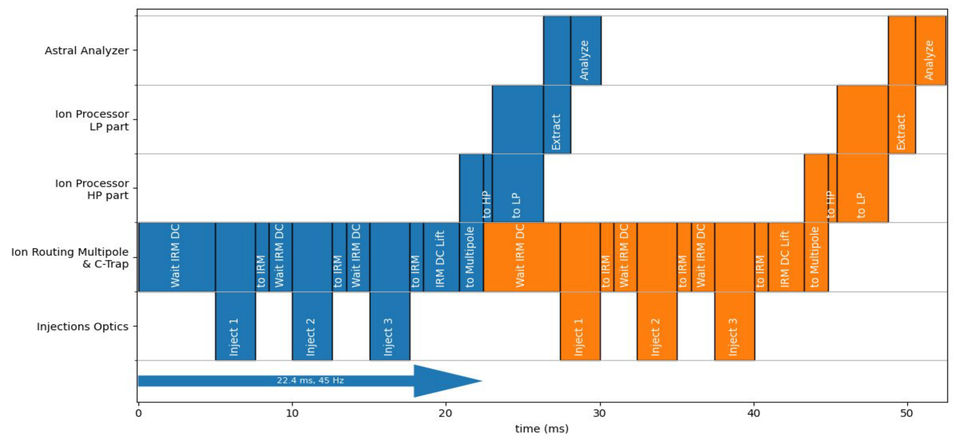

Figure 2. Stepped collision energy in the Orbitrap Astral mass spectrometer. Several serial injections with different collision energies can be performed, three in this example. After the ions of all injections are fragmented and accumulated, they are transferred to the Astral analyzer and the next scan can be started in parallel.

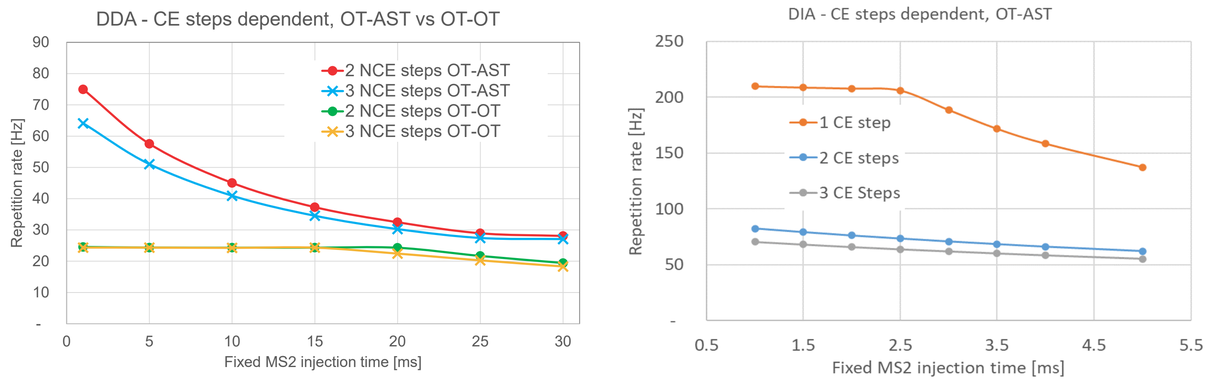

Figure 3. Comparison of repetition rate for stepped collision energy DDA (upper) and DIA (lower) MS2 experiments conducted for one, two and three CE steps. For the DDA case both the Orbitrap Exploris 480 MS (OT-OT) and Orbitrap Astral MS (OT-AST) instruments are compared. The repetition rate for a single CE in DIA is up to 200 Hz depending on the injection time. DIA with stepped collision energy is only available on OT-AST (lower).

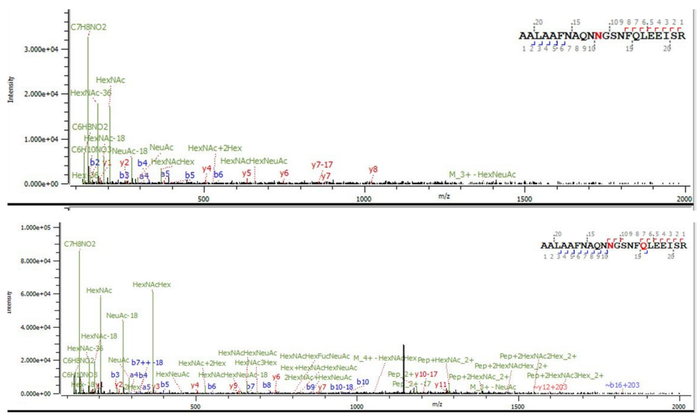

Figure 4. Comparison of single spectra for a single NCE (upper) and double NCE with two collision energies (lower).

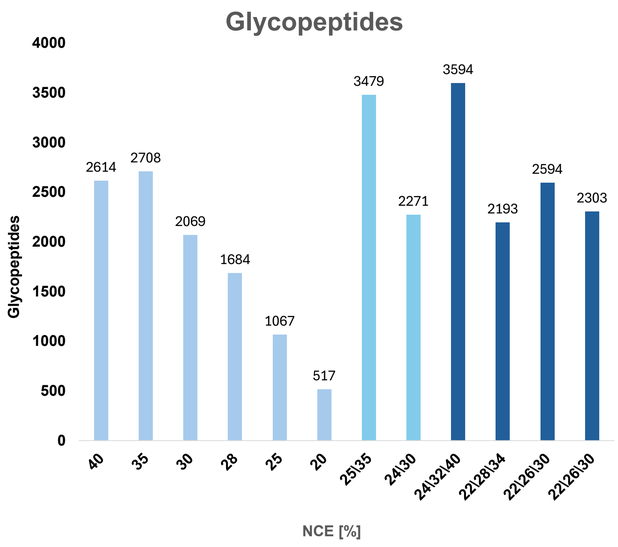

Figure 5. Glycopeptides identified with the different stepped CE settings. The PSM list from the Byonic output results were filtered with the following thresholds: presence of glycan in ‘Glycans NHFAGNa’ column, Byonic score > 200, and all duplicates from the peptide sequence tab was filtered out.

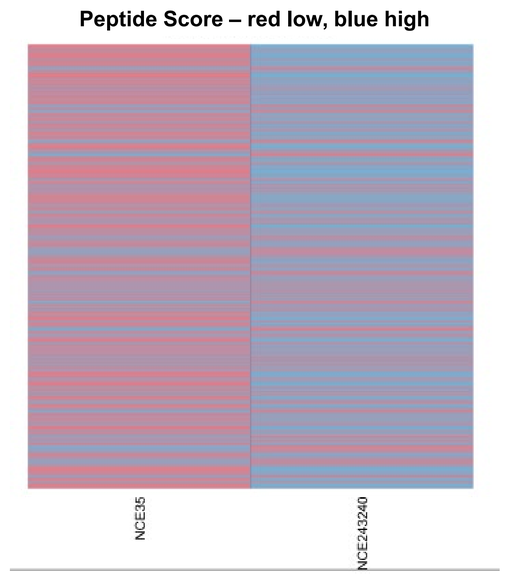

Figure 6. Heat map showing Byonic peptide scores above 200 for all uniquely identified peptides. Red indicates lower and blue higher peptide scores. The left side shows the scores for a single NCE of 35 and the right shows the scores for two stepped collision energies with NCEs of 24, 32 and 40.

Conclusions

- A glycoproteomics study using HCD stepped collision energy was successfully implemented on an Orbitrap Astral MS in which we saw a speed increase of up to 3x with respect to an Orbitrap Exploris 480 MS.
- Use of two or even three CEs as opposed to a single CE showed marker improvements in the confidence of glycopeptide IDs when analyzing enriched human plasma. Up to 33% increase in uniquely identified peptides was obtained by using stepped collision energy.
- This unoptimized study demonstrates the potential of the Orbitrap Astral MS for glycopeptide analysis by greatly increasing speed and sensitivity.

Acknowledgements

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References

1. M. Baerenfaenger et al., *Maximizing Glycoproteomics Results through an Integrated Parallel Accumulation Serial Fragmentation Workflow,* **Analytical Chemistry 96 (22),** 8956-8964 (2024).
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