State-of-the-art rapid stepped collision energy in an Orbitrap Astral Zoom Mass Spectrometer

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Abstract

Purpose:
Performance evaluation of rapid stepped collision energy in a Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer.

Methods:

- Thermo Scientific™ Pierce™ FlexMix™ Calibration Solution was used to demonstrate the scan rate enhancement of the Orbitrap Astral Zoom mass spectrometer.
- Chromatographic separation using a Thermo Scientific™ Vanquish™ Neo UHPLC system operated in a direct injection configuration.
- Data-dependent acquisition (DDA) analysis on the Orbitrap Astral Zoom MS using multiple stepped collision energies.

Results:

- The scan rate for stepped collision energy with 3 ms injection time for two and three Normalized Collision Energies (NCEs) in an Orbitrap Astral Zoom MS was 155 and 135 Hz respectively; an increase of a factor of two with respect to a Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer.
- 13.3 % and 11.5 % more glycopeptides identified for three and two NCEs respectively, compared to the Orbitrap Astral MS.
- The same number of proteins identified and quantified for a TMT TKO experiments when comparing single NCE to stepped NCE but with higher average reporter ion SNR.

Introduction

The use of electron-transfer dissociation (ETD) or a combination of ETD and HCD (i.e., EThCD) for fragmentation is commonly being used to generate rich fragment spectra which aids site localization and structural elucidation studies. Recent reports [1] have suggested that using two different serialized normalized collision energies in the same analyzed spectrum improves glycopeptide detection. Nonetheless, today's implementation of HCD and stepped HCD is time consuming, thus affecting the overall scan speed during real time data acquisition. Previously, we implemented stepped collision energy, which can be operated at up to 70 Hz in both DIA and DDA applications on the Orbitrap Astra MS [1]. This improvement in stepped HCD speed led to an improvement in identification of glycopeptide by approximately 33 % compared to the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer. The new Orbitrap Astral Zoom MS has facilitated yet another increase in the speed of stepped collision energy compared to the Orbitrap Astral MS, allowing even faster and more sensitive investigations.

Materials and methods

Sample preparation

FlexMix calibration solution was directly infused into the Orbitrap Astral Zoom MS. Lyophilized human neat plasma digest was resuspended in 0.1% FA to a total concentration of 500 ng/µl. Lyophilized Thermo Scientific™ Pierce™ TMT11plex Yeast Digest Standard was resuspended in 0.1 % FA to obtain a final concentration of 500 ng/ul.

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 either an Orbitrap Astral MS or an Orbitrap Astral Zoom 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 Zoom MS was operated in positive ion mode and configured to collect DDA MS/MS using one, two, and three collision energies per scan. For single collision energy experiments, the fragmentation is performed in the ion processor, and for multiple collision energies, stepped collision energy, the fragmentation is done in the IRM (highlighted in green, shown Figure 1). A summary of the LC and MS parameters is shown in Tables 1 and 2. All ions from each stepped collision energy injection are accumulated in the IRM and then passed to the Thermo Scientific™ Astral™ analyzer simultaneously, for analysis and detection. The schematic in Figure 2 shows how the stepped collision energy was implemented.

Figure 1. Orbitrap Astral Zoom mass spectrometer instrument layout.

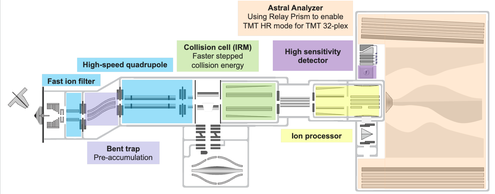


Table 1. LC gradients used for the high-throughput evaluation.

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. MS parameters used for the evaluation.

Master Scan	
Detector	Orbitrap
Orbitrap Resolution	180000
Scan Range (m/z)	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
Data-Dependent MS² scan properties	
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

Data Analysis

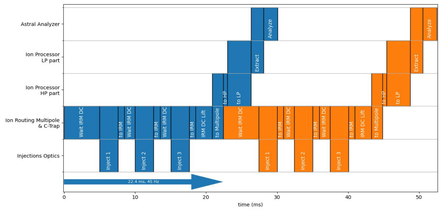
Raw data files were searched using Byonic software (v.5.3.44, Protein Metrics) against the UniProt Human FASTA (UP000005640_9606, downloaded 17 May 2024) and a built-in library of 132 human *N*-glycans, 57 human plasma N-glycan and 9 common O-glycan all set as 'rare1'. Carbamidomethylation (+57.0214) 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 a precursor tolerance of 10 ppm, a fragment tolerance (HCD) of 20 ppm and a protein false-discovery rate (FDR) of 1%. Identified glycopeptide information ('Spectra' tab of each Byonic output file) was further filtered with the following thresholds: presence of glycan in 'Glycans NHFAGNa' column, Byonic score > 200 and all reversed sequences and duplicate sequences were removed.

Results

Initial scan rate results with FlexMix

The efficiency of stepped NCE in an Orbitrap Exploris MS and Orbitrap Astral MS is limited by the voltage settling time realizable in the IRM. Settling times of several ms reduced the effectiveness of each subsequent applied collision energy due to intervening thermalization of ions. Using improved electronics in the Orbitrap Astral Zoom MS, it has become possible to significantly reduce the voltage settling times in the IRM. After utilizing robust optimization methods, voltage settling times in the IRM were reduced from typical values of 6.0 ms to 0.6 ms, hence by a factor of 10. This enabled more effective collision energy application for a given ion packet.

Figure 2. Stepped NCE example instrument sequence in the Orbitrap Astral MS and Orbitrap Astral Zoom MS. 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.



The scan rate in an Orbitrap Astral Zoom MS was evaluated using FlexMix solution with a m/z isolation of 524 ± 2. Fixed injection times of between one and 10 ms were used and the scan rate for NCEs with one, two, three, four and five steps were recorded. In the scheme shown in Figure 3, the scan rate at a fixed injection time of 1.0 ms was increased from 75 and 65 Hz (in an Orbitrap Astral MS) for two and three NCEs in previous studies [1] to approximately 230 and 180 Hz respectively for the Orbitrap Astral Zoom MS. For a fixed injection time of 3.0 ms, the scan rate was 155 and 130 Hz for the two and three NCEs respectively. Figure 4 shows that the scan rate is approximately doubled between an Orbitrap Astral MS and an Orbitrap Astral Zoom MS, for two and three NCEs.

Figure 3. The real-time, method independent scan rate in an Orbitrap Astral Zoom MS for 3 ms (left) and 1 ms (right) fixed injection times with the indicated number of stepped collision energies in red as a function of time. The red horizontal lines indicate the average scan rate for the indicated number of stepped collision energies.

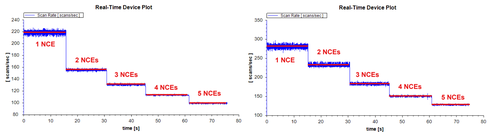
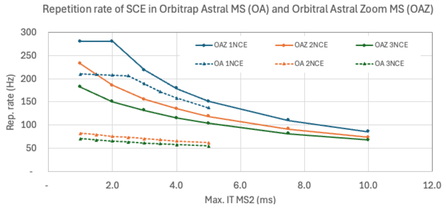


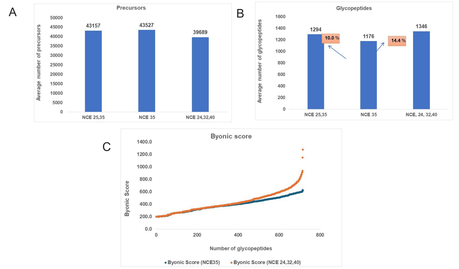
Figure 4. Overview plot showing method-independent scan rate as a function of the maximum injection time for one, two and three NCEs for an Orbitrap Astral MS (OA) and an Orbitrap Astral Zoom MS (OAZ).



Glycopeptide analysis verification

We analyzed 500 ng of neat human plasma digest using 1, 2 or 3 NCEs. As displayed in Figure 5A, while the number of precursors for 1 and 2 NCEs are very similar, this dropped for 3 NCEs by approximately 9 %. Nevertheless, 14.4 % more glycopeptides were identified with 3 NCEs compared to 1 or 2 (Figure 5B). In addition, we compared the Byonic score for 1 and 3 NCEs. Figure 5C shows that higher Byonic scores are achieved with 3 NCEs, demonstrating the importance of fast stepped collision energies for glycopeptides identification and structural determination.

Figure 5. Results from neat plasma using the Orbitrap Astral Zoom MS A) Identified precursors for different collision energies. B) Identified glycopeptides using different stepped CE settings. C) Byonic score comparison for 1 NCE (35) and 3 NCEs (24, 32, 40).



Furthermore, we compared these results to that of the Orbitrap Astral MS. In the Orbitrap Astral Zoom MS we saw 34% and 40% more identified precursors than in the Orbitrap Astral MS for two and three NCEs respectively. This translates to over 13% more glycosylated peptides in the Orbitrap Astral Zoom MS compared to the Orbitrap Astral MS.

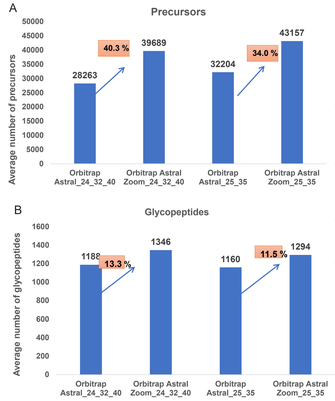
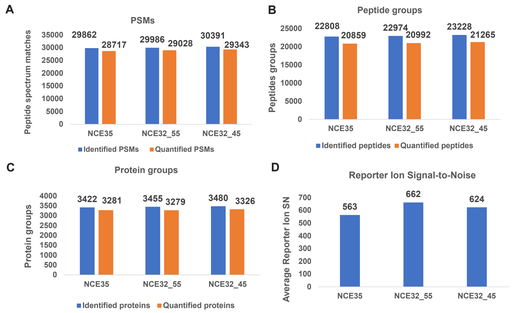


Figure 6. Results obtained on the Orbitrap Astral MS and Orbitrap Astral Zoom MS using neat human plasma digest. A) comparison of identified precursors for the same NCEs on the Orbitrap Astral MS and the Orbitrap Astral Zoom MS. B) comparison of the number of identified glycopeptides using the same conditions on the Orbitrap Astral MS and the Orbitrap Astral Zoom MS.

Evaluation of stepped collision energy for relative quantitation using TMT

In the second verification experiment, we aimed to demonstrate that rapid stepped collision energy can be used for relative quantitation experiments using TMT without sacrificing identification and quantitation. Using 500ng Thermo Scientific™ Pierce™ TMT11plex Yeast Digest Standard, we compared the percentage of PSMs, peptides and proteins used for quantitation from single and stepped collision energies. In addition, we also looked at the reporter ions signal to noise ratio (SNR). Figure 7 shows that neither identification, number of PSMs, peptides or protein groups used for quantitation drops with the use of stepped collision energy. It also shows that higher average SNR are attained with stepped collision energies, thus enhancing quantitation, as this depends on strong signals of the reporter ions.

Figure 7. TMT11plex Yeast Digest Standards analyzed with and without stepped collision energy. A) Identified and quantified spectra for the different methods. B) Identified peptides used for quantification. C) Identified and quantified protein groups. D) Average reporter ion Signal-to-Noise ratio.



Conclusions

- The scan rate for stepped collision energy with 3 ms injection time for two and three NCEs in an Orbitrap Astral Zoom MS was 155 and 135 Hz respectively.
- Orbitrap Astral Zoom MS with stepped collision energies using FlexMix, shows up to 3x higher MS2 scan speed compared to an Orbitrap Astral MS.
- The average number of identified glycopeptides increased by 10 and 14 % when using 2 or 3 stepped collision energies, respectively.
- Up to 13 % more glycopeptides are identified on the Orbitrap Astral Zoom compared to the Orbitrap Astral MS
- Improved average reporter ion SNR when using stepped collision energies for relative quantitation using TMT without sacrificing depth proteome coverage.

References

[1] J. M. Garland et al., *State-of-the-art Glycopeptide Identification Using an Orbitrap-Astral Mass Spectrometer*, HUPO2024, P-I-0115, 2024

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