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Targeted MultiNotch MS³ Approach for Relative Quantification of N-Glycans Using Multiplexed Carbonyl-Reactive Isobaric Tags

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Abstract

The recently developed and commercially available carbonyl-reactive tandem mass tags (aminoxyTMT) enable multiplexed quantification of glycans through comparison of reporter ion intensities. However, challenges still exist for collision activated dissociation (CAD) MS/MS based quantification of aminoxyTMT due to the relatively low reporter ion yield especially for glycans with labile structures. To circumvent this limitation, we utilized the unique structural features of N-glycan molecules, the common core sugar sequence (HexNAc)₂(Man)₃, and common *m/z* of Y_n ions generated from different types of precursors by MS/MS and designed a Y₁ ion triggered, targeted MultiNotch MS³ relative quantification approach based on aminoxyTMT labeling. This approach was implemented on a nanoHILIC-Tribrid quadrupole-ion trap-Orbitrap platform, which enables prescreening of aminoxyTMT labeled N-glycan precursor ions by Y₁ ion fragment ion mass in a higher-energy collisional dissociation (HCD) MS/MS scan and coisolation and cofragmentation of multiple Y_n fragment ions that carry the isobaric tags from the inclusion list in the MS/MS/MS scan. Through systematical optimization and evaluation using N-glycans released from several glycoprotein standards and human serum proteins, we demonstrated that the Y₁ ion triggered, targeted MultiNotch MS³ approach offers improved accuracy, precision, and sensitivity for relative quantification compared to traditional data-dependent MS² and Y₁ ion MS³ quantification methods.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b03289.

Parameter optimization; total ion counts; selected extracted ion chromatogram; box-and-whisker plots; Venn diagram; quantification results; data summary of H₅N₄FS (PDF)

Full list of N-glycans released from BTG and HSP (XLSX)

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Notes

The authors declare no competing financial interest.

Graphical abstract

Glycosylation of proteins is one of the most important posttranslational modifications (PTMs). It is involved in a variety of biological and physiological states. Alteration of glycosylation is associated with abnormal biological states, such as cancer progression and invasion.^{1,2} In glycobiology studies, it is observed that the significant changes of glycans could be an indicator for the disease state.³ Therefore, it is crucial to develop effective methods for relative glycan quantification.

Significant efforts have been made to develop analytical methods for relative quantification of glycans, especially for N-glycans, which are attached to asparagine residues in proteins with sequons N-X-S or N-X-T (X can be any amino acid except for Pro).¹ Among different methods for glycan quantification, mass spectrometry (MS) based approaches become popular because of the rapid advancement of MS technologies. In general, glycan abundance in different biological samples can be quantified by a label-free and chemical labeling approach by comparing MS signal intensities. The label-free approach compares relative peak intensity or spectral abundance between underivatized N-glycans. Alternatively, N-glycans can be derivatized with isotopic or isobaric labels and the intensity ratios between labels represent the relative abundance of individual N-glycans.³ Several tags have been developed by various vendors and research groups, such as Isotopic Glycan Hydrazide Tags (INLIGHT, 2-plex isotopic tag),⁴⁻⁶ stable-isotope labeled hydrazide reagents (HDEAT, 2-plex),^{7,8} isobaric aldehyde reactive tags (iARTs, 2-plex isobaric tag),⁹ Quaternary Amine Containing Isobaric Tag for Glycan (QUANTITY, 4-plex isobaric tag),¹⁰ and carbonyl-reactive tandem mass tags (aminoxyTMT, 6-plex isobaric tag).¹¹

The development of aminoxyTMT enables high-throughput quantification of carbonyl containing compounds, including N-glycans. It is a set of isobaric tags with the same chemical structure but different isotopic configurations. The amino-xyTMT reagent reacts with the reducing end of N-glycan to produce a stable oxime product. Each plex generates a unique reporter ion from m/z 126 to 131 in the low mass region upon tandem MS fragmentation.¹¹ However, the performance of collision activated dissociation (CAD) MS² based quantification is not ideal due to the low reporter ion yield from some categories of glycans, such as the complex type of N-glycans with core-fucosylation, and acidic N-glycans with labile residues.¹¹⁻¹³ As the Y_n ions are always observed to be abundant in the

CAD MS/MS of most types of N-glycans and they carry the intact aminoxyTMT tags, further isolation and fragmentation of Y_n ions can be employed for reporter ion intensity based quantification.

To improve the duty cycle of the MS^3 based quantification method, the MultiNotch MS^3 method was introduced recently for quantitative proteomics and implemented on a commercially available high resolution/accurate mass (HR/AM) instrument: Orbitrap Fusion Lumos.¹⁴ In this approach, isolation waveforms with multiple frequency notches were used to coisolate and cofragment multiple MS^2 fragment ions. It was demonstrated that the interference from coeluting peptides was minimized and the quantification accuracy was enhanced compared to MS^2 based quantification.¹⁴ Another approach developed by Niu et al.,¹⁵ utilized the intensity of Y_1 ions for detection of reporter ion interference in MS^2 scans of TMT labeled tryptic peptides. Enlightened by the previous strategies on improving accuracy of TMT based quantitative proteomics, we developed a Y_1 product-ion triggered, targeted MultiNotch MS^3 approach for more accurate and precise relative glycan quantification based on the unique structural features of N-glycans. As all N-glycans share the same conserved core, the signature Y_1 ions ([HexNAc-aminoxyTMT + H]⁺ or [HexNAcFuc-aminoxyTMT + H]⁺) generated by CAD cleavage can be used as selection criteria for amino-xyTMT labeled precursor ions to trigger a MultiNotch MS^3 scan event. Moreover, a target inclusion list including all conserved Y ions could be used for MultiNotch MS^3 to improve the specificity. In this study, we systematically optimized and evaluated the nanoHILIC-MS parameters of the targeted MultiNotch MS^3 method and compared it with the standard MS^3 and MS^2 methods for quantifying aminoxyTMT labeled N-glycans from glycoprotein standards and a protein mixture.

EXPERIMENTAL SECTION

Materials and Chemicals

All reagents were used without additional purification. Methanol (MeOH), acetonitrile (ACN), water, acetic acid (AA), and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA). Triethylammonium bicarbonate buffer (TEAB, 1.0 M) and Tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO). PNGase F was purchased from Promega (Madison, WI). AminoxyTMT reagents, bovine thyroglobulin (BTG), bovine lactoferrin (BLF), RNaseB, and human serum protein mixture (HSP) were provided by Thermo Fisher Scientific (Rockford, IL). Oasis HLB 3 cm³ (60 mg) extraction cartridges were purchased from Waters Corporation (Milford, MA). Microcon-30 kDa centrifugal filters (30K MWCO) were purchased from Merck Millipore Ltd. (Darmstadt, Germany). PolyGLYCOPLEX A beads (3 μ m) were purchased from PolyLC Inc (Columbia, MD). Fused silica capillary tubing (inner diameter 75 μ m, outer diameter 375 μ m) was purchased from Polymicro Technologies (Phoenix, AZ).

Filter-Aided-N-Glycan Separation (FANGS)

N-Glycans were released from glycoproteins following the FANGS protocol⁴ with slight modifications (Figure 1). Briefly, glycoprotein (2 μ g/ μ L dissolved in 50 mM TEAB buffer) was mixed with 4 μ L of 0.5 M TCEP, heat-denatured, and added to a 30K MWCO filter.

Heat-denaturing was performed by alternating sample tubes between 100 °C and room temperature water baths for 4 times, 15 s each. The mixture was then buffer exchanged with 200 μL of 50 mM TEAB buffer for three times (centrifuged at 14 000g for 20 min at 20 °C each time) and incubated with PNGaseF (1 μL of PNGaseF/10 μg of protein) for 18 h at 37 °C. The released glycosyamines were separated from the deglycosylated protein by centrifugation at 14 000g for 20 min at 20 °C. The pH of the flow through solution was adjusted to 3 by adding 1% acetic acid solution, and the mixture was vortexed at room temperature for 40 min. The resulting N-glycans were then evaporated to dryness under vacuum and stored at –20 °C (Figure 1).

AminoxyTMT Labeling and Cleaning Up

Amino-xyTMT labeling reaction was performed following the manufacturer's instruction (Thermo Scientific, Rockford, IL) with slight modification. Briefly, aminoxyTMT reagent was reconstituted in 200 μL of 95% MeOH with 0.1% acetic acid and mixed with acidified N-glycans released from 10 to 30 μg glycoproteins standards (dissolved in 10 to 30 μL of H_2O). The mixture was vortexed for 10 min at room temperature and evaporated to dryness under vacuum. The dried mixture was then reconstituted in 200 μL of 95% MeOH, vortexed for 10 min at room temperature, and evaporated to dryness under vacuum again. The excess labeling reagent was quenched by 100 μL of 10% acetone and vortexed for 10 min at room temperature. All labeled products were quantitatively transferred, combined into a single tube, and evaporated to dryness under vacuum. An Oasis HLB 3 cm^3 cartridge was used for sample cleanup. The cartridge was conditioned by sequentially washing with 3 mL of 95% ACN, 1 mL of 50% ACN, and 3 mL of 95% ACN. In the sample loading step, the labeled N-glycans were dissolved in 200 μL of 50% ACN and added to a conditioned cartridge which was preloaded with 3 mL of 95% ACN. The loaded cartridge was washed twice with 3 mL of 95% ACN and eluted with 2 mL of 50% ACN. The eluted sample was evaporated to dryness under vacuum and stored at –20 °C until LC-MS/MS analysis.

NanoHILIC Column Fabrication and LC Conditions

A NanoHILIC column was fabricated with 3 μm PolyGLYCO-PLEX A beads and 75 μm ID fused-silica capillary. A laser pulled fused silica capillary tubing end was etched with hydrogen fluoride for 3 min and washed with MeOH. A slurry of beads was prepared by adding 1:4 of isopropanol and methylene chloride and packed into the capillary with a pressure bomb until the desired length (30 to 40 cm) was reached. The packed column was then washed with MeOH and flushed with nitrogen.

A Dionex Ultimate 3000 nanoLC system was used for nanoHILIC separation. Mobile phase A was H_2O with 0.1% FA, and mobile phase B was ACN with 0.1% FA. A flow rate of 0.30 or 0.45 $\mu\text{L}/\text{min}$ was used. AminoxyTMT labeled N-glycans released from 1 to 2 μg of glycoproteins were loaded onto the column. Different linear and stepped gradients were tested, and the optimization results are specified in the Results and Discussion.

MS Methods and Parameters

An Orbitrap Fusion Lumos Tribrid quadrupole-ion trap-Orbitrap mass spectrometer with NanoSpray Flex ion source (Thermo Scientific, Bremen, Germany) was used for data

acquisition. The following parameters were used unless otherwise specified. The data was acquired by positive ion mode with spray voltage of 3 kV and ion transfer tube temperature of 300 °C. Full MS was acquired with an m/z range of 500–1700 and detected in Orbitrap at resolution of 120 K at m/z 200 with automatic gain control (AGC) target of 1×10^6 , maximal injection time of 100 ms, and 1 microscan. At MS² level, data-dependent acquisition (DDA) was performed by collision-induced dissociation (CID) on the top 6 most intense ions with a dynamic exclusion period of 10 s and detected in the Orbitrap with resolution of 30K at m/z 200 (MS² workflow) or in the ion trap with rapid scans (MS³ workflows). Two extra higher-energy collisional dissociation (HCD) scans were performed at different normalized collisional energies (NCE) at low (NCE 30) and high (NCE 70) settings for the MS² workflow. For the MS³ methods, the MS³ scans were triggered by appearance of either of the two Y₁ ions, [HexNAc-aminoxyTMT + H]⁺ (m/z 523.3289) and [HexNAcFuc-aminoxyTMT + H]⁺ (m/z 669.3869) above the intensity threshold of 5% of base peak intensity in HCD MS/MS scan. HCD was used for MS³ scans with NCE of 70 and detected by Orbitrap at a resolution of 120 K at m/z 200. AGC target was 5×10^4 with maximum injection time of 100 ms. In the standard MS³ method, only Y₁ ion (m/z of 523.3289) was isolated for the MS³ scan; in the MultiNotch MS³ method, the top six intense Y ions from an inclusion list including Y₁ ions (m/z 523.3289 and 669.3868), Y₂ ions (m/z 726.4084 and 872.4662), etc. were synchronously isolated in an ion trap and fragmented by HCD.

RESULTS AND DISCUSSION

A Y₁ ion triggered, targeted MultiNotch MS³ method was developed on a Tribrid quadrupole-ion trap-Orbitrap platform to improve the quantification precision and accuracy of aminoxyTMT labeled N-glycans. Quantification results from MultiNotch MS³, data-dependent MS², and standard MS³ quantification methods were compared.

Method Setup and Workflows

Three instrument methods were created in Xcalibur for comparison of MS² based and MS³ based quantification performance (Figure 2). All methods started with HR/AM full scans followed by MS² scans in a data-dependent manner. The MS² method combined HCD scans with low (NCE 30) and high (NCE 70) collisional energies (Figure 2A). Both MS³ methods started with a HR/AM full scan followed by the top 6 DDA scans by ion trap “rapid” scan mode. MS³ scans are triggered by detection of Y₁ ion at m/z 523.3289 or 669.3868 in the MS² scan. Upon detection of the Y₁ ions, the standard MS³ method would isolate the resulting fragment, Y₁ ion for HR/AM MS³ scans, while the MultiNotch MS³ method would coisolate several Y ions that matched the predefined inclusion list and cofragment them for HR/AM SPS MS³ scans (Figure 2B,C). Otherwise, the instrument continues with the top 6 DDA MS/MS scan.

MS Parameter Optimization

The MS parameters for MultiNotch MS³, standard MS³, and low and high MS² methods were optimized by directly infusing aminoxyTMT¹³⁰ labeled N-glycans released from BTG, which consists of both high-mannose and complex N-glycans. S-lens RF levels (Figure

S1A), NCEs at MS² (Figure S1B,C) and MS³ levels (Figure S1D), AGC target values at each MS level, MS² ion trap scan speeds, and isolation widths were optimized. Due to the labile glycosidic bond, the S-lens RF level was optimized to maximize ion transmission while minimizing fragmentation. The intensities of the signature Y₁ ion peak at m/z 523.3289 and the most abundant parent ion peak ([H₅N₂-aminoxyTMT + 2H + K]³⁺) at m/z 525.5482 were monitored over 3 min at each RF level. The average signal intensities at each RF level were plotted in Figure S1 with error bars representing the standard deviation. At S-lens RF level of 30%, the parent ion intensity reached a plateau while the fragment ion intensity was not at a significant level. On the basis of the results, this S-lens RF level was selected for all the following experiments.

According to previous studies, MS² quantification of aminoxyTMT labeled N-glycans, especially acidic complex N-glycans, suffered from low reporter ion yield due to the alternative fragmentation pathway. Here, the NCEs for each type of aminoxyTMT labeled N-glycans at MS² (CID and HCD) and MS³ (HCD) levels were carefully optimized. At the MS² level, a high mannose N-glycan ([H₅N₂-aminoxyTMT + 2H]²⁺) and a complex N-glycan ([H₅N₂S₂F-aminoxyTMT + 3H]³⁺) were selected to represent each class of N-glycans. The intensities of the parent ions and several fragment Y ions were monitored at different NCEs. For both N-glycan species, the fragment signals reached a plateau while the parent ion signals were still preserved at NCE 30. NCE 30 was chosen as the optimum energy to preserve backbone fragment information, and NCE 70 was chosen as the optimum energy to maximize reporter ion yield. To optimize the MS³ NCE of HCD, a pseudo MS³ method was set up: S-lens RF lens level was set to 100% to maximize fragmentation during ion transmission, and the Y₁ ion at m/z 523.3289 was isolated and fragmented at the MS² level. The reporter ion reached the highest intensity at NCE 70, which was chosen as the optimum energy for MS³.

LC Gradient Optimization

Various LC gradients and flow rates were compared to optimize the separation of aminoxyTMT labeled N-glycans released from BTG (Figure S2), including a 60 min linear gradient of 90–20% ACN at a flow rate of 300 nL/min (Figure S2A), the same gradient with flow rate of 450 nL/min (Figure S2B), a 60 min linear gradient of 80–20% ACN at flow rate of 450 nL/min (Figure S2C), and a stepped gradient of 75–60% (0–18 min), 50–40% (19–33 min), and 35–30% (34–48 min) (Figure S2D). The elution order of N-glycans mainly depends on the numbers of sialic acid residues present in the glycan. The stepped gradient improved the separation of N-glycans with the same number of sialic acids while shortening the time intervals between each group. Figure S3 demonstrated the elution sequence of some representative aminoxyTMT labeled N-glycans: the neutral N-glycans were eluted between 7 and 13 min (Figure S3A–C), followed by N-glycans with one sialic acid (23 to 24 min, Figure S3D–F) and N-glycans with two sialic acids (38 to 40 min, Figure S3G,H). The stepped gradient was used for all quantification acquisitions.

Representative Spectra for MS² and MS³ Quantification Methods

Representative spectra acquired by the three quantification methods were summarized in Figure 3. A HR/AM full scan was acquired in all three methods, and the N-glycans

corresponding to each peak could be assigned by accurate mass matching (Figure 3A). For the MS² method, HCD MS² spectra of low and high NCEs were represented in Figure 3B,C. At low NCE, the backbone fragments of the N-glycans were well preserved but the reporter ion yields were low; at high NCE, none of the backbone fragment was preserved, but the reporter ion yields were high and the ratios were much more accurate than the low energy ones. For both MS³ methods, an MS² scan was acquired in the ion trap using rapid scan mode (Figure 3D,F). In the standard MS³ run, the Y₁ ion was isolated and fragmented to generate the MS³ spectrum shown in Figure 3E. In the MultiNotch MS³ run, several Y ions were coisolated and cofragmented to generate the MultiNotch MS³ spectrum shown in Figure 3G. The average reporter ion signal intensity acquired by the Multi-Notch MS³ method (8.75×10^5) was approximately nine times higher than standard MS³ method (1.05×10^5). The spectra with zoomed in reporter ion region revealed very accurate reporter ion ratios for both MS³ methods.

Quantification Results of MS² and MS³ Methods

Approximately 15% of the MS² scans were selected for MS³ scans in both workflows. This data set suggested that a large percentage of peaks, which did not have the Y₁ ion detected, were filtered away by the system. These peaks could be either non-N-glycan peaks or N-glycan peaks with poor fragmentation efficiency. With the irrelevant or low quality peaks filtered, the workflow allows one to confidently select the N-glycan peaks for quantification and thus improve the quantification performance. The other two sets of columns represented the percentage of quantifiable scans by summarizing the number of scans with at least one reporter ion detected (middle columns) and with all reporter ions detected (right columns) within 10 ppm. With both criteria, the MultiNotch MS³ method has approximately 15% to 20% more quantifiable scans compared to the standard MS³ method.

The quantification results of 1:1:1:1:1:1 aminosyTMT labeled N-glycans released from BTG by the three methods (with four quantification approaches) were summarized in Figure 4 as box-and-whisker plots for high mannose N-glycan H₅N₂ (Figure 4A) and complex N-glycan H₅N₄S₂ (Figure 4B). The average reporter ion intensities and the average ratios were labeled in each panel. The average, median, standard deviation, and data points (*N*) were summarized in Table S1. Quantification accuracy (systematic error), precision (statistical variability), and reporter ion yield (reporter ion intensity) were used to evaluate these methods. All four methods could accurately quantify the high-mannose N-glycans with small relative error. The small relative errors observed in all four methods can be attributed to the high abundance of H₅N₂ present in the labeled N-glycan mixtures and relatively even fragmentation along the glycan backbone during CID MS/MS. In spite of low reporter ion yield by some fragmentation methods, the resulting absolute intensity of reporter ions was still abundant enough to achieve reasonable quantification accuracy. The MultiNotch MS³ method and high-energy MS² method had small signal variance and could be used for precise measurements. The standard MS³ method had slightly larger variance while the low energy MS² method had very large variance due to the low reporter ion intensities with the boxes and whiskers widely separated. In terms of reporter ion intensities, the MultiNotch MS³ method had the highest signal intensity, followed by high energy MS². The reporter ion intensity generated from the standard MS³ method was one order of magnitude lower than

that from the MultiNotch MS³ method, while the low energy MS² method had the lowest signal intensity, which was two orders of magnitude lower than the MultiNotch MS³ method. Both the MultiNotch MS³ and the high energy MS² methods were suitable for accurately and precisely quantifying the high mannose N-glycans.

A different trend was observed for the acidic complex N-glycan H₅N₄S₂ (Figure 4B). The MultiNotch MS³ method produced accurate and precise measurements (Table S1, right panel) with the highest signal intensity (8.75×10^4). All other methods produced large variances (Table S1, right panel). The high energy MS² had signal intensity of 6.07×10^4 and similar quantification accuracy as the MultiNotch MS³ but with larger variance. Both standard MS³ and low energy MS² methods have lower signal intensities and large signal variance; most of the standard MS³ data points were close to a 1:1 ratio, with some scatter outliers, which resulted in small boxes but large whiskers. The data points for low energy MS² had both large boxes and whiskers, which represented large variances throughout the whole data set. In contrast to H₅N₂, H₅N₄S₂ has lower ionization efficiency and uneven MS/MS fragmentation along the sugar backbone due to the two sialic acid residues. The resulting reporter ion intensities were ten- to a hundred-fold lower than those from H₅N₂. The lower reporter ion intensity could result in the larger error observed in H₅N₄S₂ ratios.

The quantification results of N-glycans differentially labeled with six-plex aminoxyTMT (1:2:3:4:5:6) were summarized in Figure S4 for both high mannose and complex/acidic N-glycans. Similar trends with 1:1:1:1:1:1 labeled N-glycans were observed. The targeted MultiNotch MS³ method precisely and accurately measured the relative quantitation of both types of N-glycans. Both the standard MS³ method and the low energy MS² method produced large signal variance and large quantification errors for both high-mannose and complex N-glycans. The high energy MS² method could be used to precisely measure the high mannose N-glycans with larger quantification errors; however, it did not perform well for the complex N-glycans. To conclude, the targeted MultiNotch MS³ approach was most suitable for accurately and precisely measuring different types of N-glycans.

Quantification Comparisons of MS² and MS³ Methods for a Complex Mixture

To test the performance of the MultiNotch MS³ method in a complex mixture, N-glycans released from the same amount of BTG and HSP were analyzed by nanoHILIC-MS with MultiNotch MS³ and standard MS³ and MS² (high-low HCD) (Figure 5). The N-glycans of BTG were labeled with aminoxyTMT¹²⁶, amino-xyTMT¹²⁸, and aminoxyTMT¹³⁰, while the N-glycans of HSP were labeled with aminoxyTMT¹²⁷, aminoxyTMT¹²⁹, and aminoxyTMT¹³¹. The labeled N-glycans were then mixed for quantitative analysis. A full list of glycans, which were detected and quantified by each method, was summarized in Table S2. A glycan was considered as detected when its accurate *m/z* was observed in full MS scan; it was considered as quantified when at least three reporter ions were detected in the corresponding MS² (for low or high energy MS² methods) or MS³ scans (for MultiNotch or standard MS³ methods). The Venn diagrams in Figure S5 compare the coverage of glycans detected and quantified by three data acquisition methods shown in Figure 2. There is no significant difference in terms of number of glycans detected as these data-dependent methods have similar duty cycles. We also observed that all the glycans quantified by low

energy MS² were quantified by high-energy MS², which suggests that combining high-low energy HCD MS/MS scans is the optimal choice if the instrument is only capable of MS² scans. Fourteen glycans, including four neutral and ten sialylated glycans, were quantified by MS² but not the MultiNotch MS³ method due to their overall low MS¹ signals.

Figure 5A summarized the numbers of N-glycan moieties which were putatively identified by full MS (accurate mass matching), fragmented by MS², and quantified by MS³ (at least 3 reporter ions detected). Among the three methods (MultiNotch MS³ and standard MS³ and MS²), similar numbers of N-glycan moieties were identified by full MS and fragmented by MS². At the MS³ level, the MultiNotch MS³ method quantified 35 N-glycan moieties with at least 3 reporter ions detected, while the standard MS³ method quantified 26 of them. Coisolation and cofragmentation of multiple Y ions in a targeted manner during MultiNotch MS³ runs improved the reporter ion yields, thus enabling more N-glycans, especially the low abundance ones, to be quantified.

Selected quantification results of the MultiNotch MS³ method are shown in Figure 5B: the blue columns represent the intersample ratios of N-glycans (e.g., 126/127, 128/129, 130/131), and the orange columns represent the intrasample ratios of N-glycans released from BTG (e.g., 129/127, 131/129), which serve as quality control and have a theoretical ratio of 1. Only N-glycans with all reporter ion intensities above 1000, relative error of the intrasample less than 10%, and *t*-values from the student's *t*-test above 3 are shown in this figure. Various N-glycan categories, including high mannose, complex/neutral, and complex/acidic N-glycans are quantified in the mixture. Most N-glycans detected in BTG tend to have higher concentrations than N-glycans from HSP, as they are from a purified glycoprotein rather than a protein mixture.

Box-whisker plots of the quantification of H₅N₄FS by four approaches are shown in Figure 5C. In these plots, the first, third, and fifth columns are the intersample ratios, which represent the relative amounts of H₅N₄FS between HSP and BTG; the second and fourth columns are intrasample ratios of H₅N₄FS from BTG, which serve as quality controls and have a theoretical ratio of 1. The MultiNotch MS³ method revealed accurate and precise quantification results for the two quality control data points, and the variations among the three intersample data were small (Table S3). Both the low energy MS² and standard MS³ methods had large variances of the quality control ratios, and the accuracies were complemented. Intersample ratios from these two methods were not available due to the zero reporter ion counts of HSP N-glycan. High energy MS² reported nonzero values for the intersample ratios. However, the measured log₂ ratios of the reporter ions were not as accurate or precise as the MultiNotch MS³ method. It is the second-best method among these four methods. The ability to generate sufficient intensities of reporter ions is essential for quantifying analytes with precision and accuracy, especially for sample mixtures with a wide dynamic range.

The MultiNotch MS³ method provided the most precise and accurate measurements followed by the high energy MS² method for N-glycans released from a complex protein mixture. However, the high energy MS² method generated more quantifiable glycans. Therefore, there is a trade-off between quantification performance (MultiNotch MS³) and

the number of quantifiable glycans (high energy MS²). Researchers can choose an appropriate method based on specific applications and research goals. The low energy MS² and standard MS³ approaches are not suitable for quantification of aminoxyTMT labeled N-glycans, mainly due to the low reporter ion yield. Alternatively, the high-low MS² HCD MS/MS approach could be used for identification and quantification by summing up the two MS/MS scans with different NCEs.

CONCLUSIONS

A Y₁ ion triggered, targeted MultiNotch MS³ method was developed to improve the quantification performance of aminoxyTMT labeled N-glycans released from glycoproteins and complex mixtures. By this approach, multiple Y ions were coisolated and cofragmented at the MS³ level, which resulted in significantly improved MS³ spectral quality and reporter ion intensities. Meanwhile, N-glycan composition could be confirmed by MS² scans. This method significantly improves the quantification performance and has the potential to be widely applied in quantitative glycomics analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

General sample preparation and data acquisition workflow: N-glycans were released by FANGS, labeled with 6-plex aminoxyTMT, combined and cleaned up with an Oasis HLB cartridge, and analyzed on an LC-Tribrid quadrupole-ion trap-Orbitrap MS platform.

Figure 2. MS data acquisition decision trees of MS² (A), standard MS³ (B), and MultiNotch MS³ (C) quantification methods.

Figure 3.

Representative spectra of HR/AM full MS (A), low energy HCD MS² (B), high energy HCD MS² (C), low energy CID MS² (D, F), standard MS³ (E), and MultiNotch MS³ (G) of triply charged complex N-glycan H₅N₄S₂. The spectra circled in each box represent one of the quantification methods: (B, C) for MS², (D, E) for standard MS³, and (F, G) for MultiNotch MS³. The highlighted peaks in (A), (B), (D), (F) were isolated and fragmented in the next level MS analyses.

Figure 4.

Representative box-and-whisker plots of 1:1:1:1:1 aminoxyTMT labeled N-glycans released from BTG: high mannose N-glycan H₅N₂ (A) and complex N-glycan H₅N₄S₂ (B), quantified by MultiNotch MS³, standard MS³, low energy MS², and high energy MS². The box represented the 25 and 75 percentiles, whiskers represented 5 and 95 percentiles, and the crosses represented the 1 and 99 percentiles. The average reporter ion intensities and average calculated ratios were labeled on the figure. Data quantified by each approach was highlighted in the same color. The average, median, standard deviation, and number of data points were summarized in Table S1.

Figure 5.

Quantification results of N-glycans released from BTG and HSP. The N-glycans released from BTG were labeled with aminoxyTMT¹²⁶, aminoxyTMT¹²⁸, and aminoxyTMT¹³⁰, while the N-glycans of HSP were labeled with aminoxyTMT¹²⁷, amino-xyTMT¹²⁹, and aminoxyTMT¹³¹. (A) Number of N-glycan moieties identified by full MS (orange), fragmented by MS² (blue), and quantified by MS³ with at least 3 reporter ions detected (yellow). (B) Selected MultiNotch MS³ quantified ratios of N-glycan moieties which were significantly different between BTG and HSP: columns in blue represented the averaged ratios of BTG/HSP, and the columns in orange represented the average ratios of BTG between channels 126, 128, and 130 (Filter of (B): all reporter ion intensities >1000, relative error of BTG control ratio <10%, *t*-value >3). (C) Box-and-whisker plots of H₅N₄F₅ quantified by MultiNotch MS³, standard MS³, low energy MS², and high energy MS². Supporting data (average, median, standard deviation, and number of data points) were summarized in Table S3.