Published in final edited form as:

Anal Chem. 2019 July 16; 91(14): 9206–9212. doi:10.1021/acs.analchem.9b01983.

Optimized fragmentation for quantitative analysis of fucosylated N-glycoproteins by LC-MS-MRM

Wei Yuan^{1,3}, Renhuizi Wei^{1,3}, Radoslav Goldman^{1,2,3}, Miloslav Sanda^{1,3,*}

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, 20057, USA;

²Department of Biochemistry and Molecular & Cellular Biology, Georgetown University, Washington, DC, 20057, USA.

³Clinical and Translational Glycoscience Research Center, Georgetown University, Washington, DC, 20057, USA.

Abstract

Quantitative analysis of site specific glycoforms of proteins is technically challenging but highly desirable; resolution of the fucosylated glycoforms is of particular interest due to their biological importance. In this study, we developed a sensitive and specific LC-MS-MRM quantification method that distinguishes the outer arm and core fucosylated configurations of the N-glycopeptides. We take advantage of limited fragmentation of the glycopeptides at low collision energy CID to produce linkage-specific Y-ions. We select these informative ions as MRM transitions for the quantification of the outer arm and total fucosylation of 12 fucosylated glycoforms of 9 glycopeptides in 7 plasma proteins. Our workflow showed improved sensitivity and specificity of quantification of the glycopeptides compared to oxonium ion transitions which allowed us to quantify the glycoforms directly in plasma or serum without fractionation of the samples or glycopeptide enrichment. A pilot study of fucosylation in liver cirrhosis of the HCV and NASH etiologies confirms the quantitative capabilities of the method and shows that liver cirrhosis is consistently associated with increased outer arm fucosylation of majority of the analyzed proteins. The results show that the outer arm fucosylation of the A2G2F1 glycoform of the VDKDLQSLEDILHQVENK peptide of fibrinogen increases greater than 10-fold in the HCV and NASH patients compared to healthy controls.

Graphical Abstract

*Corresponding Author: ms2465@georgetown.edu; Tel: +1 202-6876279; Fax: +1 202-6871988.

Supporting Information

The supporting Information is available free of charge on the ACS Publications website. Impact of sample load on the measurement, fold-change in total and outer arm fucosylation in the HCV and NASH patients compared to healthy controls, fold-change of the glycoforms measured in paired plasma and serum samples of HCV patients, impact of resolution setting at Q3 on the sensitivity and specificity of the quantification of total fucosylation of A3G3F1 and A3G3F2 glycoforms of the ADTHDEILEGLNFNLTETPEAQIHEGFQELLR peptide (PDF)

The authors declare no competing financial interest.

N-glycosylation of proteins is one of the most common posttranslational modifications. Alteration of N-glycans has been observed in various diseases and, specifically, fucosylation of the N-glycoproteins has been frequently associated with the diseases of the liver.^{1–7} In human tissues, fucosylation of N-glycans takes place on the innermost GlcNAc (core fucosylation) by α -1,6 linkage, or on the outer arms by an α -1,2 linkage to a galactose (often terminal), or α -1,3 or α -1,4 linkage to the mostly subterminal GlcNAc based on the activity of specific fucosyltransferases.⁸ The linkage of fucose, especially the core versus outer arm fucosylation, is an important determinant of the N-glycoprotein interactions and activities.⁹ Several published studies quantified core fucosylation in the context of liver disease^{10,11} and used either lectin affinity¹⁰ or endoglycosidase F (or H) digestion¹¹ to achieve the quantification of core fucose. However, a site- and linkage-specific quantification of the core and outer arm glycoforms was not achieved to our knowledge because of the technical challenges of these mass spectrometric assays.

Multiple reaction monitoring (MRM) workflows are widely used in quantitative proteomics due to their high sensitivity and specificity. In the recent years, this technique has been successfully employed for the quantification of glycopeptides.^{5,12–14} Mass spectrometric quantification of glycopeptides is challenging because of the substoichiometric representation of the microheterogeneous glycopeptides and their somewhat lower ionization efficiency; the reported workflows typically measure the low mass oxonium ions, such as m/z 204.1 (HexNAc), 366.1 (HexHexNAc), 138.1 (HexNAc-2H₂O-CH₂O), and 274.1 (Neu5Ac-H₂O).^{15,16} The oxonium ions are the major fragment for all N-glycopeptides under CID condition typical for peptide analysis; these ions have high sensitivity but low specificity and cannot distinguish between structural isomers. Therefore, analysis of isolated proteins, simple mixtures, or glycopeptide enrichment is typically used to reduce the sample complexity. We have shown that Y-ions carrying the peptide backbone become the major fragments of the N-glycopeptides when the collision energy (CE) is lowered to approximately 50% of the optimal CE for peptide fragmentation.^{6,17} These Y-ions not only provide improved sensitivity (signal to noise) and specificity in complex samples but they can also yield characteristic fragments for specific structural features like core or outer arm fucosylated glycoforms.^{17,18} We therefore describe the development of a MRM workflow using optimized soft fragmentation yielding Y-ions as transitions for the fucosylation study of major glycoproteins directly in plasma samples without prior enrichment of proteins or the glycopeptides.

EXPERIMENTAL SECTION

Chemicals and Reagents

Dithiothreitol (DTT), acetonitrile and water in LC-MS grade were obtained from ThermoFisher Scientific (Waltham, MA). Iodoacetamide (IAA) was from MP Biomedicals (Santa Ana, CA). Trypsin Gold (V5280) and ProteaseMax (V2071) were from Promega (Madison, WI). α 2–3,6,8,9 Neuraminidase A (P0722) and digestion buffers were from New England Biolabs (Ipswich, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Study population

All the participants were recruited under protocols approved by the Georgetown University Institutional Review Board. Blood samples were collected using BD vacutainer serum collection tube or EDTA Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) and the samples were processed within 6 h of the blood draw according to the manufacturer's protocol. Both plasma and serum samples were aliquoted and stored at -80°C until use. Participants were further grouped into healthy controls ($n=5$), cirrhotic patients of hepatitis C (HCV, $n=5$), and non-alcoholic steatohepatitis (NASH, $n=5$) etiologies. The three groups were age-matched. HCV and NASH groups had comparable degree of liver damage as measured by model for end-stage liver disease (MELD) score (HCV 14.8 vs NASH 15.4).

Tryptic digest and exoglycosidase treatment

Plasma or serum was diluted in 50 mM ammonium bicarbonate buffer, reduced with 5 mM DTT, and alkylated with 15 mM Iodoacetamide which was quenched by 5 mM DTT. The samples were digested with Trypsin Gold in a Barocycler NEP 2320 (Pressure BioScience, Medford, MA) for one hour. ProteaseMax surfactant (0.03%) was added to the samples to improve the efficiency of digestion. Tryptic digests were then deactivated at 99°C for 10 min and further treated with α 2–3,6,8,9 Neuraminidase A in GlycoBuffer 1 (50 mM sodium acetate, 5 mM CaCl_2 , pH 5.5) according to manufacturer's instructions. The Neuraminidase A treatment was conducted overnight at 37°C . At the end of digestion, Neuraminidase A was heat deactivated at 99°C for 10 min. The digests were frozen at -80°C for 30 min before centrifugation at 16,000 g for 10 min to facilitate the removal of the surfactant. The digests were subjected to LC-MS/MS analysis in randomized order without further processing to minimize sampling bias.

Glycopeptide Quantification by LC-MS-MRM

Initial analysis of glycopeptides was carried out on an Orbitrap Fusion Lumos connected to a Dionex 3500 RSLC-nano-LC (Thermo Scientific) in a data-dependent mode in order to obtain the fragmentation information of the glycopeptides. Peptide and glycopeptide separation was achieved on a $150\text{ mm} \times 75\text{ }\mu\text{m}$ C18 pepmap column by a 5 min trapping/washing step using 99% solvent A (2% acetonitrile containing 0.1% formic acid) at 10 $\mu\text{L}/\text{min}$ followed by a 90 min acetonitrile gradient at a flow rate of 0.3 $\mu\text{L}/\text{min}$: 0–3min 2% B, 3–5min from 2% to 10% solvent B (0.1% formic acid in acetonitrile); 5–60 min from 10% to 45% solvent B; 60–65min from 35% to 98% solvent B; 65–70min at 98% solvent B;

70–90min equilibration by 2% solvent B. The electrospray ionization voltage was set to 2.3 kV, and the capillary temperature was set to 275 °C. Targeted tandem mass scan were performed at low collision energy CID (20% of the normalized collision energy).

Quantitative analyses were performed in high mass positive ion mode on a 6500 Q-trap mass analyzer (AB Sciex, Framingham, MA) coupled with a nanoAcquity chromatographic system (Waters Associates, Milford, MA) consisting of a UPLC 2G Symmetry C18 TRAP column (5µm, 180 µm × 20 mm) and a BEH C18 300 capillary analytical column (1.7µm particles, 75 µm × 150 mm). Separation of the analytes was achieved by a 2 min trapping/washing step using 100% solvent A (2% acetonitrile containing 0.1% formic acid) at 15 µl/min followed by a 35 min acetonitrile gradient at a flow rate of 0.4 µl/min: 1 min from 1% to 5% solvent B (0.1% formic acid in acetonitrile) and 34 min from 5% to 50% solvent B. A cleaning gradient at a flow rate of 0.4 µL/min (10 min from 1% to 99% solvent B followed by a 10 min of isocratic run at 99% solvent B) with a 5 min sample trapping/washing step using 99% solvent B at 15 µl/min followed immediately after the peptide and glycopeptide separation in order to remove the nonpolar residues of ProteaseMax. Multiple reaction monitoring (MRM) mode was used for the quantification of glycopeptides with ion spray voltage set at 2300 V, curtain gas 11, ion source gas 1 30, and the interface heater temperature 180°C. Entrance potential (EP), collision cell exit potential (CXP), and declustering potential (DP) were set at 10 V, 13 V, and 75 V, respectively. Q1/Q3 were set at unit resolution. The plasma digest was analyzed on a 6600 TripleToF mass analyzer (AB Sciex, Framingham, MA) coupled with the nanoAcquity chromatographic system operated under the same chromatographic conditions described above. The MRM transitions for the glycoforms monitored in our experiments are listed in Table 1; oxonium ion m/z 512.2 was added to the transition list to monitor outer arm fucosylation. Collision energy (CE) for each MRM transition was optimized by a 5 V step optimization followed by a 1 V step fine tuning. Instrument control and data acquisition were performed by AB Sciex Analyst software (version 1.7.1).

Data processing and Statistical analysis

LC-MS-MRM data was processed by Multi Quant 2.0 software (AB Sciex) with manual confirmation of peak assignment. Peak areas were used for glycopeptide quantification and data normalization. The details of the MSMS transitions used for the quantification of each glycoform are listed in Table 1. Relative intensity of the outer arm or total fucosylation was calculated by normalizing the respective peak area to the corresponding non-fucosylated glycoform.

RESULTS AND DISCUSSION

Low CE (soft) MRM method development

Selection of MRM transitions—In our previous study using a data-independent method,⁶ we found a greater than 1.5 fold increase in the fucosylation of 125 glycoforms of 25 tryptic glycopeptides derived from 10 plasma proteins in cirrhotic patients compared to healthy controls. Both core and outer arm fucosylated glycoforms are elevated in the plasma of the cirrhotic patients. Based on those results, we selected 23 glycoforms of 9 tryptic

glycopeptides derived from 7 proteins (Table 1) to develop the MRM workflow for linkage specific quantitative fucosylation study in plasma. The rationale for the selection of the MRM transitions is documented in Figure 1. The figure shows that, at the optimal CE, Y-ions become the major fragments for quantification of the A3G3 and A3G3F1 glycoforms of VVLHPNYSQVDIGLIK peptide of haptoglobin. The MSMS spectrum of A3G3F1 glycoform (Figure 1A) shows that both core and outer arm fucosylation contribute to the formation of the Y-ion m/z 1188.6. Therefore, this fragment is chosen to represent total fucosylation. On the other hand, Y-ion m/z 1139.9 resulting from the loss of a fucosylated GlcNAc-Gal arm of the N-glycan is the characteristic fragment of an outer arm fucosylated glycoform. This fragment stands for outer arm fucosylation. In addition to the Y-ions, the oxonium ion at m/z 512.2 is monitored to confirm the outer arm fucosylation. Because of the lack of isotopically labeled standards of the glycopeptides, we normalize the integrated peak area of the total or outer arm fucosylated transitions to its corresponding non-fucosylated glycoform for the purpose of quantification; in the case of A3G3F1 glycoform of VVLHPNYSQVDIGLIK peptide we normalize to its A3G3 glycoform (Figure 1B). Y ion m/z 1139.9, resulting from the loss of one GlcNAc-Gal arm (dissociation of the Man-GlcNAc bond), is chosen to quantify the A3G3 glycoform. The XIC corresponding to these MRM transitions is shown in Figure 1C and the MRM transitions of all glycoforms targeted in this study are chosen based on the same principle and listed in Table 1. Transitions for sialylated glycoforms (A2G2S1 and A2G2S2) of VVLHPNYSQVDIGLIK peptide of haptoglobin are added to monitor the completion of Neuraminidase A digestion. CEs for each MRM transition were optimized to achieve maximum signal intensity. The glycoforms and their retention times were verified by running the same plasma digest on a 6600 TripleToF mass analyzer coupled with the NanoAcquity chromatographic system using the same chromatographic conditions.

Impact of resolution in Q3 on sensitivity and specificity—The settings of resolution for the Q1 and Q3 can affect both sensitivity and specificity; lower resolution results in higher sensitivity but lower specificity. In this study, Q1 was set at Unit resolution to increase the specificity of precursor ion selection. The sensitivity and specificity at different resolution settings (Unit, Low, and Open) at Q3 were compared. The intensity of analyte peaks increased when the resolution was lowered from Unit to Low while specificity remains comparable (Figure S1). The intensities of the high and moderate abundant analytes, such as the A3G3F1 and A3G3F2 in ADTHDEILEGLNFNLTETPEAQIHEGFQELLR of antitrypsin (Figure S1 A and B), increased more than 1.5 fold compared to the Unit resolution. At the same time, the background remains low so that the S/N ratios of these peaks improve compared to the Unit resolution. At Open resolution, the background signal decreased the S/N ratio and the interference peaks reduced the specificity of detection (Figure S1 B). For the low abundant analytes, such as the A3G3F1 glycoform of EHEGAIYPDNTTDFQR peptide of ceruloplasmin (Figure 3), low resolution leads to a 4 fold increase of the intensity of the targeted peaks but a minimal increase in the background noise. The resulting S/N ratios of both outer arm and total fucosylation transitions increased over 3 fold compared to the unit resolution for this analyte. We therefore chose the Low resolution setting of Q3 for the quantification of the analytes in this study.

Comparison of Y- and oxonium ion transitions—More than half of the Y-ions in this study have an m/z greater than 1250, beyond the upper limit of the low mass mode on the Qtrap 6500. We therefore developed the quantification method in the high mass mode. In general, the high mass mode is about 5 times less sensitive compared to the low mass mode. However, the specificity of the high mass fragments outweighs the loss of sensitivity in the high mass mode on this mass spectrometer. We compared the specificity and sensitivity of Y-ion transitions in high mass mode to the oxonium ion transitions in low mass mode. When the Y-ions of A3G3F1 and A3G3F2 of ADTHDEILEGLNFNLTETPEAQIHEGFQELLR of antitrypsin were compared to their corresponding oxonium ion transitions (Figure 3), the Y-ions provided up to 2-times higher S/N ratio. The less specific oxonium ion transitions produce interference peaks to a greater degree as shown in the case of A3G3F2 transitions of this glycopeptide (Figure 3D) compared to the Y-ions (Figure 3B). Similar results were observed for most of the monitored glycoforms which suggests that the use of Y-ions generated by soft fragmentation is advantageous even with the lower sensitivity of the high mass mode on this mass spectrometer.

Reproducibility

We used a quality control (QC) sample to assess the reproducibility of the method. The QC plasma sample was analyzed repeatedly and relative quantification of all the targeted glycoforms was performed by normalizing the integrated peak area of total or outer arm fucosylated transitions to their corresponding nonfucosylated glycoform (Table 2). The average RSD across the analytes is 11.6% and ranges from 1.5 to 23%. We also checked the influence of the protein load on the performance of the assays by measuring the RSDs of 0.75, 1, or 2 μ g total plasma protein. Our results show that the average RSD of the measurements is 9.4% and that RSD of any measurement is below 22% except one glycoform of ceruloplasmin (Table S1). Thus, the optimized soft fragment MRM method achieves sensitive and reliable quantification of the glycoforms in the tryptic digest of plasma without any fractionation or enrichment. At the same time, the method allows quantitative analysis of specific fucosylation structures by separate quantification of the core fucosylation linkage.

Quantification of the fucosylated glycoforms in the plasma of patients with liver cirrhosis—We used the energy-optimized MRM workflow to quantify the linkage specific fucosylation change of 12 glycoforms in 7 plasma proteins in cirrhotic patients of HCV (n=5) or NASH (n=5) etiologies compared to disease free controls (n=5). Except for the A2G2F1 glycoform of the SWPAVGNCSSALR peptide of hemopexin (HCV and NASH etiology) and the A3G3F2 glycoform of ADTHDEILEGLNFNLTETPEAQIHEGFQELLR peptide of antitrypsin (NASH etiology), both outer arm and total fucosylation increased significantly in the HCV and NASH patients compared to the healthy controls (Table S2, >1.6-fold, $p < 0.05$). We did not find significant differences between the HCV and NASH patients, in line with the notion that cirrhotic changes are the major contributor to the observed differences irrespective of the etiology. This is in agreement with the results from our^{5,6,17,19} and other labs.^{4,20,21} The observed fold changes in fucosylation of the outer arm are similar to those of total fucosylation, suggesting that core fucosylation represents a

minor contribution to the observed changes. The outer arm fucosylation is likely more sensitive to the liver damage in line with our previous studies.¹⁷

Interestingly, we observed the most significant fold change (>10-fold, $p < 0.01$) on the fucosylated outer arm of the A2G2F1 glycoform of the VDKDLQSLEDILHQVENK peptide of fibrinogen γ -chain (Asn52) (Figure 4). It was reported that sialylation of the N-glycans of fibrinogen affects its clotting behavior²² and increased fucosylation and sialylation of fibrinogen has been reported in cirrhotic patients with/out HCC²³ which is consistent with our findings. Moreover, our results suggest that outer arm fucosylation of the A2G2F1 glycoform of this peptide of fibrinogen is more sensitive to the fibrotic changes of the liver than fucosylation of other glycoproteins. The Asn52 of fibrinogen is adjacent to a central hinge point located in a non-helical segment of the γ chain, where the coiled-coils region of fibrinogen can bend around.²⁴ The coiled-coil region has been attributed to the tensile deformation of fibrin fibers.^{25–27} Fibrin is formed after enzymatic release of fibrinopeptides from fibrinogen and further polymerized to form fibrin clot. Reports on computational analysis of fibrinogen suggest that the carbohydrate moieties on Asn52 of the γ -chain and Asn364 of the β -chain affect the B-b knob-hole interactions in fibrin,²⁵ and this knob-hole interaction has been proposed to affect the susceptibility of clots to proteolytic digestion.²⁸ We also noticed an increase in both the outer arm (>6-fold, $P < 0.01$) and total (>4-fold, $P < 0.01$) fucosylation of the A2G2F1 glycoform of the GTAGNALMDGASQLMGENR (Asn364) β -chain of fibrinogen (Table S2) in the cirrhotic patients.

Serum is the commonly used blood sample obtained by the removal of the clotting factors and fibrinogen. Our results suggest that plasma which contains the fibrinogen may be the desired blood fraction for the quantification of the fibrotic liver disease. We fully acknowledge that this small proof of principle quantification study was not designed to test this hypothesis and needs to be expanded to justify the observation. Nonetheless, we evaluated whether the sample matrix (serum vs plasma) affects the quantitative results of analytes present in both serum and plasma. To this end, we analyzed paired serum and plasma of patients with cirrhosis of HCV etiology using our optimized workflow. Our results show that the fold changes measured in serum are consistent with the paired plasma samples and we did not detect any significant differences in those two sample sets (Table S3). This further confirms the reliability of our quantitative measurement and suggests that the fucosylation of fibrinogen is indeed more sensitive than the other proteins to the fibrotic changes of the liver in this small sample set.

CONCLUSION

We successfully optimized a sensitive and selective method for the quantification of linkage specific fucosylation of 12 glycoforms of 7 plasma proteins. Selecting the optimized soft fragments (Y-ions) instead of the commonly used oxonium ions as MRM transitions improved the sensitivity and specificity of quantification. Our results document that the workflow can be applied to the quantification of the glycoforms of abundant proteins directly in plasma or serum without fractionation or glycopeptide enrichment. This minimizes artifacts of sample processing and simplifies the analytical protocol. The Y-ions

also provide information on fucose linkage and allowed us to resolve partially the linkage isoforms of the fucosylated glycopeptides. This is a desired improvement allowing association of the activity of specific glycosyltransferases with biomarker discovery which results in an improved understanding of the biology of the diseases. We applied the workflow to a pilot study of fucosylation of N-glycoproteins in liver cirrhosis of the HCV and NASH etiologies. The results reveal that increased outer arm fucosylation of majority of the proteins is consistently associated with the development of liver cirrhosis. We have found that the outer arm fucosylation of the A2G2F1 glycoform of the VDKDLQSLEDILHQVENK peptide of fibrinogen increases greater than 10 times in this small pilot study of cirrhosis in the HCV and NASH patients. In summary, we report a novel LC-MS-MRM workflow for the quantification of site- and linkage- specific fucosylation based on energy optimized fragmentation of the N-glycopeptides. The quantification was achieved directly in a tryptic digest of serum or plasma proteins without fractionation or glycopeptide enrichment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

Research reported in this publication was supported by the National Institutes of Health under awards S10OD023557, UO1 CA230692, and RO1 CA135069 to RG and CCSG Grant P30 CA51008, to Lombardi Comprehensive Cancer Center, supporting the Proteomics and Metabolomics Shared Resource, Georgetown University Medical Center. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Reference List

- (1). Jia L; Zhang J; Ma T; Guo Y; Yu Y; Cui J *Front Oncol.* 2018, 8, 565. [PubMed: 30619732]
- (2). Miyoshi E; Moriwaki K; Terao N; Tan CC; Terao M; Nakagawa T; Matsumoto H; Shinzaki S; Kamada Y *Biomolecules.* 2012, 2, 34–45. [PubMed: 24970126]
- (3). Morelle W; Flahaut C; Michalski JC; Louvet A; Mathurin P; Klein A *Glycobiology* 2006, 16, 281–293. [PubMed: 16339757]
- (4). Comunale MA; Lowman M; Long RE; Krakover J; Philip R; Seeholzer S; Evans AA; Hann HW; Block TM; Mehta AS *JProteome.Res* 2006, 5, 308–315.
- (5). Yuan W; Sanda M; Wu J; Koomen J; Goldman RJ *Proteomics.* 2015, 116, 24–33. [PubMed: 25582524]
- (6). Sanda M; Zhang L; Edwards NJ; Goldman R *Anal.Bioanal.Chem* 2016, 409, 619–627. [PubMed: 27822650]
- (7). Wang M; Sanda M; Comunale MA; Herrera H; Swindell C; Kono Y; Singal AG; Marrero J; Block T; Goldman R; Mehta A *Cancer Epidemiol.Biomarkers Prev* 2017, 26, 795–803. [PubMed: 28223431]
- (8). Staudacher E; Altmann F; Wilson IB; Marz L *Biochim.Biophys.Acta* 1999, 1473, 216–236. [PubMed: 10580141]
- (9). Ferdosi S; Rehder DS; Maranian P; Castle EP; Ho TH; Pass HI; Cramer DW; Anderson KS; Fu L; Cole DEC; Le T; Wu X; Borges CR *JProteome.Res* 2018, 17, 543–558.
- (10). Cao Q; Zhao Q; Qian X; Ying W *Methods Mol.Biol* 2017, 1619, 127–137. [PubMed: 28674882]
- (11). Ma J; Sanda M; Wei R; Zhang L; Goldman RJ *Proteomics.* 2018, 189, 67–74. [PubMed: 29427759]

- (12). Sanda M; Pompach P; Brnakova Z; Wu J; Makambi K; Goldman R *Mol.Cell Proteomics* 2013, 12, 1294–1305. [PubMed: 23389048]
- (13). Song E; Pyreddy S; Mechref Y *Rapid Commun.Mass Spectrom* 2012, 26, 1941–1954. [PubMed: 22847692]
- (14). Yang N; Goonatileke E; Park D; Song T; Fan G; Lebrilla CB *Anal.Chem* 2016, 88, 7091–7100. [PubMed: 27311011]
- (15). Goldman R; Sanda M *Proteomics.Clin.Appl* 2015, 9, 17–32. [PubMed: 25522218]
- (16). Zhu R; Song E; Hussein A; Kobeissy FH; Mechref Y *Methods Mol.Biol* 2017, 1598, 213–227. [PubMed: 28508363]
- (17). Yuan W; Benicky J; Wei R; Goldman R; Sanda MJ *Proteome.Res* 2018, 17, 2755–2766.
- (18). Acs A; Ozohanics O; Vekey K; Drahos L; Turiak L *Anal.Chem* 2018, 90, 12776–12782. [PubMed: 30299079]
- (19). Benicky J; Sanda M; Pompach P; Wu J; Goldman R *Anal.Chem* 2014, 86, 10716–10723. [PubMed: 25302577]
- (20). Comunale MA; Rodemich-Betesh L; Hafner J; Wang M; Norton P; Di Bisceglie AM; Block T; Mehta A *PLoS.One* 2010, 5, e12419. [PubMed: 20811639]
- (21). Zhu J; Lin Z; Wu J; Yin H; Dai J; Feng Z; Marrero J; Lubman DM *JProteome.Res* 2014, 13, 2986–2997.
- (22). Dang CV; Shin CK; Bell WR; Nagaswami C; Weisel JW *J Biol.Chem* 1989, 264, 15104–15108.
- (23). Nagel T; Klaus F; Ibanez IG; Wege H; Lohse A; Meyer B *Anal.Bioanal.Chem* 2018, 410, 7965–7977. [PubMed: 30397756]
- (24). Marsh JJ; Guan HS; Li S; Chiles PG; Tran D; Morris TA *Biochemistry* 2013, 52, 5491–5502. [PubMed: 23875785]
- (25). Weisel JW; Litvinov RI *Subcell.Biochem* 2017, 82, 405–456. [PubMed: 28101869]
- (26). Zhmurov A; Brown AE; Litvinov RI; Dima RI; Weisel JW; Barsegov V *Structure*. 2011, 19, 1615–1624. [PubMed: 22078561]
- (27). Zhmurov A; Kononova O; Litvinov RI; Dima RI; Barsegov V; Weisel JW *J Am.Chem.Soc* 2012, 134, 20396–20402.
- (28). Doolittle RF; Pandi L *Biochemistry* 2006, 45, 2657–2667. [PubMed: 16489759]

Figure 1.

Optimized fragmentation selected for the quantification of changes in the fucosylation of the VVLHPNYSQVDIGLIK peptide of haptoglobin. A. MSMS spectrum of A3G3F1 glycoform at low CE, B. MSMS spectrum of A3G3 glycoform at low CE. C. XICs of the selected Y-ions of A3G3 (orange), outer arm fucosylation (pink) and total fucosylation (green) of A3G3F1.

Figure 2.

Impact of resolution at Q3 on the sensitivity and specificity of the measurement of A3G3F1 glycoform of the EHEGAIYPDNTTDFQR peptide of ceruloplasmin. A. Q3 at Unit resolution; B. Q3 at Low resolution; C. Q3 at Open resolution; Red: transitions for outer arm fucosylation; Blue: transitions for total fucosylation.

Figure 3. XIC of the Y-ion (A and B) and oxonium ion (C and D) transitions of A3G3F1 and A3G3F2 glycoforms of the ADTHDEILEGLNFNLTETPEAQIHEGFQELLR peptide of antitrypsin under optimized CE conditions. Pink trace: transitions for outer arm fucosylation; Blue trace: transitions for total fucosylation; Red trace: $M^{5+} \rightarrow 204.1$; Green trace: $M^{5+} \rightarrow 366.1$.

Figure 4. Outer arm fucosylation of the A2G2F1 glycoform of the VDKDLQSLEDILHQVENK peptide of fibrinogen in healthy control (n=5) and cirrhotic patients of the HCV (n=5) and NASH (n=5) etiologies.

Table 1.

MRM transitions of the targeted glycoforms.

Loss of F-arm or nonF-arm Y-ion stands for the Y-ion resulting from the loss of a fucosylated or non-fucosylated GlcNAc-Gal arm of the N-glycan, respectively. We monitor the oxonium ion m/z 512.2 transition for all fucosylated glycoforms.

Protein	C(Carbamidomethyl) peptide	Glycan Composition	Precursor m/z	Precursor Charge State	Loss of F-arm Y-ion m/z	Loss of nonF-arm Y-ion m/z	Oxonium Ion	RT (min)
Haptoglobin	M(O)VSHHNLTTGATLINEQWLLTTAK VVLPNYSQVDIGLIK	A3G3	1171.5	4	N/A	3+	N/A	23.67
		A3G3F1	1208.0	4	1440.0	1488.7	512.2	23.64
		A2G2	855.2	4	N/A	1018.2	N/A	20.84
		A2G2F1	891.7	4	1018.2	1066.8	512.2	20.74
		A3G3	946.4	4	N/A	1139.9	N/A	20.65
Serotransferrin	QQQHLFGSNVTDSCGNFCLFR	A3G3F1	983.0	4	1139.9	1188.6	512.2	20.61
		A3G3	1126.5	4	N/A	1379.9	N/A	22.06
		A3G3F1	1163.0	4	1379.9	1428.6	512.2	21.99
Antitrypsin	ADTHDEILEGLNPNLTIPEAQIHEGFQELLR	A3G3	1136.7	5	N/A	4+	N/A	29.45
		A3G3F1	1165.9	5	1329.4	1365.9	512.2	29.41
		A3G3F2	1195.1	5	1365.9	1402.4	512.2	29.38
Fibrinogen	VDKDLQSLEDILHQVENK GTAGNALMDGASQLMGENR	A2G2	937.2	4	N/A	3+	N/A	23.98
		A2G2F1	973.7	4	1127.5	1176.2	512.2	23.93
		A2G2	879.6	4	N/A	1050.8	N/A	21.28
		A2G2F1	916.1	4	1050.8	1099.5	512.2	21.22
Alpha1-acid glycoprotein	SVQEIQTFFYFTPNK	A4G4	1069.0	4	N/A	1303.2	N/A	24.09
		A4G4F1	1105.5	4	1303.2	1351.9	512.2	24.07
Ceruleplasmin	EHEGAIYPDNTTDFQR	A3G3	970.9	4	N/A	1172.5	N/A	16.62
		A3G3F1	1007.4	4	1172.5	1221.2	512.2	16.61
		A2G2	1009.8	3	2+	2+	N/A	17.62

Protein	C(Carbamidomethyl) peptide	Glycan Composition	Precursor m/z	Precursor Charge State	Loss of F-arm Y-ion m/z	Loss of nonF-arm Y-ion m/z	Oxonium Ion	RT (min)
Hemopexin		A2G2F1	1058.4	3	1331.6	1404.6	512.2	17.6
		A3G3	1131.5	3	N/A	1514.1	N/A	17.58
		A3G3F1	1180.2	3	1514.1	1587.2	512.2	17.57
QC for Neuraminidase A Haptoglobin	VVLHPNYSQVDIGLIK	A2G2S1	927.9	4	1526.7	1445.7	N/A	274.1
		A2G2S2	1000.7	4	1526.7	1344.2	N/A	274.1

Table 2. Reproducibility of the measurements expressed as RSD (%) of the quantification (n=5).

protein	peptide	glycoform	RSD(%)	
			outer arm	total
Haptoglobin	M(O)VSHHNLTTGATLINEQWLLTTAK	A3G3F1	14.4	12.5
		A2G2F1	13.8	6.1
		A3G3F1	15.1	10.0
Serotransferrin	QQQHLLFGSNVTDCSGNFCLFR	A3G3F1	20.4	21.2
Antitrypsin	ADTHDEILEGLNLFNLTEIPEAQIHEGFQELLR	A3G3F1	12.2	11.8
		A3G3F2	21.5	23.0
Fibrinogen	VDKDLQSLLEDILHQVENK	A2G2F1	11.5	N/A
		A2G2F1	13.1	14.6
Alpha-acid glycoprotein	SVQEIQAIFFFYFTPNK	A4G4F1	4.6	0.9
Ceruloplasmin	EHEGA1YPDNTTDFQR	A3G3F1	8.8	7.8
Hemopexin	SWPAVGNCSALR	A2G2F1	4.1	1.5
		A3G3F1	11.3	6.5