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Applications of Multiple Reaction Monitoring to Clinical Glycomics

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Abstract

Multiple reaction monitoring or MRM is widely acknowledged for its accuracy of quantitation. The applications have mostly been in the analysis of small molecules and proteins, but its utility is expanding. Protein glycosylation was recently identified as a new paradigm in biomarker discovery for health and disease. A number of recent studies have now identified differential glycosylation patterns associated with health and disease states, including aging, pregnancy, rheumatoid arthritis and different types of cancer. While the use of MRM in clinical glycomics is still in its infancy, it can likely play a role in the quantitation of protein glycosylation in the clinical setting. Here, we aim to review the current advances in the nascent application of MRM in the field of glycomics.

Introduction

As one of the most common post-translational modifications of proteins, present in at least 70% of plasma and membrane proteins, glycosylation is involved in many key biological processes. The glycan moiety of glycoproteins plays a role in protein folding [1] and protein stability [2] as well as cell-cell interactions, cell-matrix interactions [3] and receptor binding and activation. Given the importance of these processes in human health, it is not surprising that differential glycosylation has been observed in numerous disease states including several types of cancer [4–8], autoimmune diseases [9, 10] and diabetes [11]. Moreover, altered glycosylation profiles have been observed with aging [12, 13] and during pregnancy [14, 15]. Analytical methods suitable for fast and robust profiling of glycosylation – preferably at the level of individual proteins- are instrumental to further advance the use of glycomics in a clinical setting. Multiple reaction monitoring (MRM) has the potential to be one of the key techniques in this field.

The ability to quantitate accurately is an important aspect of clinical mass spectrometry. For many years, MRM has been the standard for quantitation in targeted applications, specifically in proteomics [16] and metabolomics [17]. MRM is commonly performed on triple quadrupole (QQQ) instruments and is widely acknowledged for its quantitative properties. In MRM with a QQQ, the first and third quadrupole are used as mass-selective filters that specifically select the analyte of interest (Q1) and a characteristic fragment ion of

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the analyte (Q3). The second quadrupole (Q2) functions as a collision cell with a broad m/z passage range, where the analyte is fragmented under collision induced dissociation (CID) conditions. A pair of analyte/fragment ions is typically referred to as a transition. Multiple transitions are detected in a single run as co-eluting background analytes (often originating from sample impurities) are filtered out in the two stages of selection resulting in high selectivity of the method. The targeted nature of the analysis provides high sensitivity and linearity over a wide dynamic range. Therefore, MRM methods are very suitable for the robust, fast, sensitive and specific quantitative analysis of multiple compounds simultaneously in the presence of other more abundant compounds (e.g. in complicated mixtures, such as biofluids).

Because MRM is a targeted approach, knowledge regarding the mass and charge state of the analyte as well as its fragmentation behavior in collision induced dissociation (CID) is essential for the development of the transitions. Several studies have focused on the fragmentation pattern of glycans and glycopeptides [18–20]. In low energy CID conditions found in standard triple quadrupole instruments, the typical fragmentation occurs at the glycosidic bonds. This allows for the determination of monosaccharide connectivity, but in this mode there is usually little that distinguishes the different linkage positions. Under low energy conditions of native compounds cross-ring cleavages are often of low abundances. Therefore, CID of N-glycans and N-glycopeptides yields primarily glycan oxonium ions at higher abundances resulting from the cleavage of glycosidic bonds.

Recent advances have been made in the use of MRM in the field of glycobiology and glycomics, however its use and application in biological studies is still in its infancy and rather limited. This review aims to provide an overview of the nascent utility of MRM for the analysis of glycans and glycoconjugates with an emphasis on its application in the clinical setting. It is structured based on the different types of analytes: quantification of glycoproteins, quantification of glycosylation at the glycopeptide level and quantification of glycans.

Quantification of glycoproteins

One of the more commonly applied strategies is the quantitation of glycosylated proteins using multiple reaction monitoring. Methods for glycoprotein or glycopeptide analysis involved extensive enrichment of target analytes from a complicated biological sample for subsequent quantitation. Several methods for enrichment have been employed either based on global glycan enrichment (e.g. [21]) or based on the enrichment of specific glycan features using lectins (e.g. [22]).

A detailed protocol for a global enrichment strategy using glycan oxidation and hydrazine-based enrichment of glycoproteins (solid-phase extraction of N-linked glycoprotein/peptide (SPEG)) has been described [23]. The proteins were first immobilized at the glycan moiety and then tryptic digested. PNGase F was then used to release the immobilized glycan from the peptide moiety, resulting in free deglycosylated peptides. Monitoring the deglycosylated peptide was performed with synthetic and isotopically labeled peptides used for both MRM optimization and quantitation of the glycoprotein [23]. While no immediate application of

the method was described, an adaptation of the method was used to evaluate the glycosylation of prostate specific antigen (PSA) in prostate cancer and non-cancer tissues [24]. Here, both the global SPEG method, as well as a sialic acid-specific method using mild oxidation conditions was used. Transitions to the deglycosylated peptide were monitored in prostate cancer tissues (n=9) and non-cancer tissues (n=9). While no differentially expressed levels of the deglycosylated peptide were observed using the global enrichment method, higher levels of the deglycosylated PSA peptide were observed when the sialic acid specific enrichment method was used suggesting, albeit indirectly, increased sialylation of PSA in prostate cancer [24]. More recently, SPEG in combination with MRM was employed for the identification of differentially expressed glycopeptides in serum of esophageal adenocarcinoma patients [25]. Here, five glycopeptides, originating from four glycoproteins were identified to be differentially expressed.

The use of lectins for glycan specific enrichment of glycoproteins in combination with MRM has also been performed. Aberrantly glycosylated TIMP-1 has previously been associated with colorectal cancer. To quantify the differentially glycosylated TIMP-1, the protein was enriched using phytohemagglutinin-L (L-PHA) - a lectin that specifically recognizes the beta-1,6-GlcNAc moiety of N-linked glycans, followed by Stable Isotope Standard Capture with Anti-Peptide Antibodies (SISCAPA) and MRM [26]. Using this method, the aberrantly glycosylated TIMP-1 was quantified from less than 2 μ L of serum. The method was subsequently extended to cover a larger fraction of the serum glycoproteins using the four lectins AAL, L-PHA, ConA and DSA. MRM transitions were developed for peptides originating from 67 proteins [27]. An average coefficient of variation (CV) of 7.9% was reported for the higher abundance proteins monitored in the study, but no clinical application was reported.

The validation of differentially fucosylated glycoproteins that were previously identified was assessed in a similar fashion [28]. Using AAL lectin, followed by tryptic digestion and MRM analysis of the respective peptides the four proteins APCS, C9, SERPINA4, and PON1 were shown to be differentially fucosylated in sera of non-small cell lung cancer patients [28] as illustrated in Figure 1 for PON1. An alternative strategy for the use of MRM in glycoprotein analysis is based on the observation that differential glycosylation may result in altered levels of missed cleavages [29, 30]. In studies by Lee et al. [30, 31], this phenomenon was used by monitoring the partially missed cleaved peptides. Lower levels of these peptides were associated with hepatocellular carcinoma [30].

Quantification of glycopeptides

Another strategy for quantitating glycosylation using MRM involves the direct analysis of glycopeptides. These methods are inherently difficult due to the lower ionization efficiencies induced by the glycan moiety on the peptide. Additionally, the heterogeneity of the glycosylation divides the intensities of the glycopeptides across several glycoforms.

The first step in this process is to determine the occupancy of a specific glycosite. Here, MRM can also be employed [32] by performing PNGaseF in the presence of ^{18}O yielding a labeled aspartic acid. MRM transitions developed to both the asparagine-containing peptide

and the labeled aspartic acid are then used to determine the degree of occupancy. Using this method, it was shown that increased activity of congenital disease of glycosylation (CDG) type 1 results in a lower N-glycan occupancy [32]. So far, only a limited number of studies on the analysis of N-glycopeptides by MRM have been published [33–37]. In the majority of these studies, characteristic oxonium ions representing hexose (m/z 163), HexNAc (m/z 204), NeuAc (m/z 292) HexHexNAc (m/z 366), HexHexNAcFuc (m/z 512) and HexHexNAcNeuAc (m/z 657), with or without loss of water were used as the reporter ions. Only one study, in which the glycan moiety was stabilized using PA-labeling, reported the use of the peptide + HexNAc as the most intense reporter ion [36].

Biofluids, particularly serum or plasma, are typically the most readily available patient samples. All reported studies on the analysis of glycopeptides with MRM have therefore focused on these substances. We recently developed a method for the direct analysis of immunoglobulin G (IgG) and its glycoforms from serum [34]. The source parameters were specifically optimized for glycans and glycopeptides, a necessary procedure as these compounds are relatively more labile than the peptides and small molecules - the standard compounds used by manufacturers for optimizing commercial triple quadrupole instruments. Transitions were developed for 26 glycopeptides from IgG to the oxonium ions m/z 366 (HexHexNAc) or m/z 204 (HexNAc). To achieve normalization of the glycosylation profile to the overall protein content, transitions were also developed for non-glycosylated peptides. The collision energies were also optimized. The resulting method yielded a limit of detection of 60 amol and a wide dynamic range extending 3 orders magnitude for IgG protein quantitation [34]. A typical MRM chromatogram of IgG is shown in Figure 2. The glycopeptides of IgG subclass 3 and IgG subclass 4 could not be distinguished using this method directly, however subclass specific analysis can be obtained during the sample preparation [38]. This method is currently being expanded to the other immunoglobulins IgA and IgM (manuscript in preparation). Altered glycosylation of immunoglobulins has been associated with multiple diseases [39], and the differential immunoglobulin glycosylation of ovarian cancer is currently being assessed using MRM (manuscript in preparation).

A similar approach was used for the differential analysis of haptoglobin in plasma of hepatocellular carcinoma (HCC) patients [35]. Fucosylation of haptoglobin is increased in HCC [40] and upon isolation of HCC and treatment with sialidase and galactosidase, 24 glycopeptides of the T3- glycopeptide containing N-glycosylation site N²⁴¹ were monitored. Transitions were developed to oxonium ions at m/z 204, m/z 366 and m/z 512 and the collision energies were optimized. Using this method, differential fucosylation of this specific haptoglobin glycopeptide in HCC patients compared to controls and cirrhosis patients was determined [35].

An alternative, not protein specific, approach has been employed by Kuroguchi et al. [36], who aimed to quantify a broad range of sialylated glycopeptides from mouse serum. To enrich for sialylated glycopeptides a reverse glycoblotting technique was used, which allows for the retrieval of glycopeptides as pyridylaminated conjugates. The enriched glycopeptides were then characterized using LC-MS/MS, followed by the development of MRM transitions. Interestingly, the peptide + HexNAc fragment was identified as the most

important reporter ion. Using this approach, 67 glycopeptides from mouse serum could be monitored and using a diabetes disease model, differently expressed glycopeptides were identified [36]. It is anticipated that a similar method could be applied to human biofluids.

A similar approach was recently used by Song et al. who first applied lectin enrichment using an SNA and AAL mixture on high abundant protein depleted serum samples, and then used MRM for glycopeptide quantifications [25]. Transitions for 57 glycopeptides were monitored to the characteristic oxonium ions at m/z 204, 274 and 366. The method was then applied to serum samples from esophageal adenocarcinoma patients (EAC), patients with high-grade dysplasia in the esophagus (HGD) and disease-free controls (DF). Four glycopeptides were identified to be differentially expressed in EAC compared to DF, while levels of 11 glycopeptides were significantly altered between HGD and DF.

The analysis of O-GlcNAc modifications is inherently difficult. Recently, the successful use of MRM for the identification of an O-GlcNAcylated peptide was reported [41]. A commercially available O-GlcNAcylated peptide was used to optimize instrument parameters and show the feasibility of LC-MRM-MS for the quantitation of the O-GlcNAc modification. Characteristic peptide fragments were shown to be most suitable for quantification. GSK-3 β is known to be O-GlcNAcylated, but the site of attachment is not clear. Using LC-MRM, the O-GlcNAcylated peptide could be identified unambiguously [41].

Quantification of oligosaccharides

One of the earliest applications of MRM for oligosaccharide analysis was in the analysis of a glycogen derived glucose tetrasaccharide that is known to be elevated in urine of those with Pompe disease[42]. Butyl-4-aminobenzoate (BAB) derivatized oligosaccharides were analyzed, and a C₁₃ labeled tetraglucose standard was added during sample preparation to increase accuracy of quantification. Transitions were monitored toward the sodium adduct of Glc₃ and the results obtained with the LC-MRM method were in good agreement with the conventional HPLC-UV method, indicating that MRM can be used for the analysis of oligosaccharides for the determination of Pompe disease.

Four additional studies have recently been reported on the use of MRM for the quantitation of human oligosaccharides [43–45], each presenting alternative methods. We recently reported on the use of MRM for the analysis and quantification of human milk oligosaccharides (HMO) in the native, reduced state. Using a porous graphitized carbon stationary phase, structural isomers can be separated and quantified individually, resulting in the simultaneous analysis of 49 HMO structures. Seven standard HMO (2'-FL, LNT, LNFP-I, 3'-SL, 6'-SL, and LSTc) were used for absolute quantitation and the LOD for each of these compounds was found to be in the low femtomole to attomole range. Using this method, significant differences were observed in the HMO composition of secretors (mothers that can excrete HMO with α 1-2 linked fucose) and non-secretors (mothers that do not produce HMO with α 1-2 linked fucose) [44] as is illustrated in Figure 3.

Two alternative strategies that are based on labeling techniques have been applied to oligosaccharides. 1-phenyl-3-methyl-5-pyrazolone (PMP) labeling has been reported to be

instrumental in the quantification of urinary oligosaccharides [43]. Under CID, a characteristic PMP-fragment at m/z 175 was consistently observed in the product ion scans, which was used as the reporter ion. Interestingly, the PMP specific stationary phase pentafluorophenylpropyl was used, and good separation was observed. Using this method, signals characteristic of CDGs were observed in the urine of CDG patients [43]. An alternative strategy for relative quantification using labeling with alanine and D₆-labeled alanine was reported for N-glycan analysis [45]. When the D₆-alanine labeled standards were used, better CVs of less than 5% were obtained compared to quantitation without heavy labeling. 42 N-glycans were observed to be differentially expressed in serum from rabbit on either a high cholesterol diet (n=5) or a control diet (n=5) [45].

Permethylation has been widely applied in mass spectrometry analysis of released glycans to stabilize sialic acid linkages and improve fragmentation efficiency. MRM was recently employed for the analysis of permethylated N-glycans released from serum proteins from two cohorts of HCC cases and liver cirrhosis controls [46]. Next to global profiling using LC-MS, transitions were developed for 117 N-glycans on QQQ-MS. using MRM, 15 glycans were shown to be differentially expressed in one of the two cohorts, of which 11 overlapped with the global profiling method. None of glycans was identified to be differentially expressed in both cohorts.

To allow absolute quantitation of oligosaccharides, standards are needed. There are some standards that are commercially available (mostly N-glycans and milk oligosaccharides), but they are typically very expensive and cover only a small range of the glycan heterogeneity. To overcome this, we determined the average response factor of seven HMO standards and applied that to all milk oligosaccharides in a mother's milk [44]. The overall total HMO amount thus calculated was in good concordance with the actual amount, indicating that such a generalization may provide results that are quite accurate.

Conclusions and future prospects

Multiple reaction monitoring is a technique that is widely acknowledged for its accurate quantitation, which is an area in glycomics that could still benefit from further methodological improvements. MRM can be used only after the identification of a compound in a given biological process which is typically performed using other mass spectrometry based detection techniques such as time-of-flight or ion trap mass spectrometry. Quantitation with validation of the compound is then performed using MRM. A benefit of this strategy is that it allows for the identification of the typical charge state and characteristic fragment ions during the identification process, which can then be used in the development of the MRM transitions, thereby enabling faster method development of MRM analysis.

Application of MRM in the field of glycomics can be subdivided in three area's: quantification of glycoproteins, glycopeptides and oligosaccharides, with each of them having their intrinsic difficulties. When quantifying glycoproteins, the selection of glycopeptides for monitoring is of importance [47]. Most importantly, one should take the presence of amino acid modifications such as oxidation of methionine and deamidation of

glutamine and asparagine into account. If possible, peptides containing such modifications should be avoided, as their quantitation is typically less accurate.

The choice of protease also largely influences the MRM analysis of both glycoproteins and glycopeptides [48, 49]. Typically tryptic digestion is used for the generation of peptides or glycopeptides, but, due to the uneven distribution of lysine and arginine residues this may result in long peptides that are not readily detectable by MS. Furthermore, when analyzing glycopeptides, such long peptides can result in doubly glycosylated glycopeptides thereby complicating the resolution of the glycosylation profile. This was observed for one of the tryptic glycopeptides from haptoglobin, and one of the reasons why it was decided to monitor the glycosylation profile on one of the other glycopeptides [35]. However, such a strategy cannot be typically employed, as the glycosylation pattern is different by site of glycosylation. Therefore the use of alternative proteases should be considered, as was illustrated by Maury et al. who used Glu-C to generate O-GlcNAcylated glycopeptides [41].

Labeled standards are often used in mass spectrometry to increase the accuracy of both absolute and relative quantitation. Such labels are now often multiplexed and isobaric, but provide characteristic fragment ions that differ in mass by 1 da (e.g. [50]). However, due to the large inclusion windows of the quadrupoles (typically at least 0.5 da), these strategies are not suitable in MRM. Alternative strategies may include the use of non-isobaric labeling [51], and this has been applied in MRM. A D₆ labeled alanine was used for the labeling of glycans, resulting in a m of 6 da, which was shown to be largely sufficient in MRM [45]. Similar approaches for peptide and glycopeptide analysis are available and would be suitable for use in MRM-based quantitation.

Compared to peptides and metabolites, the MRM of glycans and glycoconjugates is still in its infancy. Thus far, there have been limited clinical applications of MRM in the glycomics field; however, the initial studies using MRM have shown its potential for clinical applications and especially in the validation of earlier findings. While the lack of standards, both glycans and glycoconjugates, is a major issue, which hinders the field, the applications of MRM within the field of glycomics are expected to increase as its utility becomes better known. It is therefore anticipated that MRM will play a more profound role in clinical glycomics applications in the near future.

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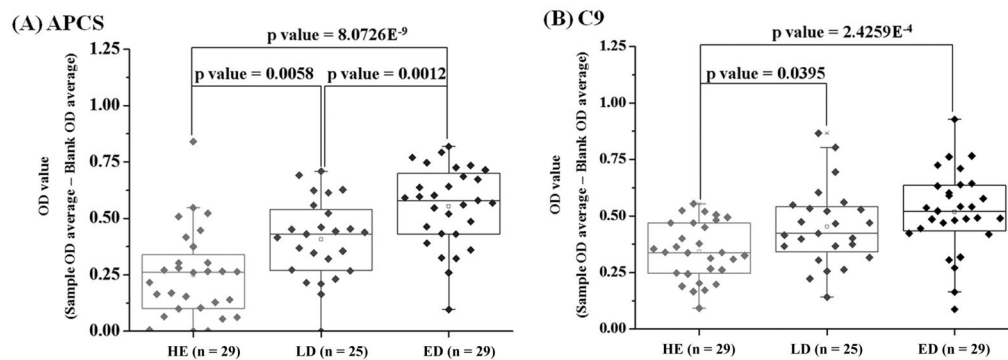


Figure 1. Differential levels of fucosylated PON1 in the serum of small cell lung cancer patients Patients had either limited disease (LD) or extended disease (ED) and were compared to healthy controls (HE). Fucosylated proteins were enriched using the AAL lectin, followed by tryptic digestion and MRM based protein quantification. Significant results (including PON1) were then validated using hybrid AAL ELISA, of which the intensities are represented. The P-values were calculated using nonparametric Kruskal-Wallis analysis of variance. Reprinted from [28].

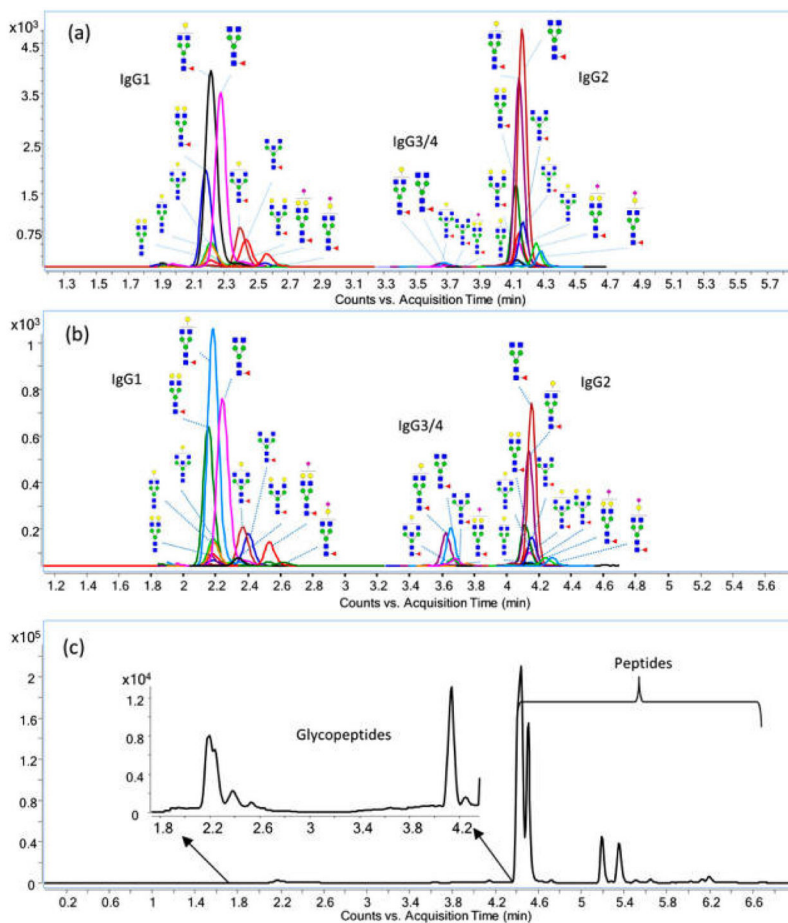


Figure 2. MRM chromatograms of immunoglobulin G and its glycopeptides

Depicted are the chromatograms of (a) glycopeptides in a tryptic digest of IgG standard (b) glycopeptides in a tryptic digest of serum and (c) peptides and glycopeptides from a tryptic digest of serum. One MRM transition was monitored for each glycopeptide with either the HexNAc or HexHexNAc oxonium ion as the reporter ion; two MRM transitions were monitored for each peptide. Reprinted with permission from [34] Copyright 2013 American Chemical Society.

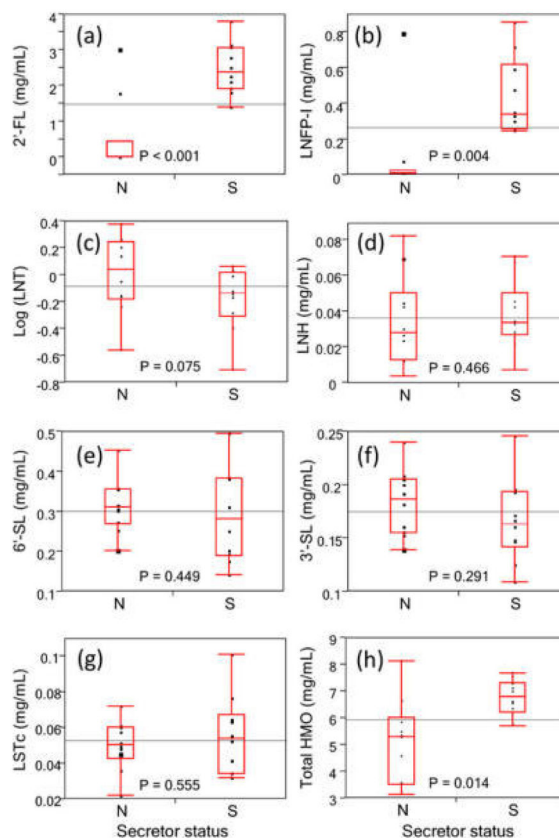


Figure 3. The profile of human milk oligosaccharides in a mothers milk is dependent on her secretor status

Shown are box-whisker plots for secretor mothers (S) and nonsecretor mothers (N). Plots are depicted for (a) 2'-FL, (b) LNFP-I, (c) LNT, (d) LNH, (e) 6'-SL, (f) 3'-SL, (g) LSTc and (h) Total HMO content, and the reported P-values are from one-tail Student t-test unless specified. Values were obtained using integrals of MRM signals of native reduced HMO in the positive ionization mode. Reprinted with permission from [44]. Copyright 2014 American Chemical Society.