Published in final edited form as:
Anal Chem. 2014 January 7; 86(1): 196–212. doi:10.1021/ac403969n.

Oligosaccharide Analysis By Mass Spectrometry: A Review Of Recent Developments

Muchena J. Kailemia[1], **L. Renee Ruhaak**[2], **Carlito B. Lebrilla**[2], and **I. Jonathan Amster**[1,*]

[1]Department of Chemistry, University of Georgia, Athens, GA 30602

[2]Department of Chemistry, University of California at Davis, Davis, CA 95616

INTRODUCTION

Carbohydrates are central players in a number of important biological processes including cell signaling, cell adhesion, and the regulation of biochemical pathways. Unlike nucleic acids and proteins, the biosynthesis of carbohydrates is not template-driven. They occur in nature as heterogeneous mixtures, often of high complexity. There is no method for amplifying the amount of a carbohydrate analogous to overexpression for proteins or polymerase chain reaction for nucleic acids, and so carbohydrate analysis is typically limited to what can be obtained from natural sources, thus the researcher must cope with small quantities of heterogeneous material. Mass spectrometry has high sensitivity and is tolerant of mixtures, and is a natural choice for the analysis of this class of molecules.

Compared to advances in protein analysis, progress in the application of mass spectrometry to carbohydrates has evolved somewhat slowly, principally because carbohydrates are a more challenging set of targets for structural characterization. In contrast to proteins, there is no database containing an inclusive and closed set of sequences representing all possible carbohydrate structures. The characterization of carbohydrates relies upon obtaining the full details of structure from the mass spectrum. Subtle differences due to isomerism or chirality can produce molecules with very different biological activities, making complete structural analysis even more demanding.

Mass spectrometry methodologies and technologies for biomolecule analysis continue to rapidly evolve and improve, and these developments have benefited carbohydrate analysis. These developments include approaches for improved ionization, new and improved methods of ion activation, advances in chromatographic separations of carbohydrates, the hybridization of ion mobility and mass spectrometry, and better software for data collection and interpretation. It thus seems timely to examine how these developments affect carbohydrate analysis. This review covers developments in the application of mass spectrometry to the analysis of carbohydrates, with an emphasis on work that has occurred from January 2011 through October 2013. The coverage is not mean to be exhaustive, but rather focuses on significant developments that, in the opinion of the authors, have advanced the field.

IONIZATION

The most widely used ionization methods for oligosaccharides are matrix assisted laser desorption/ionization (MALDI)[1] and electrospray ionization (ESI).[2] They impart little

*address for correspondence: Department of Chemistry, University of Georgia, Athens, GA 30602-2556, Phone: 706-542-2726, jamster@uga.edu.

energy to the sample, producing less fragmentation during the ionization process compared to methods previously used for ionization of carbohydrates, such as fast atom bombardment (FAB). Ions can be generated either in positive or negative ion mode, depending on the nature of the sample. Oligosaccharides containing acidic groups (sulfate, carboxylate, or phosphate) are readily analyzed using negative ion mode. Both ionization modes are used for native oligosaccharides. Chemical methylation (permethylation) of –OH, -NH_2 and -COOH groups in which a hydrogen atom is replaced with a methyl group enables uniform ionization for both acidic and basic oligosaccharides.[3] Methylation improves LC analysis by reducing the polarity of glycans, making their separation more reproducible and quantitative. Derivatized oligosaccharides display different fragmentation patterns by MS/MS analysis compared to their underivatized counterparts. Alkali adducted methylated oligosaccharides produce both glycosidic and cross-ring fragments by MS/MS, yielding fine structural details.[4–10]

MALDI Analysis

To generate ions by MALDI, the sample is dissolved by an organic solvent, mixed with a solution of a matrix, dried and then spotted on a MALDI target. The dry mixture spot is then irradiated using a ultraviolet laser and the matrix absorbs and transfers some of the energy to the analyte which ionizes.[11] Detailed information about the application of MALDI to glycan analysis, including matrices that are of particular use for carbohydrates, can be found in a comprehensive review by Harvey.[11] MALDI, compared to ESI, has higher sensitivity for glycans, ionizes well even at higher mass range, and it is more tolerant to contaminants. Spectra from this method are less complex than ESI spectra because a majority of ions generated in both negative and positive mode are singly charged through protonation or deprotonation. Singly charged ions are also formed as adducts with alkali or alkaline earth metals, and these kind of ions have been found to generate useful fragment ions during tandem mass spectrometry analysis.[4] MALDI imparts more internal energy into the analyte than does ESI, and can cause in-source fragmentation of labile groups such as sulfates, phosphates, or sialic acids.

Permethylation (described above) stabilizes the labile bonds of acidic groups in glycans and glycosylated peptides making them more amenable to MALDI ionization. It is difficult to couple MALDI with online separation techniques, as methods utilizing liquid matrices have been reported, but have not been found to have sufficient sensitivity or practicability. However oligosaccharides can be separated offline and subsequently analyzed by MALDI.[5,6,9] Recently several research groups have developed methods aimed at improving MALDI ionization and some of them are highlighted here. Incorporating salts that contain anions such as NO_3^- and Cl^- was recently found to improve ionization of neutral N-glycans in negative ion mode, and to generate structurally-informative product ions upon tandem mass spectrometry.[12] Other anion complexes, such as I^- and HSO_4^-, produce abundant [M +anion]$^-$ peaks, but do not yield useful fragments.

The choice of the matrix has a profound effect on the type of ions that are generated by MALDI.[13] Conventional MALDI relies on dried droplet and thin layer methods to generate ions. Recent work has shown that incorporating diamond nanoparticles (DNPs) into the matrix can assist in enhancing the underivatized carbohydrate signal even in the presence of protein.[14] These results were obtained by sandwiching the DNPs between the matrix and the sample containing a 1:1 mixture of protein and carbohydrate.[14]

Improved sensitivity, better quantitation, and more structurally useful tandem mass spectra of N-glycans have been obtained through labeling using on-target methods, for example by reductive amination using 3-aminoquinoline.[15] Anion doped liquid matrices have also been found to improve the sensitivity of N-glycans.[16] A frozen aqueous mixture of carbohydrate

and matrix has also been found to improve MALDI spectra. An aqueous/acetonitrile solution of the carbohydrates and 2,5- dihydroxybenzoic acid matrix frozen at 100K was ionized by UV-MALDI.[17] Enhanced sensitivity and reduced fragmentation from post source decay of oligosaccharides was observed when using the frozen mixture compared to conventional dry matrix.[17]

ESI analysis

With ESI, analyte ions are generated by passing a dilute solution (1–10 μM) through a thin diameter needle placed near the MS inlet capillary, at a potential of 1–4kV. The potential difference between the tip and the capillary and high temperature generate fine charged droplets and then drying gas vaporizes the solvent from the ions as they are aspirated into the mass spectrometer.[18] Both positive and negative ions can be generated using this method, and multiple charging is generally observed. The pH of the sample and the presence of salts have a profound influence on the formation of molecular ions and their anionic or cationic adducts. ESI couples well with online liquid flow separation methods like HPLC or capillary electrophoresis (CE) techniques.

Generally, ESI sensitivity decreases as the mass of the glycan increases. Conventional ESI is known to be less effective for neutral oligosaccharides due to poor ionization efficiency. Smaller sized droplets obtained in both static or flow nano-electrospray increase the sensitivity and show increased tolerance to salts and other contaminants compared to conventional electrospray.[4,19] Permethylation and reducing end modifications such as reductive amination and hydrazine tagging helps in LC separations and improves the sensitivity for both neutral and acidic oligosaccharides. Permethylation also enables acidic oligosaccharides to be analyzed in positive mode where they are detected as metal adducts.[7,20]

Derivatization of N-linked gycans using hydrophobic reagents via hydrazone formation have been shown to increase detection sensitivity [21] and improve the efficiency of chromatographic separations. A simple and faster methylation method for heparin disaccharides that overcomes some of the challenges associated with the current glycosaminoglycans methylation procedure was recently reported.[22] The addition of ionic liquids 2, 5-dihydroxybenzoic acid butylamine (DHBB) and α-cyano-4-hydroxycinnamic acid butylamine (CHCAB) have been found to enhance the ESI mass spectra of maltotriose polysaccharides. [23]

ION ACTIVATION

The fine details of oligosaccharide structure may have a profound effect upon their biological function. Progress has been slow in obtaining these structural details using tandem mass spectrometry due to the heterogeneity and structural complexity of naturally occurring carbohydrates, a result of their non-template driven biosynthesis. Recent developments in ionization and fragmentation methods are revealing more details about oligosaccharides structure, thus increasing the understanding of these essential biomolecules.[5,6,10,24–26]

For the purpose of mass spectrometry analysis, oligosaccharides are most often ionized by electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI). Compositional information can be derived from a single stage of MS, particularly with a high resolution, accurate mass measurement. The details of the structures of oligosaccharide molecules are obtained by tandem mass spectrometry (MS/MS or MS^n). In tandem mass spectrometry, a molecular ion is selected by a first stage of MS, undergoes activation and fragmentation, and the product ions are analyzed to provide information about the sequence,

monosaccharide compositions, linkages and locations of various modifications.[5,6,10,24,25] MS^n approaches can distinguish closely related isomeric structures, for example those that vary by linkage positions.[27] Such an approach to structure analysis requires considerable expertise in selecting the appropriate product ions for further stages of tandem mass spectrometry, which has hindered the widespread application of MS^n for carbohydrates. However, there are efforts underway to automate this approach for high throughput applications.[28]

Low energy fragmentation

There are two broad categories of tandem mass spectrometry techniques that have been applied to oligosaccharide analysis.[29–34] The first category comprise of threshold activation methods, including low energy collision induced dissociation (CID) [29] and infrared multiphoton dissociation (IRMPD).[30] CID is the most commonly employed fragmentation method, and has been implemented on many different MS platforms. However, CID ruptures the weakest bonds (also true for IRMPD), which can be detrimental to glycopeptides analysis, or for the analysis of oligosaccharides with labile modifications. Recently, methods have been developed that can improve the utility of this method by modifying the ESI spray solutions, derivatization, for example, by methylation or acetylation, and also coupling the labile groups with metals.[26,35–38]

Low energy CID fragmentation of positively charged ions, particularly native N-linked glycans, favor glycosidic fragmentation and may cause residue rearrangement which can lead to the wrong structural assignment.[39] Metal adducted oligosaccharides ions do not undergo rearrangement and provide more fragment ions, including cross-ring fragments which provide more detailed information about the oligosaccharides.[10,39] For gangliosides, both negative and positive precursor ions provide extensive fragmentation that complement each other.[8,40] Negative ionization and MS/MS has also been shown to produce informative fragment ions for acidic biomolecules especially glycosaminoglycans and other acidic glycans containing sialic acids.[41] For underivatized neutral oligosaccharides, fragment ions obtained from positive and negative mode may complement each other, enhancing structural assignment.[42,43]

Electron aided fragmentation

A second category of ion activation is electron based methods.[31,32] These methods entail the transfer of an electron to or from a selected multiply charged molecular ion, yielding a radical ion which then undergoes fragmentation. These methods are gaining attention because they often result in backbone fragments that give detailed structural information about peptidoglycans and oligosaccharides, at the same time preserving labile modifications that would otherwise be lost using threshold methods. These methods include electron capture dissociation (ECD),[31] electron transfer dissociation (ETD),[33] electron detachment dissociation (EDD),[32] negative electron transfer dissociation (NETD),[34] and negative ion electron capture dissociation (niECD).[44] EDD and NETD are suited for multiply charged negative ions especially from acidic oligosaccharides or glycopeptides.[45,46–49] ECD and ETD are used to fragment multiply positively charged ions and they have been useful for mapping sites of glycosylation from glycopeptides by producing backbone fragments while retaining the glycan within the peptide chain.[50,51] niECD is a recently reported electron based fragmentation method in which negatively charged ions capture ~5 eV electrons and increase their charge state, in contrast to the aforementioned methods of EDD, NETD, ECD, and ETD, which lead to a reduction in charge state. The presence of charge-increased ions confirms electron capture, and these radical ions initiate fragmentation similar to the ones observed in ECD/ETD. This method requires a zwitterionic structure, presumably for electron capture by the positively charged region of the ion. niECD produces extensive

backbone fragmentation and has been applied to characterize peptides [44] including O-sulfopeptides.[52] Although it has not yet been applied to carbohydrates, it would appear to have utility for negatively charged oligosaccharides, such as glycosaminoglycans.

The Domon and Costello nomenclature [53] is widely accepted as a convenient way to present the details of carbohydrate fragmentation. Two major types of product ions are produced by the fragmentation of oligosaccharides, those resulting from glycosidic fragmentation (B, Y, C and Z) and those from cross-ring fragmentation (A and X). The ions that retain charge on the reducing end are X, Y, and Z while the ones that retain the charge on the non-reducing end are A, B and C. Internal fragments may also be produced, but these are generally less informative regarding structure. A complete set of glycosidic fragments give details about the sequence, monosaccharide compositions and branching while cross-ring fragments locate modifications and determine the position of glycosidic linkage within individual residues.[10]

Several recent reviews address tandem mass spectrometry of oligosaccharides [5,10,24,54] and specific classes of oligosaccharides, such as GAGs,[55] glycopeptides,[56] O- and N-linked glycans,[9,25,26,57] and milk oligosaccharides.[58] This section of the review will highlight some of the recent developments in ion activation methods for the last two years focusing on sulfated oligosaccharides.

MS/MS of GAGs

Glycosaminoglycan (GAG) sequencing has predominantly relied on tandem mass spectrometry of short GAG chains resulting from enzymatic or chemical depolymerization (bottom-up). Recently, the first top-down MS/MS assignment of intact glycan chains from a GAG proteoglycan was obtained using FTICR and FT-orbitrap data.[59] The O-linked glycans were released from the proteoglycan, bikunin. These full-length glycans are a complex mixture, which have been characterized previously by mass spectrometry and were known to have a modest degree of polymerization (dp20–dp45) and a low degree of sulfation.[60] The glycan mixture was resolved partially by capillary gel electrophoresis, and the fractions were amenable to MS/MS analysis. One of the interesting observations was that CID yielded useful MS/MS data, contrary to the general observation for a GAG glycan.

This was a result of the low degree of sulfation, which allowed the charge state of each precursor to be greater than the number of sulfo modifications within a chain. A particularly surprising result of this analysis is that each composition yielded a single sequence, and that there was a conserved pattern of sulfation modifications among all the glycan chains that were examined. This was unexpected since it was believed that GAGs modifications take place in a random manner during their biosynthesis. This important work provides scientists and medical researchers with a new understanding of these vital biomolecules.[59]

It is believed that a similar approach may be applied to other more complex GAGs to learn whether they also exhibit a simple pattern of sulfate and acetyl modifications. However, more complex chains would pose challenges in separation and CID analysis due to presence of a greater number of compositions, a higher degree of polymerization, a higher level of sulfation and more alkali metal-hydrogen heterogeneity. Having more compositions with close structural features will introduce separation issues, while higher sulfation often leads to unproductive SO_3 loss. Higher alkali metal-hydrogen heterogeneity will lead to reduction of signal and isolation issues due to overlap of multiple peaks within the spectrum. Previous reports have shown that having charge state equal or higher than the number of sulfates affords structural informative fragments and reduces SO_3 loss.[47,61]

Another biologically relevant aspect of GAG structure is the C5 uronic acid stereochemistry. There have been of studies directed at determining uronic acid stereochemistry in different classes of GAGs from their MS/MS data. EDD was found to produce unique fragments for distinguishing glucuronic acid (GlcA) from iduronic acid (IdoA) in heparan sulfate tetrasaccharides.[46] In a more recent work, EDD was combined with principal component analysis (PCA) and used to differentiate the uronic acid stereochemistry of four synthetic heparan sulfate GAG diastereomers, varying only in the uronic acid C_5 stereochemistry. The developed method explored the possibility of using PCA to quantitate the abundance of two epimers in a binary mixture.[62] CID has also been used for distinguishing chondroitin sulfate A (CS-A) and dermatan sulfate (DS), which differ only by their uronic acid stereochemistry.[63] More recent CID work utilizing MS^3 showed that glycosidic fragments were more diagnostic of uronic acid stereochemistry than the ones obtained by MS/MS.[64] EDD fragmentation in combination with statistical analysis has also been used to establish unique product ions for distinguishing GlcA from IdoA.[65] These analytical methods could potentially be utilized to assign uronic acid stereochemistry of CS or DS oligomers and to quantify the relative amount of each in the mixture.

Even with many ion activation methods for biologically useful glycosaminoglycans (GAGs), the structural characterization of highly sulfated heparin and heparan sulfate has lagged behind other classes of GAGs due to the increased lability of sulfo modifications as the number of such modifications increases in GAG oligomers.[66] Increasing the deprotonation of the sulfate groups helps to stabilize them during ion activation, but for densely sulfated GAGs charge-charge repulsion prevents the possibility of ionizing every sulfate groups. Another way to stabilize these acidic groups is to replace protons with metal cations, but until recently, such an approach has had only moderate success in improving MS/MS data.[36] Recently, both charging and Na^+/H^+ exchange was used to ionize all the acidic groups within the highly sulfated pentasaccharide, Arixtra,[37] as well as in longer, highly sulfated GAGs oligomers.[67] Full deprotonation of the ionizable groups (sulfates and carboxylates) was found to make the sulfate modification stable during CID experiments, producing complete sets of both glycosidic and cross-ring fragment ions which located all SO_3 modifications within the highly sulfated chains. This approach will be useful for other highly sulfated GAGs, such as those with specific protein binding behavior.

EDD has been proven to be also very useful for analysis of N-glycans and other oligosaccharides.[48] EDD of chloride-adducted neutral and sialylated species was found to generate more cross-ring and glycosidic fragment ions compared to the ones obtained from EDD of deprotonated molecular ions.[49] Positive ion mode electron based methods can also be used for sulfated glycans. Recent work demonstrated the efficacy of ECD for sulfated carbohydrates complexed with divalent metal ions. When complexed with Ca^{2+}, kappa-carrageenan sulfated oligosaccharides containing one to four sulfate groups produced fragment ions that established the position of the sulfates within the chain residues.[68]

Photodissociation

Ultraviolet photodissociation (UVPD) has been shown to be useful for fragmenting sialylated oligosaccharides and other glycans.[69] Photodissociation at 193 nm in a linear ion trap successfully fragmented deprotonated sialylated oligosaccharides and glycans from fetuin. Compared to CID which produced mostly glycosidic fragments, UVPD produced an extensive series of cross-ring cleavage ions as well as sialic acid residue triol site specific fragments. Investigation of the UVPD fragment ions of doubly deprotonated molecular ions indicated electron photodetachment could be responsible for the observed extensive fragmentation, similar to electron based methods.[70]

MS/MS of anion complexes

In contrast to negatively charged ions, neutral N-glycans have a tendency to produce positively charged molecular ions that produce mostly glycosidic fragments, and thus do not provide the structural information required to determine branching. Efforts have previously been made to generate negative ions from these molecules using ESI by adding salts with anions that might complex with a carbohydate, such as ammonium nitrate. [9] Nitrates are preferred because of their stability and they have been found to show better sensitivity than the $[M-H]^-$ ions. In order to tap the benefits associated with both MALDI ionization and fragmentation of N-glycans in negative mode,[10,71] a more recent work used negative charged anions like SO_4^-, NO_3^-, and Cl^- to dope the 2,4,6-trihydroxyacetophenone (THAP) MALDI matrix and help to generate negatively charged molecular ions. CID of these ions on a MALDI-LIFT-TOF/TOF mass spectrometer generated abundant fragment ions especially from nitrate adducted molecular ions $[M-NO_3]^-$. During fragmentation, first HNO_3 is eliminated, and the resulting $[M-H]^-$ ion fragments to produce structurally informative ions. The work was applied to glycans varying in length from dp 7–13, and the results were comparable to those obtained using ESI-CID on a Q-TOF instrument.[12] In related work, the same method was coupled with an ion mobility separation which helped with sample clean up and isomer separation.[71]

N-linked and O-linked oligosaccharides

Electron-based activation produces abundant glycosidic and cross-ring fragments that provide detailed structural information about the glycans, and in addition retains labile modifications such as sulfation, and also retains glycans within glyproteins or glycopeptides during ion activation. Details of the effect of electron energy on fragmentation have been previously reported for glycosaminoglycans,[72] and similar efforts have been applied to other families of glycans. Permethylated maltoheptaose was used to study the effect of electron energy on the fragmentation of metal adducted molecular ions. For electrons having 1.5 eV of kinetic energy, electron capture dissociation (ECD) took place, while at an intermediate energy of 9 eV hot ECD (HECD) is observed. Electron excitation (EED) fragmentation occurred at 14eV energies. Li^+ adducted molecular ions were found to produce more structural information than larger metal cations (K^+, Rb^+, or Cs^+) that produced less informative fragment ions and more metal losses. Li^+ was thus used for the fragmentation of permethylated N-linked, $Man_5GlcNAc_2$ (Man: mannose, GlcNAc: N-acetyl glucosamine and EED was able to identify five of six glycosidic linkage positions and provided more structural information compared to low energy ECD and CID.[73]

Blood groups Le^a, Le^x, Le^b, and Le^y, which vary in the glycan modification of the proteins presented on the surface of red blood cells, have been characterized using tandem mass spectrometry.[43] Recently, a method employing negative ESI and CID was developed to characterize blood-group A-, B-, and H- determinants on type 1 and type 2 and type 4 globosides. This work identified ^{0,2}A-type cleavages as diagnostic fragment ions which are important for assigning blood-group and chain types. These diagnostic fragments only occur under the negative-ion conditions for reducing sugars but not for reduced alditols or under positive-ion conditions. The method is useful for other reducing free oligosaccharides obtained from polysaccharides by acid hydrolysis, glycolipids by endoglycoceramidase or by ozonolysis or N-glycans released by peptide-N-glycanases or by hydrazinolysis.[74]

Milk oligosaccharides have been analyzed using other methods such as CID and IRMPD.[38,75] Recent work which used ETD compared the results with other activation methods such as CID, and MS^3 by combining both CID and ETD in series on milk oligosaccharides. ETD generated various types of abundant cross-ring cleavage ions which enabled the clarification of different linkage types and branching patterns of the

representative milk sugar samples. This work shows that ETD can contribute substantially to confident structural analysis of a wide variety of oligosaccharides.[50]

It is challenging to map the sites of O-linked glycan modifications within a given peptide or a protein. Most often, the glycan is cleaved during ion activation and this hinders the efforts to locate the modified sites. Using a free electron laser (FEL) as a tunable IR light source, IRMPD has been used for the analysis of the protonated O- glycopeptide in which amino acid fragments b/y and glycan glycosidic fragments B/Y types were observed in the range of 5.7–9.5 μm. The spectrum of the sodiated glycopeptide showed major peaks of photoproducts of the B/Y type in the range of 8.4–9.5 μm. This work indicates that a tunable laser can help to generate product ions from both the peptide and the glycan, thus affording more structural details from O-glycosylated peptides.[76]

Combining multiple activation methods with complementary fragmentation products can increase the information content of MS/MS data for glycopeptides.[77] In a recent study, higher-energy collisional dissociation (HCD) was combined with ETD to analyze O-linked β-N-acetylglucosamine (O-GlcNAc) within peptides. HCD generated HexNAc signature ions while ETD produced sequence-informative peptide fragment ions while retaining the labile GlcNAc modifications necessary for establishing its location at the amino acid level. This method was used to characterize O-GlcNAc modified proteins enriched from HEK293T cells in which 83 sites of O-GlcNAc modifications were identified. This work shows that combining HCD and ETD increases the useful fragmentation necessary for identifying posttranslational modifications within proteins.[51]

QUANTIFICATION

The identity and the level of glycan expression within animal cells and tissues are known to affect physiological and pathological processes and to correlate with the health of an individual.[78] The level of expression of these vital biomolecules varies between individual species and they are known to change during the development and progression of many diseases.[78] In order to understand more about glycan expression and its impact on biological processes, it is important to perform quantitative analysis of glycans in cells and tissues in addition to structural characterization.[79] Methods of quantitative analysis of glycans are less established than those for proteins or peptides but considerable effort has recently been made in this research area.

Label-free quantitation

Both label-free and heavy isotope labeling are used for glycan quantification. Label-free methods have the advantage of experimental simplicity, while labeling methods potentially provide a more accurate result. With label-free methodologies, glycoconjugates are processed to detach the glycan from its protein and analyzed in their native state or after derivatization.[80] Samples from different sources can be compared by dividing the intensities of a selected glycan by the glycan total ion current. This method is susceptible to errors from differences in the ionization efficiencies of various glycans, instrument performance variability, and sample handling differences. Use of internal standards that have MS characteristics similar to the ions of interest can be used to standardize the procedure thus producing more reliable results.[80]

Another challenge to glycan quantification is in-source fragmentation, especially when using MALDI- MS, leading to incorrect interpretation of the results. Oligosaccharides containing acidic residues are particularly susceptible to this problem. The ionization efficiency of glycans can be improved by methylation, which allows both acidic and basic glycans to be analyzed simultaneously, and with uniform sensitivity.[5,80] Including a separation step, such

as LC or CE, improves the quantification process by separating isomers that cannot be differentiated my mass alone. Methylation and reducing end derivitization also improves LC separations since they make glycans more hydrophobic.[5]

A comprehensive label free procedure to identify and quantify milk oligosaccharides and other glycans in infant feces and urine using nano-LC-TOF MS was recently developed.[81] During this study, MALDI-FTICR was used for accurate mass measurement compositional analysis. Using deuterated human milk oligosaccharides (HMO) internal standards, the oligosaccharides were identified and quantified from mother-infant dyads and from infants consuming infant dietary formula and then monitored in the infant feces and urine.[81]

Similar approaches may be used to monitor changes in the profiles of ingested and excreted oligosaccharide for diagnostic purposes. A method using graphite carbon HPLC- MS indicated that milk oligosaccharides differ between lactating mothers and also vary during the lactating period.[82] An LC-MS/MS method was used to investigate and quantify glycosaminoglycans in the urine of patients with MPS I, II, and VI.[83]

Chip-based nano-LC/TOF-MS was used to profile and quantify N-linked gycans, to correlate with the prognosis of prostate cancer patients.[84] Nano-LC allows the analysis of small sample amounts (100 μL serum volumes), and generally improved sensitivity with minimal ion suppression. Differences in glycan abundance between patients was found to have a statistically significant correlation to their prognosis.[84] This method could be useful as a diagnostic tool and could be used to monitor the progression of diseases.

Isotopic labeling approaches

Chemical or metabolic labeling approaches incorporate a heavy isotope into a glycan for comparative analysis. These techniques reduce some of the issues associated with label free analysis such as instrument response and differences in ionization efficiencies. In chemical labeling approaches, heavy isotopes are introduced during methylation or reductive amination procedures. Differential deuteration or ^{13}C-labeling can be introduced by methylation reactions. CH_3I and CD_3I have been used in comparing N-glycans profiles for healthy and breast cancer patients using MALDI-MS.[85] Recently, a method was reported for isotopic labeling by methylation, with LC-ESI-MS for comparative analysis of N-glycans.[86] Methylation was carried out using CH_2DI and CD_3I heavy isotopes and CH_3I light isotope. The work compared N-glycans from several esophageal diseases and healthy samples and found that there are significant glycan profile differences between them. $^{13}CH_3I$ and $^{12}CH_3I$ was used to analyze mixtures of N-glycans derived from glycoproteins.[87]

A $^{13}CH_3I$ and $^{12}CH_2DI$ pair whose mass difference is only 0.002922 Da was used to analyze N-linked oligosaccharides from glycoprotein and human serum.[88] Due to the very small mass difference between the $^{13}CH_3I$ and $^{12}CH_2DI$ pair, high resolution mass spectrometry with more than $m/\Delta m=30,000$ resolution is required in order to resolve the two isotopic species. This method, called QUIBL for quantitative isobaric labeling, is particularly useful for quantitating isomers by tandem mass spectrometry. The precursor mass of the derivatized compounds have the same nominal mass, and thus undergo ion activation simultaneously, removing one significant source of experimental error in the quantitative measurement. The same isomer from two samples are distinguished and quantitated by comparing the abundance of the isobarically-labeled species, while isomers are distinguished by differences in product ions during MS/MS.

Using reductive amination, fluorophores or chromophores such as, 2-aminobenzamide (2-AB) and 2-aminopyridine (2-AP) can be attached to the reducing end of the glycan, to enable their UV detection.[3,20] Reductive amination is useful for N-glycans and other

oligosaccharides with a free reducing end. The release of O-glycans from proteins through β-elimination produces a hydroxyl group at the reducing end, which is not suitable for reductive amination. However, isotopic labeling can be achieved by using $NaBD_4$ during β-elimination to introduce deuterium labels.[89] O-linked glycosaminoglycans have been examined by enzymatic depolymerization to produce tetrasaccharides that have normal reducing ends. Reductive amination was then used to incorporate four differentially labeled aniline tags (D_0,D_4,D_8,D_{12}) that enabled simultaneous analysis of four chondroitin sulfate glycosaminoglycans samples using LC-MS and tandem mass spectrometry.[90] The isotope overlap due to the 4 mass unit difference between the tags requires deconvolution. This tetraplex stable-isotope coded tagging method allows four samples to be compared in a single experiment, which offers a considerable advantage over more conventional methods that compare only two samples at a time.

A method derived from proteomic technology, tandem mass tags, has recently been introduced to glycan analysis.[91] This glycan version is called stable isotope labeled carbonyl-reactive tandem mass tags (glyco-TMTs), and has been used to profile N-glycans in primary tumors and metastatic tumors in human colon carcinoma cells.[91] The reagents consist of a reporter and reactive end, linked by a mass normalizer. Two reactive end chemistries were examined, hydrazide and aminooxy, which form a hydrazine or oxime, respectively. The mass spectrum of the differentially labeled glycans have less spectral complexity than in a conventional labeling experiment, due to the isobaric nature of the labeled components. Ion activation during MS/MS releases a low molecular weight reporter ion, which can be used to quantitate the abundance of the glycans from each sample that is being compared.

A metabolic labeling method that takes advantage of the biosynthetic pathways of amino sugar residues was recently developed.[92] The isotopic detection of amino sugars with glutamine (IDAWG)[92] utilizes amide-^{15}N labeled glutamine as the sole source of nitrogen for hexosamines. The glycans expressed in cells grown under these conditions increase in molecular weight by one amu per amino sugar. This technique has been used for the analysis of both N-linked and O-linked glycans from murine embryonic stem cells. Since the ‘light’ and the ‘heavy’ glycans are produced by the same procedure, errors associated with the sample preparation are minimized.[92] This method can be used to study the glycans turnover to better understand the role of these molecules in biological systems.[80]

Triple quadrupole mass spectrometers can be used for selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) analysis to quantify known target analytes. A specific precursor is selected in the first quadrupole and fragmented in the second quadrupole and then specific fragment ions (transitions) are monitored in the third quadrupole.[93] These methods have been found to increase the signal-to-noise of the analyte of interest by reducing the chemical background, making it possible to identify low abundance glycans. More precise results are obtained from MRM analysis since multiple transitions are monitored unlike a SRM method which monitors a single transition.[94] More recently MRM was examined with energy-resolved structural analysis. Oligosaccharide oxonium fragment ions from a glycopeptide were monitored by collision induced dissociation (CID) over a wide range of energies, and a structure-unique fragmentation pattern was deduced. This method was found to distinguish purified immunoglobulin isomeric glycopeptides.[95]

A study describing site specific quantification of core fucosylated glycoprotein was developed recently.[96] Three optimum transitions were identified for quantification and seven glycopeptides from six proteins were quantified. [96] Another related study used MRM combined with liquid chromatography and tandem mass spectrometry to quantify

glycoproteins from both model glycoproteins and depleted human blood serum using oxonium ions as transitions.[97]

SEPARATIONS

Mass spectrometry is a powerful tool for glycosylation analysis, but it cannot distinguish structural isomers, which have the same mass, but not the same structural features. Therefore, mass spectrometry is often combined with a second mode of separation that is typically based on liquid chromatography (HPLC) but may also include electrophoresis (e.g. capillary electrophoresis). The mode of separation used depends on the type of sample analyzed (glycans, GAGs, glycolipids, glycopeptides or glycoproteins) and the sample preparation (e.g. types of enzyme or release procedure, native versus permethylation, and chromophore label). Thus far, there is no consensus on a single method that yields both identification and accurate quantitation in a rapid manner. Because of the structural diversity of glycans, there may be no such method even in the future. For this reason, the different modes of separation used in glycomics analysis will be discussed here individually.

Reverse phase separations

Reversed Phase (RP) chromatography has traditionally been widely used for the analysis of glycans and glycoconjugates. To allow retention of glycans on RP stationary phases, they have to be derivatized either as permethylated species or by the attachment of a single chromophoric tag for spectrophotometric detection. [20] Native oligosaccharides are generally not retained in RP stationary phases, however perderivatizing the compound or the addition of a single tag with greater hydrophobic character is often sufficient to allow separation. For example, it was recently found that the use of the aminobenzoic acid (2-AA) label for the analysis of released glycans from commercial antibodies using a C_{18} separation yielded better separation and peak shape compared to aminobenzamide (2-AB). [98]

A chemical derivatization method involving methylation of the –OH, –NH groups and replacement of the sulfite groups with acetyl or trideuteroacetyl groups followed by LC-MS n was used for structural characterization of GAGs recently.[99] This method showed improved RPLC separation and the ions are detected in positive mode. The method is found to determine the structures of heparin and heparan sulfate GAGs[100] as well as distinguishing CS/DS isomers.[99]

The separation of digests of glycosaminoglycans may also be performed using reversed phase. The use of C18 was recently reported for the analysis of AMAC-labeled disaccharides originating from heparan, HS and low molecular weight heparan. Up to 12 disaccharides were separated using a 60 minute gradient.[101] In a similar approach but with UPLC, up to 17 disaccharides originating from HS/HP, CS/DS and HA were separated. [102]

An alternative strategy for the analysis of GAG disaccharides is through reversed phase ion pairing (RPIP)-LC-MS. In RPIP-LC, lipophilic ions are added as ion-pairing reagents to the mobile phase to enhance the retention of charged molecules on the hydrophobic stationary phase. The method was reported for the separation of heparin/HS digests,[103,104,105] but was also successfully applied for the separation of amphiphilic sulfated oligosaccharides,[106] heparin-like glycosylaminoglycans (HLGAGs) [107] and glycol-split heparins. [108] RPIP separations strongly rely on the choice of ion pairing reagent as well as on the pH of the separation. Moreover, to allow coupling of RPIP to mass spectrometry, the use of volatile ion-pairing reagents in the mobile phase is required. Initial studies have demonstrated the separation of anomeric disaccharide standards using a traditional method with 20 mM tributylamine (TrBA) and 2.5 mM NH_4COOH at pH6.5. It was shown that the anomer separation can be diminished using a higher concentration of 30 mM TrBA at pH 3.5. [105]

In a different study, the use of TrBA, dibutylamine and pentylamine (PTA) as ion pairing reagents were assessed in conjunction with acetonitrile and methanol based mobile phases for the separation of LMW heparin.[103] PTA was found be superior to TrBA and dibutylamine when used in acetonitrile based mobile phases, yielding the best resolution. [103] It is anticipated that further optimization of the ion pairing parameters in the near future will further enhance the use of RPIP-LC for the separation of all types of glycosaminoglycans.

HILIC separations

Hydrophilic interaction chromatography (HILIC) has gained renewed interest for the analysis of glycans and glycoconjugates. Its use in glycomic analysis was recently reviewed.[109] Novel applications of HILIC stationary phases were described for the analysis of milk oligosaccharides,[110,111] galactooligosaccharides,[112] linear oligosaccharides from plant cell walls[113] and glycopeptides. [114] The technology has earlier been applied for the analysis of glycans[109,115] and glycosaminoglycans.[116] HILIC is not only used for analytical separations, but often also for SPE sample preparation, especially for the enrichment of glycopeptides. [117,118,119] The increased interest in the application of HILIC for glycomics analyses has further sparked the development of novel HILIC materials. Especially zwitterionic hydrophilic interaction chromatography (ZIC-HILIC), whereby both positively and negatively charged groups are attached to the analytical surface resulting in a relatively weaker interaction with charged analytes, has been reported in glycan analysis.[118,119,120] For example, recently the application of click chemistry to couple aspartic acid to a silica stationary phase, thus providing a ZIC-HILIC material, was evaluated for the purification of glycopeptides from a tryptic IgG digest.[118] It was shown that good recovery of glycopeptides was obtained, together with low levels of peptide contamination, especially compared to sepharose, a more traditional HILIC support.

The use of HILIC for the separation and analysis of N-glycans upon labeling with 2-AB is now widely accepted. Recently, this method has also been employed for the separation of bovine milk oligosaccharides.[111] Using exoglycosidases, the structures of 37 BMO could be confirmed, which revealed the separation of several structural isomers. In this work, a glucose standard is used to convert the retention time to standardized glucose values (GU). A method using 4-aminobenzoic acid ethyl ester (ABEE) labeled dextran hydrolysate was recently proposed to serve as an internal standard for the determination of the GU units. [121] This strategy was shown to provide more accurate GU values, since variations in the delivery of the LC gradient can be corrected for using the internal standard.

Graphitized carbon separations

Porous graphitized carbon (PGC) has also been recognized as a valuable stationary phase for the analysis of native oligosaccharides and small glycopeptides, [122] mostly because of the extensive separation of isomers that may be obtained. While initial methods for the separation of N- and O- glycans[123] as well as milk oligosaccharides[124] and GAG digests[125] have been described earlier, several novel applications of PGC have been described recently. First, a library describing the retention times, structure and CID fragmentation patterns of N-glycans derived from blood serum proteins was developed.[126] This library illustrates the separating power of PGC for glycans, and is an excellent resource for further glycan profiling studies. Moreover, PGC-LC-MS was recently described as the stationary phase of choice for N-glycan analysis.[57] Recent applications include the analysis of N-glycans from dried blood spot specimens[127] as well as the identification of candidate biomarkers for several types of cancer.[84,128] The separating power of PGC was recently also used for the development of a fluoride mediated fragmentation strategy[129], and the identification of fucosylated high-mannose type glycans in mushrooms.[130] PGC has also been applied for the analysis of O-glycans upon beta-elimination.[131,132]

The use of pronase for the non-specific digestion of glycoproteins, resulting in short-chain glycopeptides is a powerful method for the determination of site-specific glycosylation profiles. PGC has been shown to provide an excellent stationary phase for the analysis of such digests, as was recently reported.[132,133]

PGC is also widely applied for the analysis of milk oligosaccharides: Initially a library containing mass, retention time and fragmentation of neutral milk oligosaccharides was reported[134], which was recently complemented with a library for sialylated HMO[75] and bovine milk oligosaccharides.[135] The excellent separating power of PGC was used for the development of a quantitative method for HMO using a single quadrupole mass spectrometer.[82]

While the use of one specific type of stationary phase may already provide sufficient separating power, recent developments have shifted toward the coupling of two stationary phases, especially in the area of glycopeptides analysis. Initially, this has been performed using HILIC SPE prior to RP-LC-MS analysis. However, to allow for the detailed analysis of site-specific protein glycosylation, a 2-D separation comprising a HILIC column followed off-line by C18 was reported.[136] 57 glycopeptides were identified on haptoglobin and 14 for hemopexin, indicating very good coverage.

A reverse type coupling has also been attempted: glycopeptides from the CD44 fusion protein were first fractionated using RP-LC, followed by HILIC-LC-MS characterization.[114] While this method seems to have been successful, it is perhaps less predictable, as both peptides and glycopeptides are retained on RP material, but their elution is mostly depends on the peptide moiety. HILIC however retains glycopeptides much more strongly than peptides, and thus separates glycopeptides more completely from non-glycosylated peptides.

In general, several stationary phases can be used for the separation of glycans and glycocunjugates. The choice for a specific stationary phase often depends largely on the sample (GAG, glycans or glycopeptides) and the sample preparation. This is illustrated by several recent studies that have been designed to compare stationary phases. The separation of neutral oligosaccharides without derivatization was evaluated using high-pH anion exchange (HPAEC), RP, HILIC and PGC. PGC was shown, perhaps not surprisingly, to provide the best separation of isomeric structures, while HILIC was determined to best for the separation of compounds of different composition.[137] In a study to separate 2-AB labeled N-glycans from standard proteins, it was concluded that HILIC, RPIP and PGC each had individual advantages. [138] A comparison between ZIC-HILIC separation of 2-AB labeled glycans and PGC for the separation of reduced N-glycans from monoclonal antibodies showed that higher sensitivities were obtained using PGC, and that PGC provided excellent separation of isobaric glycans.[139] Interestingly, because PGC is effective for separating isomers, it is the best suited for coupling with mass spectrometry as the latter yields a second dimension of separation, namely mass. It is therefore expected that PGC will find even great use for the separation of native glycans and glycoconjugates.

ION MOBILITY

Ion mobility (IM) is a fast separation technique that is based on the interaction of the gas-phase ion with a buffer gas in a varying or fixed electric potential through a drift region. Analytes are ionized using standard methods and are separated based primarily on their shape and intrinsic charges, thereby yielding an additional level of structural detail. When combined with mass spectrometry, ions are separated and can be presented by shape (collision cross section) in one dimension and mass to charge ratio (m/z) in a second dimension. The early experiments were performed primarily on unique, home-built instruments, however, with the development of the first commercial ion mobility mass

spectrometery (IM-MS) instrument in 2006, the Synapt, the technology has become more widely available. This has sparked a large number of efforts in the application of IM-MS not only as an ion-chemistry tool, but also as an analytical platform for areas such as proteomics and glycomics. [140]

Spatial dispersion IM

There are two major approaches whereby ions may be separated using IM, either through space or time. High field asymmetric waveform ion mobility (FAIMS) also known as differential mobility spectrometry utilizes differences of ion mobility in alternating high and low electric field applied between two electrodes at atmospheric pressure. Ions are pushed through the FAIMS device by a carrier gas which could be pure or a mixture of gases. The applied field from an asymmetric, periodic waveform, disperses the ions within the device toward one of the electrodes, depending on their differential mobility, and they end up neutralized. When a small DC potential (compensation voltage, CV) is applied, ions of a particular differential mobility assumes a stable trajectory through the device and are thus detected.[141] FAIMS- LTQ Orbitrap was recently used for the separation of isomeric O-linked glycopeptides differing only at the site of glycosylation (GalNAc), and that were difficult to separate using RPLC.[142] The site of glycosylation was determined from ETD fragment ions obtained from separated peaks at a given CV.

In another study, FAIMS-FTICR was used to separate ESI generated isobaric charge state ions resulting from different length of chondroitin sulfate A GAGs.[143] Additionally, epimeric mixture of heparan sulfate tetrasaccharides differing only at one stereocenter was separated and their ions activated using EDD to obtain structural information. Multiple peaks obtained from a single species were found to be due to the ability of FAIMS to separate anomers. Alkylating the reducing end or using reduced species eliminated most of the extra peaks.[143] Since FAIMS separate ions spatially rather than temporally, it is a useful tool particularly when coupled to mass spectrometers in which multiple ion activation methods can be used to fragment separated molecular ions.

Time dispersion IM

Methods based on separation through time-dispersion have been widely applied for glycomics. These methods can either be performed using drift tube ion mobility (DTIM), where ions are separated in an electrostatic field by collisions with a buffer gas, or traveling wave ion mobility (TWIM), where ions are separated using a buffer gas in an electrodynamic field.

Because of the homogeneous electric field in DTIM instruments, kinetic theory can be used to readily determine the collision cross section (CCS) of an analyte based on the drift time. Conversely, the electric field in the TWIM instrument is not homogeneous, thereby limiting the use of this technology for obtaining gas-phase structure information. To alleviate this limitation, external calibrants, for which the CCS has been determined using DTIM instrumentation and which are of similar molecular identity, can be used to determine the CCS of analytes by TWIM.

IM-MS has been used to separate isobaric species belonging to different biomolecular classes. DTIM-MS has been used to examine the CCS of lipids, peptides, nucleotides and carbohydrates.[144] Because their respective CCS are sufficiently distinctive, the drift times have been used to differentiate the diverse classes of biomolecules.[144] These earlier findings suggested that IM-MS could be used for the analysis of carbohydrates from complex mixtures without the need for purification. The method was applied to a PNGaseF digest of RNAseB, where good separation of glycans from the protein was observed, [145] and to milk

where the separation of oligosaccharides from lipids was achieved.[145] The ability to obtain oligosaccharide compositional profiles from complex mixtures without pre-purification would provide an important advantage, as sample work-up is often responsible for loss or degradation of oligosaccharide structures.

In theory, gas phase separation of glycans holds potential for the differentiation of structural oligosaccharide isomers. Indeed initial studies on N- and O-linked glycans have indicated that some separation of isomers can be achieved, but the full potential of IM-MS for oligosaccharide isomer separation has not yet been determined. In recent years, several studies have been performed to further explore the potential role of IM-MS in oligosaccharide analysis.

The collisional cross sections of sodiated N-glycans, release from commercially available glycoproteins, were determined using DTIM-MS, to allow for the calibration of TWIM-MS instruments.[146] It was observed that the repeatability of the CCS for the N-glycans is very high, with a %CV of 1–2%. While the separation was not optimal, the results gave good indications that carbohydrate isomers can be distinguished using IM-MS based based on their CCS or drift time.

Released, native N-glycans from proteins were characterized using MALDI-TWIM-MS, and it was observed that THAP matrix in combination of an ammonium nitrate dopant provided good separation of analytes and matrix, and allowed for N-glycan fragmentation. Some separation of isomers of smaller N-glycans was obtained in the study, but often isomers were poorly resolved.[71,147]

The separation of permethylated mannose 7 (man7) isomers, obtained from RNAseB using DTIM in positive ionization mode provided a relatively broad drift time distribution with four local maxima. These four maxima are attributed to different isomers of man7. Using fragmentation studies, one of the maxima could be assigned, while the structures of the three other isomers could not be determined.[148] Pyridylamine (PA)-labeled N-glycans obtained from IgG were also examined for separation by IM-MS. Using TWIMS, the G1F isomers were separated with reasonable resolution. However, to simplify the IM separation, TWIMS-MS was coupled with a HILIC-HPLC separation, thus providing superior separation of isomeric glycans in under 15 minutes.[149]

While IM-MS for the analysis of carbohydrates is still in its infancy, its first application toward the discovery of cancer biomarkers was recently reported. Altered drift time profiles were observed for 9 of the 17 glycans in a study aimed at distinguishing healthy individuals from cirrhosis patients and individuals with liver cancer. Using principal component analysis, the subjects were well separated.[150] In a similar study by the same group, altered profiles for esophageal cancer were determined. [151] It has to be noted, however, that these results were not tested in a second sample set, which would be necessary to confirm the utility of the markers. [152]

While the majority of IM-MS research in glycomics has been dedicated toward N-glycans, GAGs and HMO have also been studied. Octasaccharides originating from heparin were measured using TWIM-MS.[153] Using standards, differential travelling times could be observed, indicating that it is likely that IMS can separate heparin-derived octasaccharides. However, no application to an actual heparin digest was reported. Similarly, the CCS of some HMO have been determined using DTIM-MS,[154] and separation of isomers of smaller HMO may be obtained using drift time.

Overall, it may be concluded that the use of IM-MS has potential for the rapid profiling of glycans directly from samples without pre-purification. Given that the gas-phase separations

are fast (milliseconds), such a method would be highly useful in high throughput profiling analyses, where just glycan compositional data is required. However, while IM has the potential for oligosaccharide isomer separation, this is currently not yet up to par with HPLC or CE methods. In particular, porous graphitized carbon stationary phase, which provides very good isomer separation [75,122,126,134] cannot yet be matched by IM. An advantage of the use of IM would be its very fast separation times, which would enable the analysis of glycosylation in a high-throughput fashion. Therefore, we strongly believe that further instrument developments, as well as application specific instrument settings, such as the use of CO_2 as the drift gas instead of N_2, which was recently shown to increase the resolution of the separation of the disaccharides sucrose, maltose, lactose and cellobiose, [155] would eventually allow for isomer separation of oligosaccharides. Such improvements would open up the use of IM for a large number of glycomics applications. A separate field of application that would benefit from the additional dimension of separation that is provided by IM, is glycoconjugate analysis. So far, very few studies have focused on the analysis of glycopeptides, glycolipids and other glycoconjugates using IM. Further exploratory studies towards this application are needed to evaluate the potential of IM in this field of study.

SOFTWARE

Mass spectrometry is currently the most effective technology for the analysis of glycans and glycoconjugates. To allow more efficient evaluation of the mass spectrometric data generated, software tools have been developed for the analysis of MS as well as MS^n data. The current state of the art of software tools for the evaluation of N- and O-glycans and glycopeptides was recently reviewed. [156] A separate review has focused on the available databases and the need for uniform codes for sharing glycomics data, mostly related to UPLC-fluorescence data.[157] The development of software tools in glycosylation research is currently undergoing rapid changes, yet remains insufficient, especially in the fields of GAGs, glycopeptides and other glycoconjugates. We focus this discussion on the tools and applications that have been released since the beginning of 2011.

Several methods for the evaluation of N-glycan data had already been reported prior to 2011, and the identification of N-glycans is typically not problematic, however some novel tools have recently been revealed. The Glycolyzer is a newer software tool that allows the user to annotate N-glycan MALDI-FTICR-MS mass spectra. The software integrates the Fourier transform, calibration and deisotoping prior to glycan annotation and integration.[158] Because the tool is focused on biomarker discovery, discriminant analysis may be performed to identify differential glycan signals. While several other tools have already been developed for the annotation of N-glycan mass spectra, the Glycolyzer software allows for the identification, integration and differential analysis combined, thus largely facilitating biomarker discovery.

The annotation of N-glycan MS spectra with glycan compositions is generally routine. New separation methods, however, have made it possible to resolve isomeric compounds thereby allowing the identification of complete structures. The evaluation of N-glycan data obtained using these hyphenated methods such as porous graphitized carbon (PGC)-LC-MS remains difficult. The challenge is to annotate hundreds if not thousands of MS spectra, which require deconvolution of multiply charged spectra, assigning m/z values and recognizing retention times. In addition, retention time drifts over multiple runs require the alignment of chromatographic peaks. To facilitate the data evaluation of PCG-LC-MS data reduction of N-glycan and other glycans are necessary. A reduced N-glycan library from human serum was recently constructed with complete and partial structures recognized by retention time, accurate mass and tandem MS.[126] A database with normalized retention times for HILIC-

separated 2AB-labeled glycans has previously been developed,[159] which was recently extended to include average masses.

A project was recently launched to build a database containing glycan structures, their origin, tandem MS spectra and chromatographic retention times, based on the existing glycomeDB database.[160] The database will contain both N- glycans and O-glycans. While the chromatographic stationary phase was not specified, it contains mainly HILIC separation data. Such databases can be extremely useful for the structural annotation of large sample sets, but chromatographic retention times depend on the sample preparation method, the stationary phase, the mobile phase and precise gradient. A lack of a specific separation protocol will likely prohibit precise identification of structures.

Identification of N-glycans in LC-MS/MS data provides a somewhat different challenge as annotation may be facilitated by the fragmentation spectra that are obtained. GlycanID is a software tool that was recently described for the identification of N-glycans as permethylated alditols. [161] The program first extracts MS and MS/MS features, matches different adducts in the MS mode and annotates the MS features with their respective MS/MS fragmentation, thus providing the most probable set of identified N-glycans. GlycanID was successfully applied for the identification of N-glycans released from IgG and cell surface proteins extracted from human fibroblasts. [161]

Glycan analysis is challenging in its own right, yet determining specific sites of glycosylation adds an additional level of complexity. MS/MS is required for determining sites of glycosylation in glycopeptides, but data analysis remains a considerable challenge. Two new software tools aimed for the evaluation of glycopeptides LC-MS/MS data have been reported recently. GlycoPeptide Finder (GP Finder) is a tool that was developed for the correct assignment of glycopeptides independent of the protease used (whether specific or non-specific).[162] A self-consistency scoring algorithm is used in combination with a targeted-decoy approach to determine the false discovery rate (FDR). The method was successfully applied to protein standards and non-complex mixtures of glycoproteins such as gel bands. A second tool that was recently developed for the assignment of glycopeptides is Glycopeptide search (GPS). Using indexed glycan databases, MS/MS spectra are paired with glycopeptides matches and a decoy library is used to determine the FDR. So far, this tool only supports tryptic digestions. Similar to GP Finder, this software requires a pre-defined protein sequence or a list of peptides. This is a drawback to the routine application of this software, as separate protein identification software, or maybe even an additional digestion and accompanying LC-MS/MS run is needed to provide the list of protein sequences.

Most software tools that are designed for N-glycan analysis could potentially also be used for the evaluation of GAG data, but need to be adapted specifically. Two software tools have recently been described for the targeted evaluation of GAG MS data. The first, Manatee, uses a pre-defined library of digested GAG compositions and empirical HILIC-LC retention times to extract the relevant glycan information.[163] GlycReSoft is a tool, which uses deconvoluted LC-MS data, out of which, after denoising, candidate glycans are extracted. Using a scoring algorithm, glycan compounds are identified.[164] The advantage of GlycReSoft over Manatee would be the inclusion of noise reduction and confidence measures, however Manatee provides faster analysis.

While it is clear that there is tremendous progress with respect to the development of software tools for the evaluation of glycomics mass spectrometry data, there are still some limitation that need be addressed in the next generation of tools. Most of the tools currently developed for N-glycan, O-glycan and GAG analysis are not universal to all sample

preparation methods and all analytical methods. We are aware that the development of tools often relies on libraries and/or fragmentation patterns, which are largely affected by the sample preparation method of choice. However, it would be highly desirable to have one software tool that would allow for the analysis no matter what sample preparation or analytical strategy used in the analysis. To this extent, GP Finder, which allows for the analysis of native N-glycans as well as non-specific glycopeptides, might currently be the most versatile.

In studies that are aimed toward the identification of glycans in a certain biological context, the use of the currently available tools is often sufficient. However, often the glycosylation profile of one sample or set of samples has to be compared to another, e.g. in the case of biomarker studies. Most software tools do not allow for the automated analysis of multiple samples in a batch-type manner, and thus the user is required to perform the data evaluation on a sample-by-sample basis. It would be highly beneficial if batch-processing would be more widely available in glycomics software tools.

CONCLUSIONS

Future progress in carbohydrate analysis will rely of the continued development of the analytical technologies discussed in this review. Given the heterogeneous nature of carbohydrate samples derived from natural sources, improvements in the methods for online separation of oligosaccharides will be crucial to advances in carbohydrate analysis. This includes not only chromatographic methods but also ion mobility, which is still an underexplored methodology with respect to carbohydrate analysis. Subtle structural differences in carbohydrates greatly impact their biological activity, and much work remains to be accomplished to be able to obtain this information by mass spectrometry. Developments in ion activation will continue to drive progress in the mass spectrometry characterization of carbohydrates, with advances in electron-aided methods of activation and photodissociation likely to produce the most impact in the future. While MS/MS methods are yielding remarkable levels of detail for carbohydrates, MS^3 and higher dimensional MS^n analyses can provide far more structural detail. In order for these approaches to find widespread application, it will be critical to develop automated methods for the selection of intermediates in the chain of reactions. Carbohydrate specific software developments are needed for the acquisition of mass spectra and processing and interpretation of these data.

Advances in mass spectrometry technology have enabled progress in its application to the analysis of carbohydrates. This has led to some remarkable accomplishments in the last two years, such as the sequencing of full length glycan chains in proteoglycans.[59] To a large degree, the field of mass spectrometry is driven by proteomics applications, which has led to the proliferation of higher resolution and more sensitive instrumentation, as well as the development of elaborate hybrid mass spectrometers capable of sophisticated tandem mass spectrometry measurements on complex mixtures. These developments also benefit the field of carbohydrate analysis. In many respects, carbohydrates present more challenging targets than proteins, given their heterogeneity, their subtle differences in structure, and the lability of modifications that are present. The need for improved methods to analyze carbohydrates will serve as a driver for further developments in mass spectrometry.

References

1. Hillenkamp F, Karas M, Beavis RC, Chait BT. Anal Chem. 1991; 63:1193A–1203A. [PubMed: 1897719] Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T. Rapid Commun Mass Spectrom. 1988; 2:151–153.
2. Fenn J, Mann M, Meng C, Wong S, Whitehouse C. Science. 1989; 246:64–71. [PubMed: 2675315]

3. Harvey DJ. J Chromatogr, B: Anal Technol Biomed Life Sci. 2011; 879:1196–1225.
4. Zhou W, Hakansson K. Curr Proteomics. 2011; 8:297–308. [PubMed: 22389641]
5. Zaia J. OMICS. 2010; 14:401–18. [PubMed: 20443730]
6. Zaia J. Mass Spectrom Rev. 2004; 23:161–227. [PubMed: 14966796]
7. Wuhrer M. Glycoconj J. 2013; 30:11–22. [PubMed: 22532006]
8. Park Y, Lebrilla CB. Mass Spectrom Rev. 2005; 24:232–264. [PubMed: 15389860]
9. Harvey DJ. Proteomics. 2005; 5:1774–1786. [PubMed: 15832364]
10. Harvey DJ. Proteomics. 2001; 1:311–328. [PubMed: 11680878]
11. Harvey DJ. Mass Spectrom Rev. 1999; 18:349–450. [PubMed: 10639030]
12. Domann P, Spencer DIR, Harvey DJ. Rapid Commun Mass Spectrom. 2012; 26:469–479. [PubMed: 22279023]
13. Rodrigues, JA.; Taylor, AM.; Sumpton, DP.; Reynolds, JC.; Pickford, R.; Thomas-Oates, J. Advances in Carbohydrate Chemistry and Biochemistry. Horton, D., editor. Vol. 61. 2008. p. 59-141.
14. Wu CL, Wang CC, Lai YH, Lee H, Lin JD, Lee YT, Wang YS. Anal Chem. 2013; 85:3836–3841. [PubMed: 23496007]
15. Kaneshiro K, Fukuyama Y, Iwamoto S, Sekiya S, Tanaka K. Anal Chem. 2011; 83:3663–3667. [PubMed: 21506551] Nishikaze T, Kaneshiro K, Kawabata S-i, Tanaka K. Anal Chem. 2012; 84:9453–9461. [PubMed: 23072501]
16. Nishikaze T, Fukuyama Y, Kawabata S-i, Tanaka K. Anal Chem. 2012; 84:6097–6103. [PubMed: 22725700]
17. Liang CW, Chang PJ, Lin YJ, Lee YT, Ni CK. Anal Chem. 2012; 84:3493–3499. [PubMed: 22443117]
18. Karas M, Bahr U, Dülcks T. Fresenius J Anal Chem. 2000; 366:669–676. [PubMed: 11225778]
19. Yang B, Solakyildirim K, Chang Y, Linhardt RJ. Anal Bioanal Chem. 2011; 399:541–557. [PubMed: 20853165] Fabrik I, Cmelik R, Bobalova J. Int J Mass spectrom. 2012; 309:88–96.
20. Ruhaak LR, Zauner G, Huhn C, Bruggink C, Deelder AM, Wuhrer M. Anal Bioanal Chem. 2010; 397:3457–3481. [PubMed: 20225063]
21. Walker SH, Lilley LM, Enamorado MF, Comins DL, Muddiman DC. J Am Soc Mass Spectrom. 2011; 22:1309–1317. [PubMed: 21953184]
22. Heiss C, Wang Z, Azadi P. Rapid Commun Mass Spectrom. 2011; 25:774–778. [PubMed: 21337639]
23. Chang YL, Lee YC, Yang WB, Chen CH. J Mass Spectrom. 2011; 46:367–375. [PubMed: 21438086]
24. Zaia J. Chem Biol. 2008; 15:881–92. [PubMed: 18804025]
25. An HJ, Lebrilla CB. Mass Spectrom Rev. 2011; 30:560–578. [PubMed: 21656841]
26. Khoo, K-H.; Yu, S-Y. Methods in Enzymology. In: Fukuda, M., editor. Glycomics. Vol. 478. 2010. p. 3-26.
27. Jiao J, Zhang H, Reinhold VN. Int J Mass spectrom. 2011; 303:109–117. [PubMed: 21686090]
28. Reinhold V, Zhang H, Hanneman A, Ashline D. Mol Cell Proteomics. 2013; 12:866–873. [PubMed: 23438731]
29. Cody R, Burnier R, Freiser B. Anal Chem. 1982; 54:96–101.
30. Little DP, Speir JP, Senko MW, O'Connor PB, McLafferty FW. Anal Chem. 1994; 66:2809–2815. [PubMed: 7526742]
31. Zubarev RA, Kelleher NL, McLafferty FW. J Am Chem Soc. 1998; 120:3265–3266.
32. Budnik BA, Haselmann KF, Zubarev RA. Chem Phys Lett. 2001; 342:299–302.
33. Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Proc Natl Acad Sci U S A. 2004; 101:9528–9533. [PubMed: 15210983]
34. Herron WJ, Goeringer DE, McLuckey SA. J Am Chem Soc. 1995; 117:11555–11562.
35. Zaia J, Miller MJC, Seymour JL, Costello CE. J Am Soc Mass Spectrom. 2007; 18:952–960. [PubMed: 17383193]

36. Zaia J, Costello CE. Anal Chem. 2003; 75:2445–2455. [PubMed: 12918989]
37. Kailemia MJ, Li L, Ly M, Linhardt RJ, Amster IJ. Anal Chem. 2012; 84:5475–5478. [PubMed: 22715938]
38. Xie Y, Lebrilla CB. Anal Chem. 2003; 75:1590–1598. [PubMed: 12705590]
39. Wuhrer M, Deelder AM, van der Burgt YEM. Mass Spectrom Rev. 2011; 30:664–680. [PubMed: 21560141]
40. Lee H, An HJ, Lerno LA Jr, German JB, Lebrilla CB. Int J Mass spectrom. 2011; 305:138–150. [PubMed: 21860602] McFarland M, Marshall A, Hendrickson C, Nilsson C, Fredman P, Månsson JE. J Am Soc Mass Spectrom. 2005; 16:752–762. [PubMed: 15862776]
41. Harvey DJ, Rudd PM. Int J Mass spectrom. 2011; 305:120–130.
42. Rohmer M, Baeumlisberger D, Stahl B, Bahr U, Karas M. Int J Mass spectrom. 2011; 305:199–208.Chai W, Lawson A, Piskarev V. J Am Soc Mass Spectrom. 2002; 13:670–679. [PubMed: 12056567] Harvey DJ, Bateman RH, Green MR. J Mass Spectrom. 1997; 32:167–187. [PubMed: 9102200]
43. Chai W, Piskarev V, Lawson AM. Anal Chem. 2001; 73:651–7. [PubMed: 11217777]
44. Yoo HJ, Wang N, Zhuang S, Song H, Håkansson K. J Am Chem Soc. 2011; 133:16790–16793. [PubMed: 21942568]
45. Wolff JJ, Amster IJ, Chi L, Linhardt RJ. J Am Soc Mass Spectrom. 2007; 18:234–244. [PubMed: 17074503] Wolff JJ, Leach FE, Laremore TN, Kaplan DA, Easterling ML, Linhardt RJ, Amster IJ. Anal Chem. 2010; 82:3460–3466. [PubMed: 20380445]
46. Wolff JJ, Chi L, Linhardt RJ, Amster IJ. Anal Chem. 2007; 79:2015–2022. [PubMed: 17253657]
47. Wolff JJ, Laremore TN, Busch AM, Linhardt RJ, Amster IJ. J Am Soc Mass Spectrom. 2008; 19:790–798. [PubMed: 18499037]
48. Adamson JT, Håkansson K. J Am Soc Mass Spectrom. 2007; 18:2162–2172. [PubMed: 17962039]
49. Kornacki JR, Adamson JT, Håkansson K. J Am Soc Mass Spectrom. 2012; 23:2031–2042. [PubMed: 22911097]
50. Han L, Costello CE. J Am Soc Mass Spectrom. 2011; 22:997–1013. [PubMed: 21953041]
51. Zhao P, Viner R, Teo CF, Boons GJ, Horn D, Wells L. J Proteome Res. 2011; 10:4088–4104. [PubMed: 21740066]
52. Hersberger KE, Håkansson K. Anal Chem. 2012; 84:6370–6377. [PubMed: 22770115]
53. Domon B, Costello CE. Glycoconjugate J. 1988; 5:397–409.
54. Sekiya S, Iida T. Trends Glycosci Glyc. 2008; 20:51–65.Han L, Costello C. Biochemistry Moscow. 2013; 78:710–720. [PubMed: 24010834]
55. Laremore, TN.; Leach, FE., III; Solakyildirim, K.; Amster, IJ.; Linhardt, RJ. Methods in Enzymology. In: Fukuda, M., editor. Glycomics. Vol. 478. 2010. p. 79-108.
56. Wuhrer M, Catalina MI, Deelder AM, Hokke CH. J Chromatogr B. 2007; 849:115–128.Vékey K, Ozohanics O, Tóth E, Jekő A, Révész Á, Krenyácz J, Drahos L. Int J Mass spectrom. 2013; 345–347:71–79.
57. Jensen PH, Karlsson NG, Kolarich D, Packer NH. Nat Protoc. 2012; 7:1299–1310. [PubMed: 22678433]
58. Bird B, Romeo MJ, Diem M, Bedrossian K, Laver N, Naber S. Vib Spectrosc. 2008; 48:101–106. [PubMed: 19768107]
59. Ly M, Leach FE III, Laremore TN, Toida T, Amster IJ, Linhardt RJ. Nat Chem Biol. 2011; 7:827–833. [PubMed: 21983600]
60. Laremore TN, Leach FE 3rd, Amster IJ, Linhardt RJ. Int J Mass spectrom. 2011; 305:109–115. [PubMed: 21860600] Chi L, Wolff JJ, Laremore TN, Restaino OF, Xie J, Schiraldi C, Toida T, Amster IJ, Linhardt RJ. J Am Chem Soc. 2008; 130:2617–25. [PubMed: 18247611]
61. McClellan JE, Costello CE, O'Conno PB, Zaia J. Anal Chem. 2002; 74:3760–3771. [PubMed: 12175164]
62. Oh H, Leach F III, Arungundram S, Al-Mafraji K, Venot A, Boons G-J, Amster IJ. J Am Soc Mass Spectrom. 2011; 22:582–590. [PubMed: 21472576]

63. Zaia J, Li X, Chan S, Costello C. J Am Soc Mass Spectrom. 2003; 14:1270–1281. [PubMed: 14597117]
64. Bielik AM, Zaia J. Int J Mass spectrom. 2011; 305:131–137. [PubMed: 21860601]
65. Leach F III, Ly M, Laremore T, Wolff J, Perlow J, Linhardt R, Amster IJ. J Am Soc Mass Spectrom. 2012; 23:1488–1497. [PubMed: 22825742]
66. Jones CJ, Beni S, Limtiaco JFK, Langeslay DJ, Larive CK. Annu Rev Anal Chem. 2011; 4:439–465.
67. Kailemia MJ, Li L, Xu Y, Liu J, Linhardt RJ, Amster IJ. Mol Cell Proteomics. 2013; 12:979–90. [PubMed: 23429520]
68. Liu H, Hakansson K. Int J Mass spectrom. 2011; 305:170–177.
69. Devakumar A, Thompson MS, Reilly JP. Rapid Commun Mass Spectrom. 2005; 19:2313–20. [PubMed: 16034827]
70. Ko BJ, Brodbelt JS. Anal Chem. 2011; 83:8192–8200. [PubMed: 21913695]
71. Harvey DJ, Scarff CA, Crispin M, Scanlan CN, Bonomelli C, Scrivens JH. J Am Soc Mass Spectrom. 2012; 23:1955–1966. [PubMed: 22993039]
72. Leach FE III, Wolff JJ, Laremore TN, Linhardt RJ, Amster IJ. Int J Mass spectrom. 2008; 276:110–115. [PubMed: 19802340]
73. Yu X, Huang Y, Lin C, Costello CE. Anal Chem. 2012; 84:7487–94. [PubMed: 22881449]
74. Zhang H, Zhang S, Tao G, Zhang Y, Mulloy B, Zhan X, Chai W. Anal Chem. 2013; 85:5940–9. [PubMed: 23692402]
75. Wu S, Grimm R, German JB, Lebrilla CB. J Proteome Res. 2011; 10:856–868. [PubMed: 21133381]
76. Fukui K, Takahashi K. Anal Chem. 2012; 84:2188–2194. [PubMed: 22300132]
77. Hogan JM, Pitteri SJ, Chrisman PA, McLuckey SA. J Proteome Res. 2005; 4:628–632. [PubMed: 15822944]
78. Dube DH, Bertozzi CR. Nat Rev Drug Discov. 2005; 4:477–488. [PubMed: 15931257] Ohtsubo K, Marth JD. Cell. 2006; 126:855–867. [PubMed: 16959566]
79. Nairn AV, York WS, Harris K, Hall EM, Pierce JM, Moremen KW. J Biol Chem. 2008; 283:17298–17313. [PubMed: 18411279] Prescher JA, Bertozzi CR. Cell. 2006; 126:851–854. [PubMed: 16959565]
80. Orlando R. Methods Mol Biol. 2013; 951:197–215. [PubMed: 23296532]
81. De Leoz MLA, Wu S, Strum J, Ninonuevo M, Gaerlan S, Mirmiran M, Niñonuevo M, German JB, Mills D, Lebrilla C, Underwood M. Anal Bioanal Chem. 2013; 405:4089–105. [PubMed: 23468138]
82. Bao Y, Chen C, Newburg DS. Anal Biochem. 2013; 433:28–35. [PubMed: 23068043]
83. Auray-Blais C, Bherer P, Gagnon R, Young SP, Zhang HH, An Y, Clarke JTR, Millington DS. Mol Genet Metab. 2011; 102:49–56. [PubMed: 20934363]
84. Hua S, An HJ, Ozcan S, Ro GS, Soares S, DeVere-White R, Lebrilla CB. Analyst. 2011; 136:3663–3671. [PubMed: 21776491]
85. Kang P, Mechref Y, Kyselova Z, Goetz JA, Novotny MV. Anal Chem. 2007; 79:6064–6073. [PubMed: 17630715]
86. Ma H, Miao X, Ma Q, Zheng W, Zhou H, Jia L. Iubmb Life. 2013; 65:409–422. [PubMed: 23441047]
87. Alvarez-Manilla G, Warren NL, Abney T, Atwood J, Azadi P, York WS, Pierce M, Orlando R. Glycobiology. 2007; 17:677–687. [PubMed: 17384119]
88. Atwood JA, Cheng L, Alvarez-Manilla G, Warren NL, York WS, Orlando R. J Proteome Res. 2007; 7:367–374. [PubMed: 18047270]
89. Ninonuevo MR, Ward RE, LoCascio RG, German JB, Freeman SL, Barboza M, Mills DA, Lebrilla CB. Anal Biochem. 2007; 361:15–23. [PubMed: 17181994]
90. Bowman MJ, Zaia J. Anal Chem. 2010; 82:3023–3031. [PubMed: 20230064]
91. Hahne H, Neubert P, Kuhn K, Etienne C, Bomgarden R, Rogers JC, Kuster B. Anal Chem. 2012; 84:3716–3724. [PubMed: 22455665]

92. Orlando R, Lim JM, Atwood JA, Angel PM, Fang M, Aoki K, Alvarez-Manilla G, Moremen KW, York WS, Tiemeyer M, Pierce M, Dalton S, Wells L. J Proteome Res. 2009; 8:3816–3823. [PubMed: 19449840]

93. Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK. J Chromatogr B. 2009; 877:1229–1239.

94. Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM. Proc Natl Acad Sci USA. 2007; 104:5860–5865. [PubMed: 17389395]

95. Toyama A, Nakagawa H, Matsuda K, Sato TA, Nakamura Y, Ueda K. Anal Chem. 2012; 84:9655–9662. [PubMed: 23004563]

96. Zhao Y, Jia W, Wang J, Ying W, Zhang Y, Qian X. Anal Chem. 2011; 83:8802–8809. [PubMed: 21970473]

97. Song E, Pyreddy S, Mechref Y. Rapid Commun Mass Spectrom. 2012; 26:1941–1954. [PubMed: 22847692]

98. Higel F, Demelbauer U, Seidl A, Friess W, Soergel F. Anal Bioanal Chem. 2013; 405:2481–2493. [PubMed: 23371526]

99. Huang R, Pomin VH, Sharp JS. J Am Soc Mass Spectrom. 2011; 22:1577–1587. [PubMed: 21953261]

100. Huang Y, Mao Y, Shao C, Zaia J. Glycobiology. 2012; 22:1609–1609.

101. Galeotti F, Volpi N. Anal Chem. 2011; 83:6770–6777. [PubMed: 21780812]

102. Yang B, Chang Y, Weyers AM, Sterner E, Linhardt RJ. J Chromatogr. 2012; 1225:91–98.

103. Langeslay DJ, Urso E, Gardini C, Naggi A, Torri G, Larive CK. J Chromatogr A. 2013; 1292:201–10. [PubMed: 23352830]

104. Yang B, Weyers A, Baik JY, Sterner E, Sharfstein S, Mousa SA, Zhang F, Dordick JS, Linhardt RJ. Anal Biochem. 2011; 415:59–66. [PubMed: 21530482] Wang B, Buhse LF, Al-Hakim A, Boyne MT II, Keire DA. J Pharm Biomed Anal. 2012; 67–68:42–50.

105. Jones CJ, Beni S, Larive CK. Anal Chem. 2011; 83:6762–6769. [PubMed: 21780769]

106. Fenner AM, Kerns RJ. Carbohydr Res. 2011; 346:2792–2800. [PubMed: 22015170]

107. Xue B, Alves S, Desbans C, Souchaud M, Filali-Ansary A, Soubayrol P, Tabet JC. J Mass Spectrom. 2011; 46:689–695. [PubMed: 21744418]

108. Alekseeva A, Casu B, Torri G, Pierro S, Naggi A. Anal Biochem. 2013; 434:112–22. [PubMed: 23201389]

109. Zauner G, Deelder AM, Wuhrer M. Electrophoresis. 2011; 32:3456–66. [PubMed: 22180202]

110. Fong B, Ma K, McJarrow P. J Agric Food Chem. 2011; 59:9788–9795. [PubMed: 21790206]

111. Marino K, Lane JA, Abrahams JL, Struwe WB, Harvey DJ, Marotta M, Hickey RM, Rudd PM. Glycobiology. 2011; 21:1317–1330. [PubMed: 21566017]

112. Hernandez-Hernandez O, Montanes F, Clemente A, Javier Moreno F, Luz Sanz M. J Chromatogr. 2011; 1218:7691–7696.Hernandez-Hernandez O, Calvillo I, Lebron-Aguilar R, Moreno FJ, Sanz ML. J Chromatogr. 2012; 1220:57–67.

113. Leijdekkers AGM, Sanders MG, Schols HA, Gruppen H. J Chromatogr. 2011; 1218:9227–9235.

114. Gilar M, Yu YQ, Ahn J, Xie H, Han H, Ying W, Qian X. Anal Biochem. 2011; 417:80–8. [PubMed: 21689629]

115. Ruhaak LR, Huhn C, Waterreus WJ, de Boer AR, Neususs C, Hokke CH, Deelder AM, Wuhrer M. Anal Chem. 2008; 80:6119–26. [PubMed: 18593198] Rudd PM, Guile GR, Kuster B, Harvey DJ, Opdenakker G, Dwek RA. Nature. 1997; 388:205–207. [PubMed: 9217165]

116. Huang Y, Shi X, Yu X, Leymarie N, Staples GO, Yin H, Killeen K, Zaia J. Anal Chem. 2011; 83:8222–8229. [PubMed: 21923145] Staples GO, Bowman MJ, Costello CE, Hitchcock AM, Lau JM, Leymarie N, Miller C, Naimy H, Shi X, Zaia J. Proteomics. 2009; 9:686–95. [PubMed: 19137549]

117. Li J, Li X, Guo Z, Yu L, Zou L, Liang X. Analyst. 2011; 136:4075–82. [PubMed: 21858273] Jensen PH, Mysling S, Hojrup P, Jensen ON. Methods Mol Biol. 2013; 951:131–44. [PubMed: 23296529] Wan H, Yan J, Yu L, Sheng Q, Zhang X, Xue X, Li X, Liang X. Analyst. 2011; 136:4422–30. [PubMed: 21897947] Selman MH, Hemayatkar M, Deelder AM, Wuhrer M. Anal Chem. 2011; 83:2492–9. [PubMed: 21366235] An Y, Cipollo JF. Anal Biochem. 2011; 415:67–

80. [PubMed: 21545787] Liu J, Wang F, Lin H, Zhu J, Bian Y, Cheng K, Zou H. Anal Chem. 2013; 85:2847–52. [PubMed: 23384158]

118. Li X, Shen G, Zhang F, Yang B, Liang X. J Chromatogr, B: Anal Technol Biomed Life Sci. 2013; 941:45–49.

119. Parker BL, Thaysen-Andersen M, Solis N, Scott NE, Larsen MR, Graham ME, Packer NH, Cordwell SJJ. Proteome Res. 2013Kuo CW, Wu IL, Hsiao HH, Khoo KH. Anal Bioanal Chem. 2012; 402:2765–76. [PubMed: 22287049]

120. Gimenez E, Sanz-Nebot V, Rizzi A. Anal Bioanal Chem. 2013; 405:7307–19. [PubMed: 23846592] Mauko L, Nordborg A, Hutchinson JP, Lacher NA, Hilder EF, Haddad PR. Anal Biochem. 2011; 408:235–241. [PubMed: 20887707] Mauko L, Pelzing M, Dolman S, Nordborg A, Lacher NA, Haddad PR, Hilder EF. J Chromatogr. 2011; 1218:6419–6425.

121. Neville DC, Alonzi DS, Butters TD. J Chromatogr A. 2012; 1233:66–70. [PubMed: 22391491]

122. Ruhaak LR, Deelder AM, Wuhrer M. Anal Bioanal Chem. 2009; 394:163–74. [PubMed: 19247642]

123. Karlsson NG, Wilson NL, Wirth HJ, Dawes P, Joshi H, Packer NH. Rapid Commun Mass Spectrom. 2004; 18:2282–2292. [PubMed: 15384149] Chu CS, Ninonuevo MR, Clowers BH, Perkins PD, An HJ, Yin H, Killeen K, Miyamoto S, Grimm R, Lebrilla CB. Proteomics. 2009; 9:1939–1951. [PubMed: 19288519]

124. Ninonuevo M, An H, Yin H, Killeen K, Grimm R, Ward R, German B, Lebrilla C. Electrophoresis. 2005; 26:3641–3649. [PubMed: 16196105]

125. Karlsson NG, Schulz BL, Packer NH, Whitelock JM. J Chromatogr B Analyt Technol Biomed Life Sci. 2005; 824:139–147.Gill VL, Wang Q, Shi X, Zaia J. Anal Chem. 2012; 84:7539–7546. [PubMed: 22873817]

126. Aldredge D, An HJ, Tang N, Waddell K, Lebrilla CB. J Proteome Res. 2012; 11:1958–68. [PubMed: 22320385]

127. Ruhaak LR, Miyamoto S, Kelly K, Lebrilla CB. Anal Chem. 2012; 84:396–402. [PubMed: 22128873]

128. Ruhaak LR, Taylor SL, Miyamoto S, Kelly K, Leiserowitz GS, Gandara D, Lebrilla CB, Kim K. Anal Bioanal Chem. 2013Hua S, Williams CC, Dimapasoc LM, Ro GS, Ozcan S, Miyamoto S, Lebrilla CB, An HJ, Leiserowitzf GS. J Chromatogr. 2013; 1279:58–67.Ruhaak LR, Nguyen UT, Stroble C, Taylor SL, Taguchi A, Hanash SM, Lebrilla CB, Kim K, Miyamoto S. Proteomics Clin Appl. 2013

129. Ni W, Bones J, Karger BL. Anal Chem. 2013; 85:3127–3135. [PubMed: 23398125]

130. Grass J, Pabst M, Kolarich D, Poeltl G, Leonard R, Brecker L, Altmann F. J Biol Chem. 2011; 286:5977–5984. [PubMed: 21169363]

131. Kenny DT, Gaunitz S, Hayes CA, Gustafsson A, Sjoblom M, Holgersson J, Karlsson NG. Methods Mol Biol. 2013; 988:145–67. [PubMed: 23475718]

132. Kolarich D, Jensen PH, Altmann F, Packer NH. Nat Protoc. 2012; 7:1285–98. [PubMed: 22678432]

133. Nwosu CC, Aldredge DL, Lee H, Lerno LA, Zivkovic AM, German JB, Lebrilla CB. J Proteome Res. 2012; 11:2912–2924. [PubMed: 22439776] Nwosu CC, Huang J, Aldredge DL, Strum JS, Hua S, Seipert RR, Lebrilla CB. Anal Chem. 2013; 85:956–963. [PubMed: 23215446]

134. Wu S, Tao N, German JB, Grimm R, Lebrilla CB. J Proteome Res. 2010; 9:4138–51. [PubMed: 20578730]

135. Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D. Glycobiology. 2013; 23:664–676. [PubMed: 23436288]

136. Pompach P, Chandler KB, Lan R, Edwards N, Goldman R. J Proteome Res. 2012; 11:1728–40. [PubMed: 22239659]

137. Brokl M, Hernandez-Hernandez O, Cristina Soria A, Luz Sanz M. J Chromatogr. 2011; 1218:7697–7703.

138. Melmer M, Stangler T, Premstaller A, Lindner W. J Chromatogr A. 2011; 1218:118–23. [PubMed: 21122866]

139. Mauko L, Lacher NA, Pelzing M, Nordborg A, Haddad PR, Hilder EF. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 911:93–104.

140. Harvey SR, Macphee CE, Barran PE. Methods. 2011; 54:454–61. [PubMed: 21669288] Konijnenberg A, Butterer A, Sobott F. Biochim Biophys Acta. 2013; 1834:1239–56. [PubMed: 23246828]

141. Purves RW, Guevremont R, Day S, Pipich CW, Matyjaszczyk MS. Rev Sci Instrum. 1998; 69:4094–4105.

142. Creese AJ, Cooper HJ. Anal Chem. 2012; 84:2597–2601. [PubMed: 22280549]

143. Kailemia M, Park M, Kaplan D, Venot A, Boons G-J, Li L, Linhardt R, Amster IJ. J Am Soc Mass Spectrom. 2013:1–11.

144. Fenn LS, Kliman M, Mahsut A, Zhao SR, McLean JA. Anal Bioanal Chem. 2009; 394:235–44. [PubMed: 19247641]

145. Fenn LS, McLean JA. Methods Mol Biol. 2013; 951:171–94. [PubMed: 23296531]

146. Pagel K, Harvey DJ. Anal Chem. 2013; 85:5138–45. [PubMed: 23621517]

147. Harvey DJ, Scarff CA, Edgeworth M, Crispin M, Scanlan CN, Sobott F, Allman S, Baruah K, Pritchard L, Scrivens JH. Electrophoresis. 2013; 34:2368–78. [PubMed: 23712623] Harvey DJ, Sobott F, Crispin M, Wrobel A, Bonomelli C, Vasiljevic S, Scanlan CN, Scarff CA, Thalassinos K, Scrivens JH. J Am Soc Mass Spectrom. 2011; 22:568–81. [PubMed: 21472575]

148. Zhu F, Lee S, Valentine SJ, Reilly JP, Clemmer DE. J Am Soc Mass Spectrom. 2012; 23:2158–2166. [PubMed: 23055077]

149. Yamaguchi Y, Nishima W, Re S, Sugita Y. Rapid Commun Mass Spectrom. 2012; 26:2877–2884. [PubMed: 23136018]

150. Isailovic D, Plasencia MD, Gaye MM, Stokes ST, Kurulugama RT, Pungpapong V, Zhang M, Kyselova Z, Goldman R, Mechref Y, Novotny MV, Clemmer DE. J Proteome Res. 2012; 11:576–85. [PubMed: 22148953]

151. Gaye MM, Valentine SJ, Hu Y, Mirjankar N, Hammoud ZT, Mechref Y, Lavine BK, Clemmer DE. J Proteome Res. 2012; 11:6102–10. [PubMed: 23126309]

152. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. J Natl Cancer Inst. 2001; 93:1054–61. [PubMed: 11459866] Pepe MS, Feng Z, Janes H, Bossuyt PM, Potter JD. J Natl Cancer Inst. 2008; 100:1432–8. [PubMed: 18840817]

153. Seo Y, Andaya A, Leary JA. Anal Chem. 2012; 84:2416–2423. [PubMed: 22283665]

154. Fenn LS, McLean JA. PCCP. 2011; 13:2196–2205. [PubMed: 21113554]

155. Fasciotti M, Sanvido GB, Santos VG, Lalli PM, McCullagh M, de Sa GF, Daroda RJ, Peter MG, Eberlin MN. J Mass Spectrom. 2012; 47:1643–1647. [PubMed: 23280753]

156. Woodin CL, Maxon M, Desaire H. Analyst. 2013; 138:2793–803. [PubMed: 23293784] Dallas DC, Martin WF, Hua S, German JB. Brief Bioinform. 2013; 14:361–74. [PubMed: 22843980] Desaire H. Mol Cell Proteomics. 2013; 12:893–901. [PubMed: 23389047]

157. Artemenko NV, McDonald AG, Davey GP, Rudd PM. Methods Mol Biol. 2012; 899:325–50. [PubMed: 22735963]

158. Kronewitter SR, De Leoz ML, Strum JS, An HJ, Dimapasoc LM, Guerrero A, Miyamoto S, Lebrilla CB, Leiserowitz GS. Proteomics. 2012; 12:2523–38. [PubMed: 22903841]

159. Campbell MP, Royle L, Radcliffe CM, Dwek RA, Rudd PM. Bioinformatics. 2008; 24:1214–6. [PubMed: 18344517]

160. Hayes CA, Karlsson NG, Struwe WB, Lisacek F, Rudd PM, Packer NH, Campbell MP. Bioinformatics. 2011; 27:1343–1344. [PubMed: 21398669]

161. Peltoniemi H, Natunen S, Ritamo I, Valmu L, Rabina J. Glycoconj J. 2013; 30:159–70. [PubMed: 22707226]

162. Strum JS, Kim J, Wu S, De Leoz MLA, Peacock K, Grimm R, German JB, Mills DA, Lebrilla CB. Anal Chem. 2012; 84:7793–7801. [PubMed: 22897719]

163. Dreyfuss JM, Jacobs C, Gindin Y, Benson G, Staples GO, Zaia J. Anal Bioanal Chem. 2011; 399:727–735. [PubMed: 20953780]

164. Maxwell E, Tan Y, Tan Y, Hu H, Benson G, Aizikov K, Conley S, Staples GO, Slysz GW, Smith RD, Zaia J. PLoS One. 2012; 7:e45474. [PubMed: 23049804]

Biographies

Muchena J. Kailemia completed his bachelor of science in education (B.Sc.Ed) degree at Maseno University, Kenya. After receiving his M.Sc. degree in analytical chemistry from East Tennessee State University in 2009, he entered the graduate program in Chemistry at the University of Georgia, where he is completing his Ph.D. research in the laboratory of Dr. Jonathan Amster. His research focuses on developing mass spectrometry, ion mobility and chemometrics methods for the structural characterization of glycosaminoglycans.

L. Renee Ruhaak earned her M.Sc. degree in Bio-Pharmaceutical Sciences from Leiden University, The Netherlands in 2005. During her masters education she performed research in the lab of Dr. Robert Verpoorte and completed a 1-year study abroad program at Uppsala University, Sweden, in the lab of Dr. Lars Bohlin. She then performed her Ph.D. studies at the Leiden University Medical Center, the Netherlands, under supervision of Dr. André Deelder, Dr. Eline Slagboom and Dr. Manfred Wuhrer, which resulted in a thesis on the development and application of high throughput methods for glycan analysis. Renee then joined the laboratory of Dr. Carlito Lebrilla at the University of California, Davis in 2010, where she aims to understand the role of oligosaccharides in cancer biology and develop biomarkers for cancer detection. Renee was selected as a UC Davis Graduate School of Management Business Development fellow 2012–2013.

Carlito B. Lebrilla completed his B.S. in 1981 from University of California, Irvine and his PhD in 1985 from University of California, Berkeley. He was a NATO-NSF and an Alexander von Humboldt postdoctoral fellow with Prof. Helmut Schwarz at the Technical University in Berlin. He was also a UC President's postdoctoral fellow at the University of California, Irvine. He has been on the faculty at UC Davis since 1987 and is currently a Distinguished Chair in the Chemistry Department and in Biochemistry and Molecular Medicine. He has served as Chair of Chemistry and has been on the Board of Directors of the American Society for Mass Spectrometry. He is on the editorial board of several mass spectrometry journals. His research focus is on bioanalytical chemistry with emphasis on oligosaccharides and glycoconjugates.

I. Jonathan Amster earned his B.A. (1977), M.S. (1983), and Ph.D. (1986) in Chemistry from Cornell University. He was a postdoctoral researcher at the University of California-Irvine from 1987–1988. In 1988, he joined the faculty of the University of Georgia, where he is currently Professor and Head of Chemistry. He has served on the Board of Directors of the American Society for Mass Spectrometry, is a member of the editorial board of the European Journal of Mass Spectrometry, and is an elected Fellow of the American Association for the Advancement of Science. His research interests include developments in Fourier transform mass spectrometry and its applications to the structural analysis of macromolecules, with particular focus on the tandem mass spectrometry analysis of glycosaminoglycans.

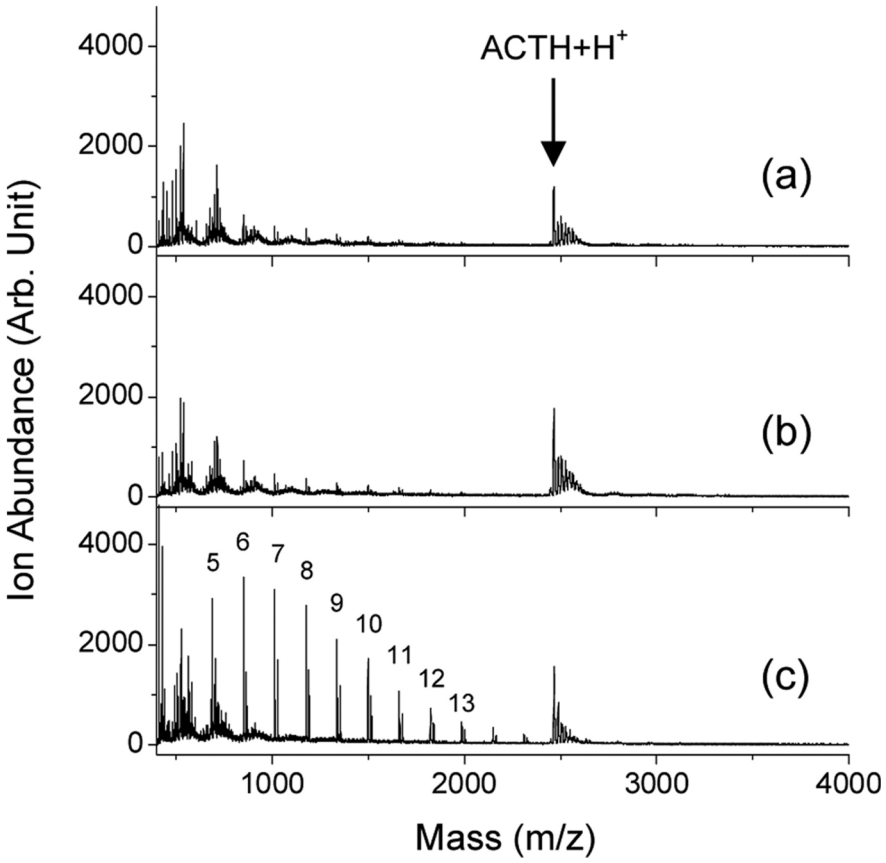

Figure 1.

Mass spectra of 5 pmol each of dextran and ACTH fragment 18–39 obtained from various samples, including (a) dried-droplet, (b) thin-layer, and (c) trilayer samples. The numbers indicate the degrees of polymerization (*n*) of sodiated dextran. Reproduced from Chieh-Lin Wu; Chia-Chen Wang; Yin-Hung Lai; Hsun Lee; Jia-Der Lin; Yuan Tseh Lee; Yi-Sheng Wang; *Anal. Chem.* **2013,** 85, 3836–3841.

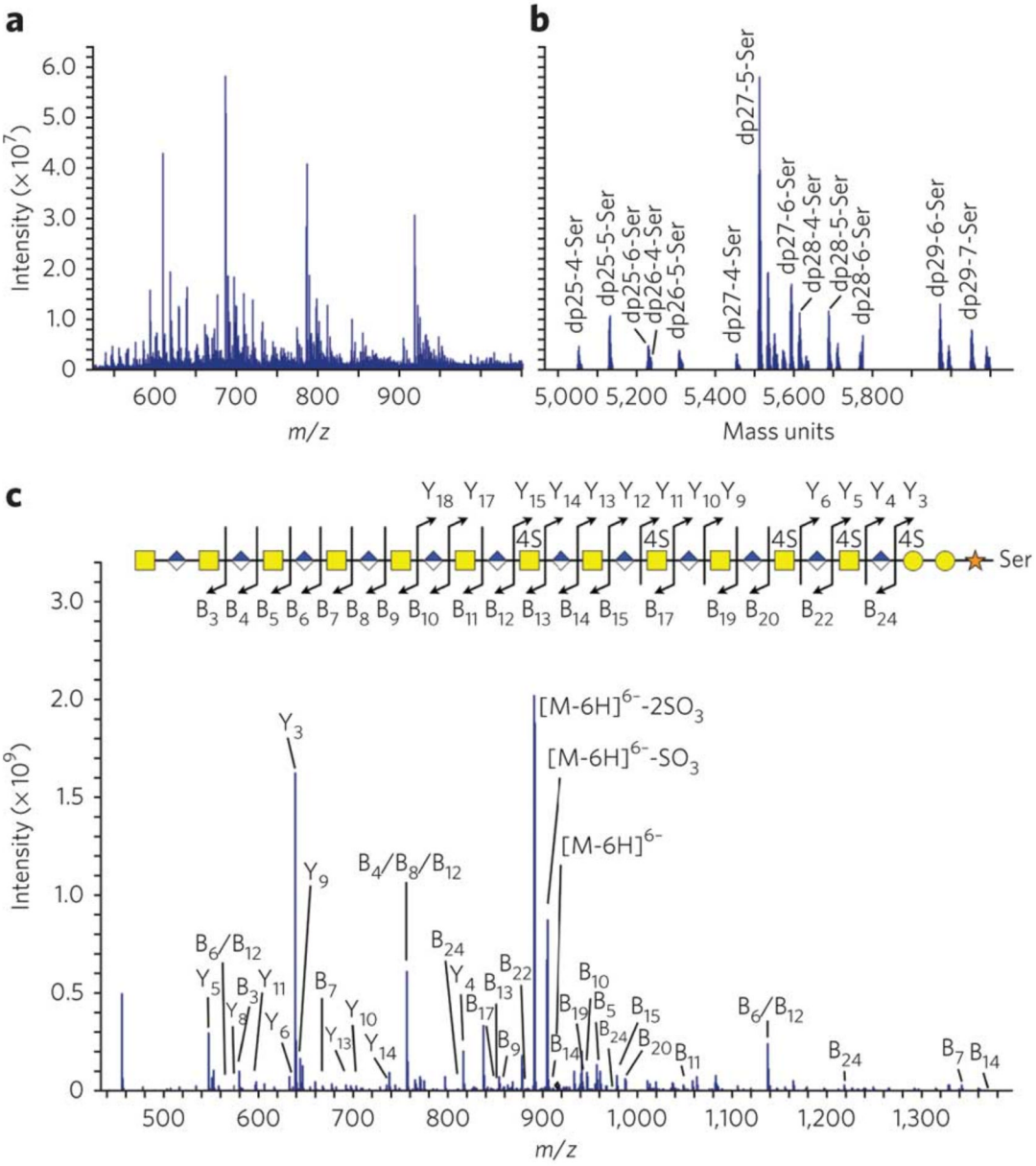

Figure 2.

(**a**) FT-ICR negative-ion mass spectrum of 5.80-kDa MR fraction by PAGE with 18 isobars and 63 parent ions. (**b**) Deconvolution of spectrum **a**. (**c**) CID-FT-ICR-MS/MS spectra of parent-ion *m*/*z* = 917.38 (*z* = 6) and annotated fragment-ions providing sequence with dp27-5-Ser fragmentation pattern assigned from spectrum. Reproduced from Mellisa Ly; Franklin E. Leach III; Tatiana N. Laremore; Toshihiko Toida; I. Jonathan Amster; Robert J. Linhardt; *Nat. Chem. Biol.* **2011,** *7*, 827–833. Copyright 2011 Nature Publishing Group.

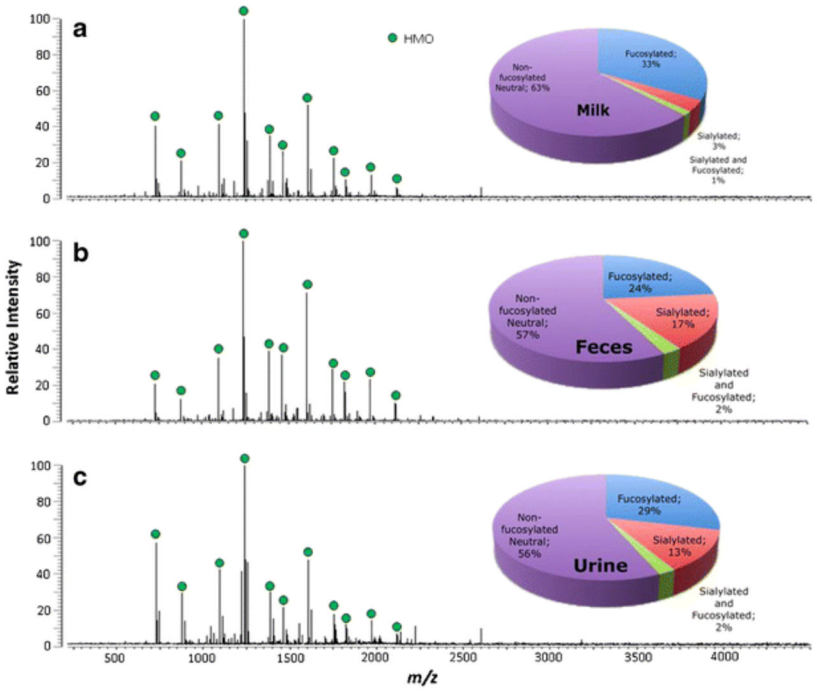

Figure 3.

MALDI FT-ICR MS profiles in positive ion mode of the HMOs in milk (**a**), feces (**b**), and urine (**c**) of a mother–preterm infant dyad. Milk, feces, and urine samples are 5, 1, and 150 ug, respectively. HMOs are marked with *green dots*. Distributions of fucosylated and sialylated glycans are based on HMO intensities normalized against the total HMO intensities. Pie charts represent nano-LC MS data. Reproduced from Maria Lorna A. De Leoz; Shuai Wu; John S. Strum; Milady R. Niñonuevo; Stephanie C. Gaerlan; Majid Mirmiran; J. Bruce German; David A. Mills; Carlito B. Lebrilla; Mark A. Underwood; *Anal. Bioanal. Chem.* **2013,** *405*, 4089–4105. Copyright 2013 Springer-Verlag.

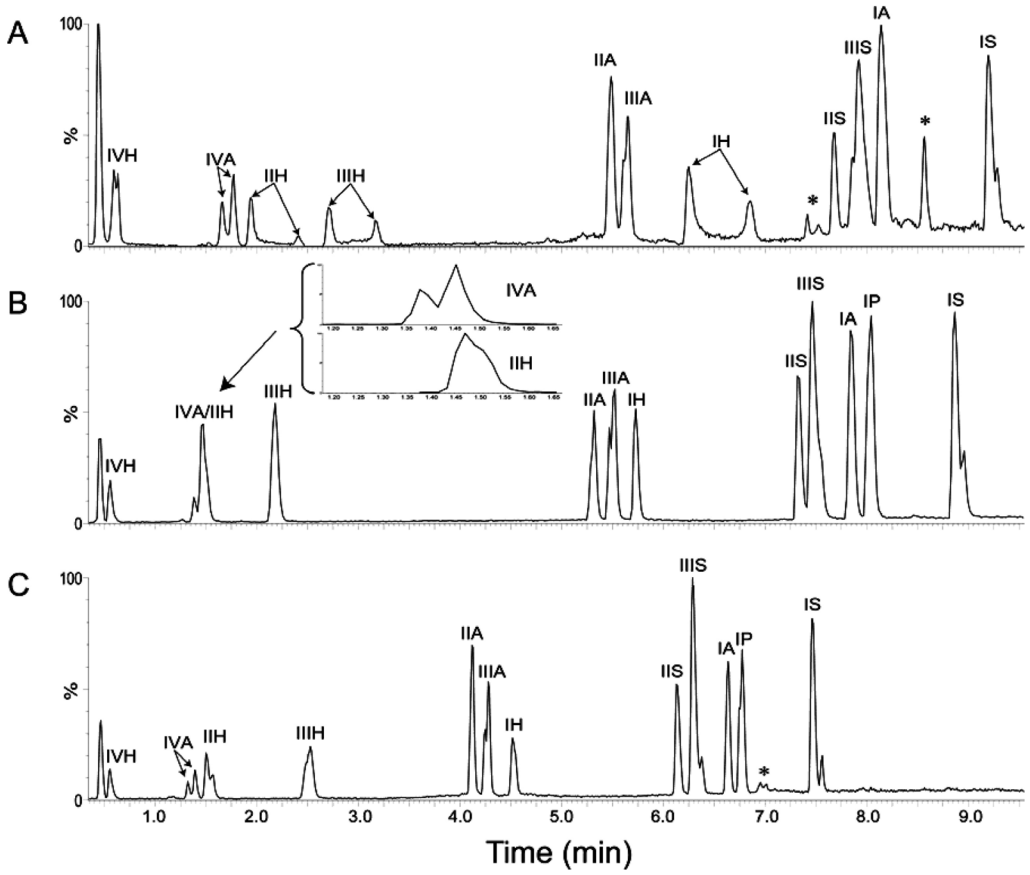

Figure 4.

TICs for the 11 heparin disaccharides studied and the internal standard ΔUA(2S)-GlcNCOEt(6S) (IP) using (A) previously published disaccharide separation method (ref 36) with 20 mM TrBA, 2.5 mM NH4COOH buffers at pH 6.5 and column temperature of 40 °C, (B) the same separation conditions at pH 3.5 with the inset showing the extracted ion chromatograms of IVA and IIIH, and (C) 30 mM TrBA and column temperature of 25 °C at pH 3.5 with a new gradient profile optimized for the higher IPR concentration. The peaks marked with an asterisk are impurities. Reproduced from Christopher J. Jones; Szabolcs Beni; Cynthia K. Larive; *Anal. Chem.* **2011,** 83, 6762–6769.

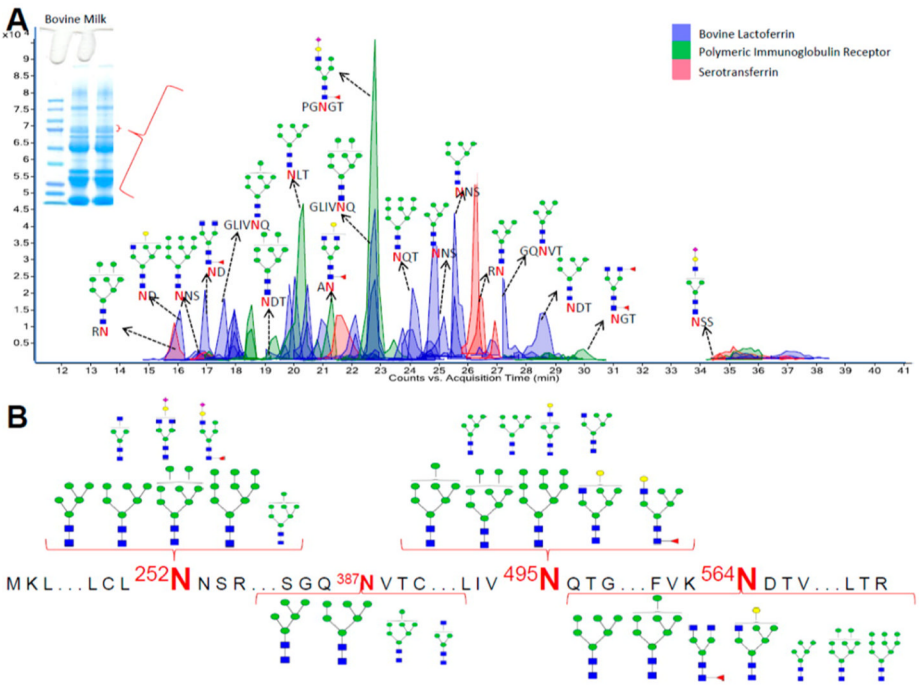

Figure 5.

(A) Extracted compound chromatogram (ECC) of glycopeptides generated from INPEG analysis of a bovine milk gel band containing bovine lactoferrin, polymeric immunoglobulin receptor, and serotransferrin. (B) Detailed site heterogeneity and detected glycoform abundance of bovine lactoferrin following INPEG analysis. Reproduced from Charles C. Nwosu; Jincui Huang; Danielle L. Aldredge; John S. Strum; Serenus Hua; Richard R. Seipert; Carlito B. Lebrilla; *Anal. Chem.* **2013,** 85, 956–963.

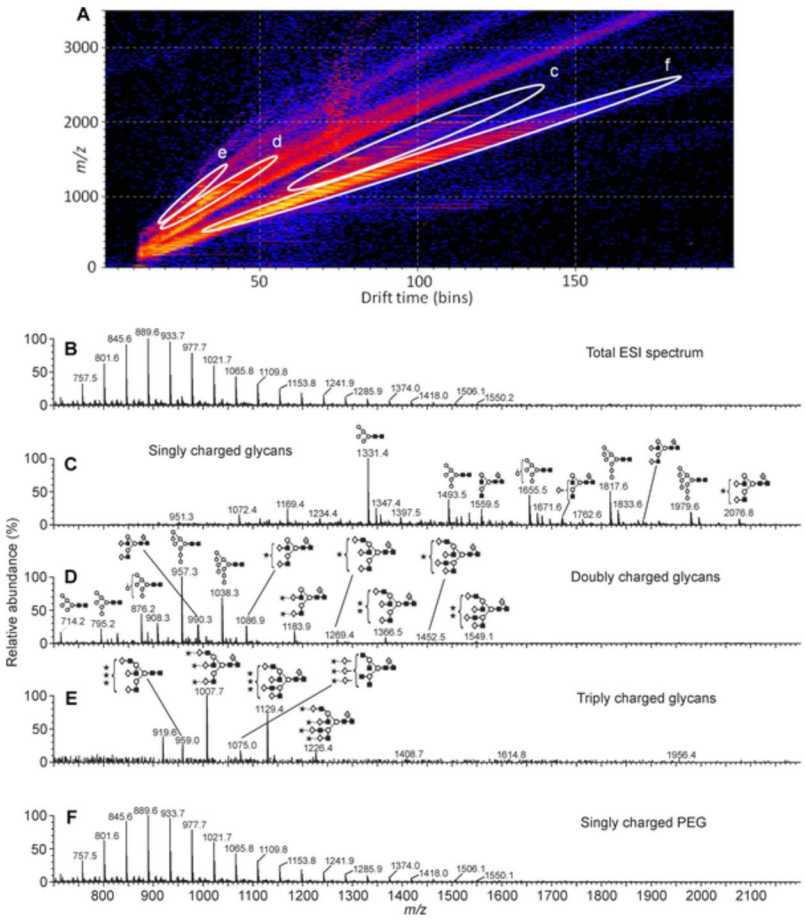

Figure 6.

(A) Driftscope (*m/z*:drift time, log scale) display of the negative ions from a sample of released gp120 glycans contaminated with PEG. Circled regions are labeled with those of the panels below. (B) Total electrospray spectrum. (C) Extracted singly charged *N*-glycan ions ($[M+H_2PO_4]^-$ except *m/z* 2076, which is $[M-H]^-$). (D) Extracted doubly charged *N*-glycan ions (High-mannose (Man) glycans are $[M+(H_2PO_4]_2)^{2-}$, glycans with one sialic acid are $[M-H+H_2PO_4]^{2-}$ and the di-sialylated glycans are $[M-H_2]^{2-}$). (E) Extracted triply charged *N*-glycan ions ($[M-H_3]^{3-}$). (F) Extracted singly charged PEG ions. Reproduced from David J. Harvey; Charlotte A. Scarff; Matthew Edgeworth; Max Crispin; Christopher N. Scanlan; Frank Sobott; Sarah Allman; Kavitha Baruah; Laura Pritchard; James H. Scrivens; *Electrophoresis* **2013,** *34,* 2368–2378. Copyright 2013 Wiley-VCH Verlag GmbH & Co.