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Original Research

Analysis of Milk Oligosaccharides by Mass Spectrometry, Protein Identification, and Other Uses of Protein Mass Spectrometry

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Abstract:

Carbohydrates serve a pivotal role in biochemical pathway control, cell adhesion, and cell signalling, among many other critical biological functions. Carbohydrate biosynthesis does not rely on templates like protein and nucleic acid production does. In nature, they are found in complicated heterogeneous mixes. Carbohydrate analysis is usually constrained to what can be sourced from nature, meaning researchers have to deal with small amounts of heterogeneous material. This is in contrast to methods like polymerase chain reaction or overexpression, which can increase the amount of proteins or nucleic acids, respectively. Mass spectrometry is an ideal analytical tool for this class of compounds due to its great sensitivity and tolerability of mixtures. Mass spectrometry's usage to carbohydrates has progressed more slowly than protein analysis, mostly due to the fact that carbohydrates are a more difficult class of targets to structurally characterise. Unlike proteins, carbohydrates do not yet have a comprehensive and closed set of sequences represented in any database. Completing the structural features from the mass spectra is crucial for carbohydrate characterisation. Completing structural analyses becomes considerably more challenging when small variations caused by isomerism or chirality result in compounds with vastly varied biological activity. Carbohydrate analysis has benefited from the fast evolution and improvement of mass spectrometry methodology and technologies for biomolecule analysis. Improvements in ionisation techniques, ion activation methods, carbohydrate chromatographic separations, ion mobility/mass spectrometry hybridization, and data collection/interpretation software are all part of these innovations. Because of this, it is appropriate to investigate the effects of these changes on carbohydrate analysis. While not an attempt at a thorough survey, this article does its best to highlight key advances that have, according to the writers, pushed the field forward.

Proteomics relies on precise protein identification as a statistical foundation for the study and use of this technology in various biological and medical fields. Recent years have seen proteomics, a subfield of biochemistry, expand and advance the science of precisely defining the biology and relationships of protein clusters, or proteomes. Although other methods, like as affinity-based identifications, continue to play important roles, mass spectrometry (MS)-based approaches have traditionally been the mainstay of protein identification in proteomics. In this article, we provide a brief introduction to MS so that readers can grasp the fundamentals of data generation and the parameters utilised to guide computational techniques employed for protein identification. After that, we go over all the bioinformatics and computational approaches used for protein identification in proteomics, including the most up-to-date metrics that everyone agrees on to confirm an identification.

In post-data analysis, protein identities are the sole determinants of biological inference, making accurate protein identification crucial. It is possible to classify the majority of postidentification analyses as either qualitative or quantitative. When conducting qualitative studies, it is common to combine the following methods: (i) analysing gene ontology terms to understand their cellular components, molecular functions, and biological processes; (ii) learning about post-translation modifications that control various cellular pathways and result in protein isoform entries in databases; and (iii) using domain and motif analysis to predict the structurally essential sites in a newly discovered protein based on information about conserved folds of domains and motifs in previously annotated proteins. (iv) protein-protein interactions: after proteins are identified, one of the last aims is to determine which other proteins interact with them. These other proteins are then responsible for controlling various biological processes. Because a protein can't do its job well without a steady connection with the proteins that bind to it, this is of the utmost importance; (v) study of biological pathways entails investigating all relevant proteins and chemical processes; (vi) Phylogenetic analysis: using top-down MS-based de novo analysis, one can find protein isoforms that differ from one other based on some changed site or due to single or multiple nucleotide polymorphisms. Phylogenetic analysis is used to appraise these mutations as they have progressed through evolution. Quantitative research also makes crucial use of precise protein identification. These compare the level of protein expression in a "normal" state with that following therapy or in a specific environmental or illness condition.

Introduction:

In every living thing, proteins play a crucial role in maintaining the system's functionality. Biochemistry has thus been deeply occupied with the pursuit of understanding their function and the functions they play in biology and medicine [1,2]. Accurately and rigorously identifying any given protein is a basic prerequisite to understanding function, as it allows one to avoid making incorrect claims and drawing incorrect inferences. There are tens of thousands of possible variants in a single protein because, unlike their genetic parental DNA, which is structured like a linear sequence of amino acids, proteins can fold into an infinite number of forms influenced by the more than 28 known post-translational modifications (PTMS). Mass spectrometry (MS)-based proteomics emerged as a solution to these and other problems that had previously made protein identification extremely difficult. Proteomics, a subfield of biochemistry that has made significant contributions to methods and tools for protein identification, was initially named in the early

1990s. Evidenced by the >68 000 publications—PubMed search—proteomics has grown tremendously in the past two decades [3,4]. Proteomics encompasses a wide range of research methods, but at its core, it boils down to one thing: determining which cells, tissues, or organisms express certain protein suites (proteomes) in response to specific environmental and temporal cues. There have traditionally been two main ways to accomplish identification and quantification [5]. The first employs antibody or affinity-based technology to identify antigens on proteins, which are proteins; the second uses MS and related technologies to sequence proteins. This review will help the reader understand the basics of various approaches, with an emphasis on MS and related technologies, so they may choose the one that works best for their experimental design in terms of methodology, equipment, and parameters. While other evaluations have focused on certain aspects of MS protein identification, none of them have covered the topic from every angle like this one. Not only does bringing together analytical and computational features in one location provide a fresh perspective on the field's future, but it also aids in the holistic identification of potential hazards and opportunities [6, 7]. Since MS is now more commonly used in proteomics, we will be concentrating on it rather than the other most popular method of protein identification in proteomics, which is based on antibodies. The following section will provide a brief overview of other methods that do not use MS since they have already been thoroughly discussed elsewhere.

Recognition of proteins:

When it comes to detecting, identifying, and quantifying proteins, antibody-based approaches are among the most used. These are an effective affinity reagent that have numerous uses in protein identification, such as Western blotting, immunohistochemistry, immunofluorescence, immunoprecipitation, flow cytometry, and enzyme-linked immunosorbent assays. By fusing a single antibody-producing B lymphocyte from an inoculated host with a tumour cell from a mouse, a process known as a "hybridoma," Kohler and Milstein became the first to create monoclonal antibodies in 1975. Over three million antibodies are now available from more than 300 commercial suppliers, attesting to the fast expansion of the worldwide antibody business since then (https://www.biocompare.com/Antibodies/).

Nevertheless, a number of publications in the past several years have brought attention to the major drawbacks and issues associated with the use of various commercial antibodies by individuals who lack knowledge or expertise in the field [8, 9]. Problems with sensitivity and specificity, low characterisation, cross-reactivity with isotypes, limited repeatability, and non-specific binding are all part of these difficulties. Actually, more than five thousand commercial antibodies have been analysed by the Human Protein Atlas, which is a crucial component of the Human Proteome Project. For applications requiring precise protein detection or identification, they have demonstrated that fewer than half of the antibodies meet their standards [10, 11]. In addition, a bioinformatics firm investigated more than 6,000 commercial antibodies from 26 suppliers and found that more than 75% of them were not specific to the specified antigens and frequently were not useful. For this reason, before beginning an experiment to identify a protein using antibodies, the selection and verification of antibodies should be prioritised.

Mass spectrometry-based protein identification:

Gaseous analytes can be identified and quantified using mass spectrometry (MS) in a vacuum by calculating their mass-to-charge ratio (m/z). The advent of rapid atom bombardment (FAB) and plasma desorption (PD) ionisation techniques in the 1980s allowed for the initial introduction of large polar biomolecules and biopolymers to MS. In subsequent years, cutting-edge electrospray ionisation (ESI) and matrix-assisted laser desorption (MALDI) methods were developed to ionise oligonucleotides, lipids, proteins, peptides, glycolipids, and glycoproteins. These methods have found extensive application in the fields of

protein localization and spatiotemporal expression, as well as in the quantification and characterization of protein sequences [12–14]. Using computational and bioinformatics techniques, it deciphers a protein's whole amino acid sequence and analyses the mass/charge ratio of peptides. It can also decipher numerous PTMS. In recent years, MS and related computation have been used to better understand protein interaction networks—essential for understanding organismal systems biology—and to better understand the three-dimensional structure of proteins. Rapid proteome identification and quantitation with enhanced sensitivity and comprehensive data generation have been made possible by MS, in contrast to traditional protein detection and identification methods like SDS-PAGE, 2DE, and Western blotting [15] (discussed above). While it would go beyond the scope of this review to provide a comprehensive description of each instrument, we will provide extensive examples of how they work and what skills they require, since these are the fundamental building blocks of the computational approaches that follow.

Carbohydrate Analysis via Mass Spectrometry:

Complex biomolecules, including (glyco)proteins and carbohydrates, are best analysed by mass spectrometry (MS). Recent 20 years have seen the development of techniques that form the basis of high throughput proteomics workflows; these techniques examine the protein backbone utilising peptides that are produced from proteolysis. Tandem MS fragmentation opens up new possibilities for protein and peptide identification and sequencing. Protein identification and the efficacy of these procedures are contingent upon knowing the genome sequence. Structural identification from tandem MS spectra is more problematic for oligosaccharides compared to peptides because they are not typically formed in a linear fashion and do not follow a template driven biosynthesis [16–18]. Oligosaccharide building components are isomeric mono-saccharides, which cannot be distinguished by mass alone. Furthermore, monosaccharide analysis in and of itself does not reveal anything about the structures of the oligosaccharides that are produced. The analyst can take heart in the fact that eukaryotic milk oligosaccharides are synthesised by Nature from a relatively small repertory of monosaccharides, rather than the whole variety that could theoretically exist. Furthermore, the protein-bound glycan and oligo-saccharide production processes are very conserved, which aids in the structure assignment using MS and tandem MS data. Even if MS technology has capabilities that were unimaginable even twenty years ago [19], the first sample preparation is still the most important step in a successful analysis. The most important thing to optimise for every sample matrix is the process of enriching the target molecules while simultaneously reducing the contaminating substances.

People, Dairy, and Glycemic Index:

In the beginning, and among the most crucial nutrients for all mammals, there is mother's milk. Breast milk provides several components that help the developing immune system as well as the correct quantity and composition of nutrients for child growth in the first stage of life. Contributing to a decreased risk of immunological and gastrointestinal problems in newborns include proteins, glycoproteins, free glycans, and glycolipids, which form the phalanx of immune active components. Animal milk products, including cow's milk, have been staples in the diets of many different nations throughout history [20]. There is a dizzying array of milk-derived products that occupy a lot of shelf space in supermarkets, and many different kinds of bacteria, yeasts, and fungi have been utilised in their creation. According to dairyco.org.uk, there were 560 million tonnes of cow's milk produced globally in 2007. This figure does not account for milk produced from other animals, such as goats, sheep, or buffalo. Consequently, animal milk is an important part of our nutrition, and there is a lot of scientific and ecological interest in understanding the various components and the biological roles they perform. Oligosaccharides and glycoconjugates, including glycoproteins and free

oligosaccharides, are abundant in all types of milk. Both nutritionally and in terms of the antipathogenic protection they provide to our progeny, these components are of particular relevance [21–23]. Because different types of carbohydrates affect the growth of different strains of bifidobacteria, milk also influences the flora that lives in the stomach.

Olegosaccharides Encased in Proteins:

The biosynthetic enzymes and monosaccharide building blocks used by free and protein bound oligosaccharides are similar, however the structures of the glycans attached to proteins are different. Protein linkage allows them to be categorised as either N-linked or O-linked glycans.2 A specific sequence on the protein, Asn-X Ser/Thr (where X can be any amino acid except Pro), is necessary for the attachment of N-linked glycans [24, 25]. This sequence is where a high mannose structure is connected to the Asn residue during protein synthesis.2 The complex type oligosaccharide structures are constructed in the Golgi apparatus after this high mannose structure has undergone regulated destruction and complex reconstruction. Typically, sialic acid is added to the non-reducing ends of the common chitobiose penta-saccharide core, which is then partially adorned with fucoses in various places and linked with GlcNAc-Gal disaccharides to varying degrees. So yet, no distinct amino acid sequon has been identified for O-Linked glycans; however, they are connected to proteins via O-glycosidic bonds to Ser or Thr residues. In the Golgi apparatus, a GalNAc residue—though alternative attachments like fucose have been documented—is typically attached to the protein for the initial O-glycosylation phase. Several different types of glycosyltransferases then proceed to incrementally lengthen these residues. Eight distinct core structures have been identified for O-glycans, in contrast to the N-glycans' shared core [26-28]. Proteins in human milk fat globule membranes are glycosylated differently from those in cow's milk, both in terms of the quantity and type of glycosylation. Specifically, core 2 type O-linked structures are the only ones seen in human milk. On the other hand, at peak lactation, the majority of the proteins in bovine milk have core 1 type structures. In order for the protein-bound O-glycans to undergo complex extension, which involves the attachment of fucose and sialic acid residues and varying amounts of LacNAc repeats, core 2 or higher type structures must first be formed. Lewis type antigens, which are recognised by bacterial lectins, are based on these types of structures. An example of the wide diversity of structures that can be found in human milk is the identification of free glycans with a very high amount of fucosylation and a large size (M43000 Da).

Alternative Methods for Preparing Free Milk Oligosaccharide For Testing:

There have been a lot of studies done on the free oligosaccharides in milk, and so far scientists have found about 200 different structures in human milk. 6 Most documented techniques begin by removing the fat component since unbound oligosaccharides are hydrophilic [29]. Carefully removing the fat layer from the top of the milk sample is followed by centrifugation at 4 1C for 30-60 minutes. In the residual water, there are still plenty of free glycans and other water-soluble components such proteins, glycoproteins, and salts. Overnight cold ethanol precipitation is a common method for removing glycoproteins from skim milk. Additional pre-fractionation steps or the use of other targeted sample preparation strategies are necessary prior to analysing free glycans. As an illustration, Nin˜ onuevo et al. conducted a chloroform: methanol extraction on skimmed milk to isolate the protein fraction and remove any residual lipids. The free oligosaccharides were extracted after drying the supernatant, and proteins were precipitated from the watery fraction using cold ethanol. Prior to conducting analysis using graphitized carbon liquid chromatography-mass spectrometry (LC-MS) and carbon solid phase extraction (SPE), the free oligosaccharides were decreased [30, 31]. Ion exchange chromatography allows for further fractionation of the free oligosaccharide pool in the presence of neuraminic acid, yielding acidic

and neutral oligosaccharide fractions. Using TSK gel Amido-80 high performance liquid chromatography (HPLC) separation, we were able to produce nearly pure single oligo-saccharide structures, which allowed us to elucidate their structures in detail using nuclear magnetic resonance (NMR). Since it requires a large sample size, expensive equipment, and specialised knowledge, this methodology is not typically used for characterisation research.

Methods for Collecting Protein-Linked Glycan Samples

Protein bound oligosaccharides, which are present in both the aqueous and fat components of milk, are attached to a protein carrier, in contrast to free oligosaccharides found in the aqueous layer (Kolarich et al. manuscript in progress). Cold centrifugation delipidation of milk enables easy and rapid sub-fractionation of several milk glycoproteins according to their solubility in water, which can be useful depending on the study's purpose. Proteins in skim milk have been the subject of proteomic investigations that have employed chromatographic or selectrophoretic techniques for protein separation (PAGE, one-dimensional or two-dimensional) [32, 33]. Centrifugation and buffering can separate the proteins of the milk fat globule membranes from the non-homogenized milk's fat layer. Once released from the protein backbone, protein-bound oligosaccharides are much easier to study structurally using MS. Only on purified, known protein sequences can comprehensive site-specific glycosylation studies of glycoproteins typically be successful. The deglycosylated peptide or protein and the released N-glycan can both be analysed using the enzyme peptide-N-glycosidase F (PNGase F), making it the go-to method for releasing N-glycans from animal protein backbones. After electrophoretic separation, enriched or purified milk glycoproteins can be de-N-glycosylated in solution or in gel. Then, the liberated N-glycans and degly-cosylated proteins/peptides can be examined independently [34]. Additionally, hydrazine-based chemical de-glycosylation has been employed; in a study by Wilson et al., N-glycans were released from milk proteins immobilised on a PVDF membrane using PNGase F. Using this method, it is possible to acquire a worldwide glycomic profile of the N-glycans and O-glycans on a single protein sample. For the release of O-linked glycans, there is currently no commercially available enzyme that is similar to PNGase F. Because they are all very specific to certain glycan structures, none of the currently available O-glycanases are suitable for use in a global release protocol. Although O-linked glycans can be released from their protein backbone using chemical procedures such b-elimination or hydrazinolysis, the protein is typically damaged and cannot be further studied afterward. The O-glycans are released and reduced concurrently during reductive b-elimination, which safeguards the oligosaccharides against chemical peeling processes, which degrade the released glycans. Sodium borohydride in potassium or sodium hydroxide is used for this purpose. The majority of O-glycans from glycoproteins are released by reductive b-elimination because it is easy to do and may be repeated [35]. In order to release O-glycans while keeping the reducing end intact, b-elimination with hydrazine has been extensively investigated. This is because reductive b-elimination does not permit subsequent fluorescent tagging of the reducing terminal of O-glycans.

The Use of ESI-MS for the Examination of Underivatized Glycans

We believe that MS is the most effective technique for the direct and sensitive analysis of underivatized glycans, ranging from fmol to pmol. Electrospray ionisation mass spectrometry (ESI MS) has been effectively employed to characterise released glycans from milk protein samples without additional derivatization, which minimises sample preparation procedures. In order to conduct a simultaneous mass spectrometry analysis of neutral and acidic glycans, specific factors must be considered. Under online LC-MS settings, neutral and acidic glycans are effectively ionised in ESI positive mode [37], with the

relative structure distribution throughout the sample being reflected in the signal intensities of each mass. Another benefit of online LC-ESI-MS analysis is that it simplifies samples by chromatographically separating the various oligosaccharides, which allows for separate tandem MS/MS fragmentation analyses. The most popular technique for LC-ESI-MS study of underivatized glycans is porous graphitized carbon (PGC) LC-MS, which has recently been reviewed by Ruhaak et al. and has several groups' detailed descriptions of its uses, advantages, and limitations. Although reduced oligosaccharides are not hydrophobic and do not adhere well to reversed phase chromatographic matrices, they do adhere well to PGC, allowing for the separation and independent analysis of isobaric structures that differ solely in linkage. Prior to analysis, it is preferable to decrease the N- and O-linked oligosaccharides using PGC chromatography. This is because the chromatogram that is produced has two peaks for each structure, since the carbon stationary phase separates the a- and b-anomers of the same structural isomer. Enzymatically released N-glycans or free glycans can be reduced using sodium borohydride before analysis, while O-glycans that are released through reductive b-elimination release are already in the reduced state. There is an extra perk to this step: when the sugar is reduced, it adds a mass tag of around 2 Da to it, making it easier to read MS/MS spectra by clearly identifying the glycan terminus. Emission of negative ions In comparison to its positive ion equivalent, MS is generally thought of as being less sensitive. Negative ionisation, on the other hand, results in enhanced low fmol sensitivity and provides an appealing substitute for the detection and analysis of acidic glycans that have a negative charge. Negatively charged glycans typically have stronger signals under these conditions than neutral oligosaccharide structures with the same amount of sugar. Negative ion mode is useful for ionising neutral and acidic oligosaccharides equally, and normalisation factors allow for relative quantification. Emission of negative ions Sulfated and phosphorylated glycan structures can also be detected with PGC LC-ESI-MS analysis. The N- and O-glycans released from the membranes of milk fat globules in both human and bovine milk were studied using this method with success. Despite noticeable variations in core O-glycan structures between human and bovine milk, protein-bound oligosaccharides in fat globule membranes of both types of milk were shown to be substantially sialylated. While human MUC1 primarily included core 2 structures with variable extensions on the C6 branch and sialylation on the C3 branch, bovine MUC1 at peak lactation exhibited mono- and disialylated core 1 type O-linked structures. A positive carbon Ninн onuevo et al. analysed free oligosaccharides in human milk using LC-ESI-MS. Similar to Nin˜onuevo et al., Tao et al. conducted a thorough investigation into free oligosaccharides in bovine milk using a chip time-of-flight (chip-ToF) MS system. However, they employed off-line nano ESI Fourier transform ion cyclotron resonance (FTICR) MS to achieve high mass accuracy in both positive and negative modes of analysis. Again, they proved that human milk is far more complicated in terms of oligosaccharide composition and abun-dance than cow's milk. There were around 200 recognised compositions in the human sample, while bovine milk had only about ten. Oligosaccharides made from human and bovine milk differed significantly in the degree of sialylation and fucosylation. In contrast to human milk, which contains nearly no fucosylated oligosaccharides—which constitute around 70% of all human structures—cow milk contains a higher concentration of sialylation of the free glycans. A different method for LC-ESI-MS analysis of released, underivatized, protein-bound glycans has been proposed by Wuhrer et al. They use homemade Amide-80 nano columns to separate oligosaccharides at the nanoscale using the polar interactions between the hydroxyl groups of the oligosaccharides and the stationary phase. 78 Separations of free milk oligosaccharides on a bigger scale have frequently made use of the same chromatographic material. Combining liquid chromatographic separation with online ESI-MS analysis is common, but off-line, direct injection nano ESI-MS analysis has also accomplished a lot

in elucidating oligosaccharide structures. With Na ESI-MS, you can have enough MS analysis time with good sensitivity and very little sample consumption. This method of MS analysis of milk oligosaccharides has yielded valuable information about the patterns of fragmentation of milk oligosaccharide structures under different ionisation conditions, and it is preceded by a number of off-line purification processes.Numbers 59–60, 82–86 When comparing positive mode fragmentation to low collision induced dissociation (CID) circumstances, it was discovered that negatively charged ions cause more cross-ring fragmentation cleavages under the former. Using an ion trap apparatus and nano electrospray ionisation multi stage mass spectrometry (ESI-MS n) in negative mode, Pfenninger et al. assessed the offline analysis of milk oligosaccharides. 60 out of 59 Isomeric combinations of milk oligosaccharides were identified by identifying specific fragmentation patterns. Using a Q-ToF instrument, Chai et al. analysed oligosaccharides; their results also demonstrated that milk oligosaccharides' branching pattern may be better understood with the help of certain MS/MS fragmentation masses. The structural assignment process benefits greatly from the establishment of generic fragmentation rules for oligosaccharides; nonetheless, these rules differ between instruments and ionisation settings.

To create analyte ions, electrospray ionisation (ESI) involves transferring a diluted solution (1-10 μM) through a thin-diameter needle that is positioned close to the MS inlet capillary and is subjected to a potential of 1-4kV. Before being sucked into the mass spectrometer, the solvent is vaporised by drying gas from the ions. Fine charged droplets are generated by the potential difference between the tip and the capillary, along with high temperatures. This process is capable of producing ions of both positive and negative charge, and it is common to detect double charging. How molecular ions and their anionic or cationic adducts are formed is heavily impacted by the sample's pH and the presence of salts. ESI works well with capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) for online liquid flow separation. In most cases, ESI sensitivity goes down as glycan mass goes up. The low ionisation efficiency of conventional ESI makes it ineffective for neutral oligosaccharides. When compared to traditional electrospray, the sensitivity and tolerance to salts and other pollutants are both enhanced by the smaller droplets produced by static or flow nano-electrospray. To enhance the sensitivity of LC separations for neutral and acidic oligosaccharides, permethylation and reducing end modifications such hydrazine tagging and reductive amination are employed [38]. Another benefit of permethylation is that it allows acidic oligosaccharides to be detected as metal adducts in positive mode analysis. Hydrophobic reagent derivatization of N-linked gycans through hydrazone production improves chromatographic separation efficiency and detection sensitivity. Recent reports have detailed an easier and faster way to methylate heparin disaccharides, which circumvents some of the problems with the standard process for methylating glycosaminoglycans. Maltotriose polysaccharides' ESI mass spectra were found to be enhanced by adding the ionic liquids 2, 5-dihydroxybenzoic acid butylamine (DHBB) and α-cyano-4-hydroxycinnamic acid butylamine (CHCAB).

Using MALDI-MS to Examine Underivatized Glycans

Oligosaccharide analysis without chemical modification is made possible by matrix-assisted laser desorption ionisation (MALDI) MS, a commonly used, contamination-tolerant, and robust technology. When analysing oligosaccharides with MALDI-MS, singly charged metal adduct ions are mainly created, in contrast to ESI where the main ions produced during the positive ionisation process are singly ssand multiple protonated [M þ xH] x1 and deprotonated [M yH] y ions, respectively. In most cases, the most numerous ions are sodium ions, however by doping the matrix with the right salt, this can be changed to other cationic ions. As a result of salt production, sialylated ions typically

generate a combination of ions {[M H], [M þ Na]1, [M nH þ (n þ 1Na)]1}. Additionally, these ions are more prone to in-source and post-source fragmentation, leading to the simultaneous loss of sialic acid. It has been found that metastable peaks can be produced by post-source fragmentation when using reflectron ToF sensors. Harvey and Zaia have written outstanding reviews that go into further depth about the idea of MALDI ionisation as it pertains to oligosaccharides. The most frequent MALDI matrix for oligosaccharide analysis is 2,5-dihydroxybenzoic acid (DHB), however there are many others. The optimal combination of 2,4,6-trihydroxyacetophenone (THAP) and ammonium citrate yields matrices that are more suited to sialylated oligosaccharides. Similarly, MALDI-MS analysis of neutral and sialylated oligosaccharides has been accomplished with the use of the matrix 2,6-azathiothymine. Furthermore, the oligosaccharides on the MALDI plate can be directly treated with various exoglycosidase treatments to determine specific linkages, since 2,6-azathiothymine is a non-acidic matrix. In order to find the best circumstances for milk oligosaccharide analysis, Pfenninger et al. investigated various matrices and variables. They found that the neutral oligosaccharides were best analysed using DHB, 3-aminoquinoline, 2,6-azathiothymine, and 5-chloromercapto-benzothiazole. Additives like NaCl only improved DHB as a matrix. Their go-to for acidic oligosaccharide matrices was 2,6-azathiothymine with diammonium hydrogencitrate (DAHC) added. In order to use MALDI-ToF-MS to characterise the structure of neutral underivatized oligosaccharides, Tzeng et al. utilised matrices doped with alkali-hydroxide. Their structure might be better characterised by PSD-ToF-MS because of the partial alkaline degradation that happened during laser desorption/ionization. In a somewhat unusual technique to pre-MALDI sample preparation, Dreisewerd et al. employed high performance thin layer chromatography (TLC) to separate the native glycans prior to MALDI analysis, with the TLC plate serving as the MALDI target itself.58 Prior to ionising the sample with an infrared laser and a Q-ToF as a mass analyzer, glycerol was used as a liquid matrix to ensure uniform wetting of the silica gel. By coating the plate with a composite matrix of glycerol and a-cyano-4-hydroxycinnamic acid, they were also able to use a 337 nm UV-laser. Dreisewerd et al. demonstrated "mobility profiles" by scanning the laser beam across the separated analyte bands using this innovative TLC separation step preceding MS analysis.

Spot the sample on a MALDI target after dissolving it in an organic solvent, mixing it with a matrix solution, drying it, and then you can create ions using MALDI. The next step is to use an ultraviolet laser to ionise the analyte by transferring some of the energy absorbed by the matrix from the irradiated dry mixture location. Harvey provides a thorough overview of the use of MALDI in glycan analysis, including matrices that are especially useful for carbohydrates. In comparison to ESI, MALDI is more tolerant of impurities, ionises effectively across a wider mass range, and is more sensitive to glycans. This approach produces simpler spectra compared to ESI spectra due to the fact that most ions produced in the negative and positive modes are single-charged after protonation or deprotonation. Additionally, alkali or alkaline earth metals can form adducts with singly charged ions; these ions can produce fragment ions that are useful in tandem mass spectrometry. More internal energy is imparted to the analyte by MALDI compared to ESI, and labile groups like sulphates, phosphates, or sialic acids can be in-source fragmented. Glycans and glycosylated peptides are more susceptible to MALDI ionisation after permethylation, which stabilises the labile bonds of acidic groups. Combining MALDI with online separation techniques is challenging since previous reports of methods using liquid matrices have not been successful in achieving adequate sensitivity or practicality. It is possible, however, to isolate oligosaccharides offline and then analyse them using MALDI. Some of the recent approaches to enhancing MALDI ionisation have been developed by different research groups; these methods are highlighted here. Adding anionic

salts like NO3 − and Cl− to neutral N-glycans enhances their ionisation in negative ion mode and produces structurally interesting product ions when using tandem mass spectrometry. I− and HSO4 − are two examples of anion complexes that create a lot of [M +anion]− peaks but don't produce any useful fragments. The type of ions generated by MALDI is greatly affected by the matrix choice. To produce ions, traditional MALDI uses dried droplet and thin layer techniques. Even when protein is present, the underivatized carbohydrate signal can be improved by adding diamond nanoparticles (DNPs) to the matrix, according to recent studies. These outcomes were achieved by enclosing the DNPs in a matrix and then adding a sample with a 1:1 ratio of carbohydrates to protein. By labelling N-glycans utilising on-target techniques, such as reductive amination with 3-aminoquinoline, we have achieved greater quantification, increased sensitivity, and tandem mass spectra that are more structurally relevant. The sensitivity of N-glycans can be enhanced by using anion doped liquid matrices. Another method that has been shown to enhance MALDI spectra is a frozen aqueous mixture of matrix and carbohydrate. Ionisation by UV-MALDI was performed on a 2,5-dihydroxybenzoic acid matrix and carbohydrates in an aqueous/acetonitrile solution that had been frozen at 100K. When comparing the frozen mixture to the traditional dry matrix, we found that it was more sensitive and produced less fragmentation due to post-source degradation of oligosaccharides.

Oligosaccharides with N- and O-Links

In addition to preserving glycans within glyproteins or glycopeptides and labile changes like sulfation, electron-based activation generates a large amount of glycosidic and cross-ring fragments, which give precise structural information on the glycans. Previous work has focused on glycosaminoglycans and attempted to apply the same principles to other families of glycans in order to describe the impact of electron energy on fragmentation. To investigate how electron energy affects the disintegration of metal adducted molecular ions, permethylated maltoheptaose was employed. Electron capture dissociation (ECD) occurred for 1.5 eV kinetic energy electrons, while hot ECD (HECD) was found at an intermediate energy of 9 eV. At energies of 14 eV, electron excitation disintegration (EED) took place. In comparison to bigger metal cations (K+, Rb+, or Cs+), which resulted in fewer informative fragment ions and more metal losses, Li+ adducted molecule ions generated more structural information. Therefore, Li+ was utilised to fragment permethylated N-linked Man5GlcNAc2 (Man: mannose, GlcNAc: N-acetyl glucosamine). EED yielded more structural information than low energy ECD and CID, and it was able to detect five out of six glycosidic linkage positions [36-38]. Tandem mass spectrometry has been used to characterise the four blood groups: Lea, Lex, Leb, and Ley. These groups differ in the glycan modification of the proteins observed on the surface of red blood cells.43 Type 1, type 2, and type 4 globosides can now be characterised using a new method that uses negative ESI and CID to identify blood-group A, B, and H determinants. In this study, 0,2A-type cleavages were found to be diagnostic fragment ions that play a significant role in the assignment of blood-group and chain types. For reducing sugars, these diagnostic pieces only form under negative-ion conditions; they do not form under positive-ion conditions or for reduced alditols. This method can be applied to several types of reducing free oligosaccharides, such as those produced by acid hydrolysis of polysaccharides, endoglycoceramidase or ozonolysis of glycolipids, or hydrazinolysis or peptide-N-glycanases of N-glycans. There have been analyses of milk oligosaccharides utilising alternative methodologies, like CID and IRMPD. By combining CID and ETD in series on milk oligosaccharides, recent work compared the results with other activation methods including MS3 and CID [39–40]. The diverse types of linkages and branching patterns of the representative milk sugar samples may be better understood thanks to the abundance of cross-ring cleavage ions generated by ETD. All sorts of

oligosaccharides can be confidently structurally analysed with the use of ETD, as demonstrated here. Locating the specific locations of O-linked glycan alterations in a protein or peptide is no easy task. Attempts to identify the altered locations are typically thwarted when the glycan is cleaved during ion activation [41–44]. The protonated O-glycopeptide was analysed using IRMPD with a free electron laser (FEL) set up as a variable infrared light source. The fragments of amino acids b/y and glycosidic bonds B/Y types were found to be between 5.7 and 9.5 μm in size. The sodiated glycopeptide's spectra revealed prominent B/Y photoproduct peaks between 8.4 and 9.5 μm. More structural information about O-glycosylated peptides can be obtained by using a tunable laser to produce product ions from the peptide and the glycan, as demonstrated in this work. The information value of MS/MS data for glycopeptides can be enhanced by combining various activation methods with complimentary fragmentation products. The analysis of O-linked β-N-acetylglucosamine (O-GlcNAc) inside peptides was carried out in a recent work by combining higher-energy collisional dissociation (HCD) with ETD. ETD preserved the labile GlcNAc alterations needed to determine its amino acid level placement while producing sequence-informative peptide fragment ions, whereas HCD generated HexNAc signature ions. Based on the results, 83 different sites of O-GlcNAc alterations were discovered in proteins isolated from HEK293T cells using this approach. Combining HCD with ETD improves the useful fragmentation needed to identify posttranslational changes inside proteins, as demonstrated in this work.

Mass Spectrometry for the Examination of Proteins

A protein's mass-to-charge ratio can be determined by mass spectrometry (MS) analysis, which can detect and quantify molecules in both simple and complicated mixtures. MS has proven to be incredibly useful in many different areas, including proteomics. Our understanding of protein structure, function, modification, and global protein dynamics has been greatly enhanced by the recent development of high-throughput and quantitative MS proteomics processes [45, 46]. This review provides a summary of mass spectrometry's function in proteomics, goes over MS techniques and equipment, and briefly discusses sample prep and separation using liquid chromatography before MS analysis.

A biological system (such as a cell, tissue, or organism) and its proteins are the subject of proteomics research. Due to the ever-changing nature of protein expression, studying genomes and proteomics together is far more challenging than studying either genomics or transcriptomics independently. Posttranslational modification (PTM) occurs to the vast majority of proteins, adding another layer of complexity to the proteome. The vast potential of proteomics has only just come to light, thanks in large part to advances in mass spectrometry technology, within the past fifteen years [47-50].

The mass-to-charge (m/z) ratio is the basis for mass spectrometry, a sensitive method for detecting, identifying, and quantifying compounds. The biological sciences were the first to employ MS, which was initially created over a century ago to quantify the atomic weights of elements and the natural abundance of particular isotopes, to trace the movement of heavy isotopes through biological systems. Later on, MS was employed for nucleotide structure analysis and oligonucleotide and peptide sequencing.

Protein structure may be studied using MS after the invention of macromolecule ionisation techniques, such as electrospray ionisation (ESI) and air pressure chemical ionisation (APCI). One further benefit of ionisation is that it let scientists collect mass "fingerprints" of proteins and peptides, which they could then compare to databases to find previously unidentified targets. The relative and absolute amounts of target proteins were quantified using new isotope tagging methods [51-59]. Thanks to these innovations in technology, we now have techniques that can analyse samples in their solid, liquid, or gaseous forms. Analytes can be detected

in the attomolar range (10-18) by using modern mass spectrometers, which are very sensitive.

Functional protein analysis using mass spectrometry

Using the m/z ratio of ions, mass spectrometry can detect and quantify compounds in both simple and complicated mixtures. MS has proven to be incredibly useful in many different areas, including proteomics. Our understanding of protein structure [60-63], function and modification, and global protein dynamics has been greatly enhanced by the recent advent of high-throughput and quantitative MS proteomics processes. Reviewing MS methods and equipment, this overview explains mass spectrometry's function in proteomics. It also discusses the steps needed to prepare the samples for MS analysis, including using liquid chromatography for separation [64–66].

Conclusions:

An onslaught of new biological MS applications and technologies began with the advent of soft ionisation techniques like ESI and MALDI. Along with this expansion came enormous strides in ion detection and fragmentation options, which opened up a whole new realm of biomolecules that MS can analyse in terms of both sensitivity and selectivity. We still haven't seen the full potential of this technology. The preparation of the sample is still the first and most important step in doing a good MS analysis, regardless of any advancements in equipment. While this review primarily focuses on MS methods for milk analysis, many of the same methods may be used to analyse free and protein-bound oligosaccharides from various food sources.

These investigations have a wide range of applications, including but not limited to agriculture, conservation, biotechnology, and human diagnostics and therapies. Accurate protein identifications have many downstream applications, so the HUPO has developed a commonly accepted set of parameters for accurate identification and published a set of metrics to help practitioners and users evaluate MS protein identification data. We suggest running these measurements before doing any post-processing. Protein identification using non-MS data is not yet standardised, but what is often accepted is the employment of orthogonal techniques (e.g., MS and Western blotting) to confirm an identity suitable for hypothesis testing.

Advanced analytical methods for carbohydrates, as outlined in this review, are essential for the field's future success. Advances in carbohydrate analysis will depend on better methods for online separation of oligosaccharides, because carbohydrate samples from natural sources are very diverse. This encompasses chromatographic techniques as well as ion mobility, a relatively uncharted territory in the realm of carbohydrate analysis. There is still a long way to go before mass spectrometry can reveal the intricate structural variations in carbohydrates that have such a profound effect on their biological function. Carbohydrate mass spectrometry characterisation will be driven by developments in ion activation, with future advancements in electron-aided activation and photodissociation techniques likely to have the greatest impact. Although MS/MS techniques are producing impressive carbohydrate resolutions, MS3 and higher dimensional MSn studies are capable of delivering significantly more structural information. The development of automated systems for the selection of chain reaction intermediates is crucial for the widespread implementation of these methodologies. The acquisition, processing, and interpretation of mass spectra necessitates the creation of software that is specifically tailored to carbohydrates. The examination of carbohydrates has been made possible by advances in mass spectrometry technology. The sequencing of proteoglycan full-length chains is just one of several outstanding achievements over the past two years made possible by this.59 Proteomics applications have been a major force in the field of mass spectrometry, pushing the technology to new heights of sensitivity and resolution and inspiring the creation of complex hybrid mass

spectrometers that can perform tandem mass spectrometry measurements on complicated mixtures. Carbohydrate analysis is another area that gains from these innovations. The variety, minor structural variations, and lability of changes inherent in carbohydrates make them more demanding targets than proteins in many respects. Advancements in mass spectrometry will be driven by the demand for better methods to analyse carbohydrates.

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