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REVIEW ARTICLE

Decoding Sugars: Mass Spectrometric Advances in the Analysis of the Sugar Alphabet

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ABSTRACT

Monosaccharides play a central role in metabolic networks and in the biosynthesis of glycomolecules, which perform essential functions across all domains of life. Thus, identifying and quantifying these building blocks is crucial in both research and industry. Routine methods have been established to facilitate the analysis of common monosaccharides. However, despite the presence of common metabolites, most organisms utilize distinct sets of monosaccharides and derivatives. These molecules therefore display a large diversity, potentially numbering in the hundreds or thousands, with many still unknown. This complexity presents significant challenges in the study of glycomolecules, particularly in microbes, including pathogens and those with the potential to serve as novel model organisms. This review discusses mass spectrometric techniques for the isomer-sensitive analysis of monosaccharides, their derivatives, and activated forms. Although mass spectrometry allows for untargeted analysis and sensitive detection in complex matrices, the presence of stereoisomers and extensive modifications necessitates the integration of advanced chromatographic, electrophoretic, ion mobility, or ion spectroscopic methods. Furthermore, stable-isotope incorporation studies are critical in elucidating biosynthetic routes in novel organisms.

Abbreviations: 2AA, 2-aminoacridine; 3-NPH, 3-nitrophenylhydrazine; 4-MOBHA·HCl, O-(4-methoxybenzyl)hydroxylamine hydrochloride; 9AA, 9-aminoacridine; Aci, acinetaminic acid; AMAC, 2-aminoacridone; Api, apiose; APTS, 9-aminopyrene-1,4,6-trisulfonic acid; Ara, arabinose; Araf, arabinofuranose; Arap, arabinopyranose; ATP, adenosine triphosphate; Bac2Ac4Ac, 2,4-diacetylbacillosamine; BEH, Ethylene Bridged Hybrid; BGE, background electrolyte; BTME-H-APS, bistrimethoxysilylethane hydrolysis aminopropyltrimethoxysilane; CAD, charged aerosol detector; CCS, collision cross-section; CE, capillary electrophoresis; cIMS, cyclic ion mobility spectrometry; CMP, cytidine monophosphate; CSDB, Carbohydrate Structure Database; CV, compensation voltage; CZE, capillary zone electrophoresis; DHB, 2, 5-dihydroxybenzoic acid; DIMS, differential ion mobility spectroscopy; DMA, differential mobility analyzers; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DMBA, 4,5-dimethylbenzene-1,2-diamine; DMS, differential mobility spectrometry; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; DTIMS, drift tube ion mobility spectrometry; EI, electron ionization; EOF, electroosmotic flow; ESI, electrospray ionization; FAIMS, high-field asymmetric waveform ion mobility spectrometry; Fru6P, fructose-6-phosphate; Fuc2NAc, 2-acetamido-2,6-dideoxy-D-galactose; Fus, fusaminic acid; Gal, galactose; GalA, galacturonic acid; GalN, galactosamine; GalNAc, N-acetyl-galactosamine; GC, gas chromatography; GDP, guanosine diphosphate; Glc, glucose; Glc1P, glucose-1-phosphate; Glc6P, glucose-6-phosphate; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, N-acetyl-glucosamine; GMD, golm metabolome database; GMDB, Glycan Mass Spectral DataBase; GulA, guluronic acid; HDMS, high definition mass spectrometer; HEK, human embryonic kidney; HILIC, hydrophilic interaction liquid chromatography; HMDB, Human Metabolome Database; HPAE, high performance anion exchange; HPAEC, high performance anion exchange chromatography; HPLC, high-performance liquid chromatography; HR, high-resolution; IdoA, iduronic acid; IEC, ion exchange chromatography; IEX, ion exchange; IMS, ion mobility spectrometry; IP, ion pair; IR, infrared; IRIS, infrared ion spectroscopy; IRMPD, infrared multiple photon dissociation; IT, ion trap; KDG, 2-keto-3-deoxygluconate; KDN, keto-deoxy-neuraminic acid; KDO, 2-keto-3-Deoxy-D-Mannooctanoic Acid; KEGG, Kyoto Encyclopedia of Gene and Genome database; LC, liquid chromatography; Leg, legionaminic acid; Leg5Ac7AcAla, 5-N-acetyl-7-N-acetyl-D-alanyl-legionaminic acid; LIF, laser-induced fluorescence; MALDI, matrix-assisted laser desorption/ionization; Man6P, mannose-6-phosphate; ManA, mannuronic acid; ManNAc, N-acetyl-mannosamine; MMC, mixed mode chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; MSI, imaging mass spectrometry; MSTFA, N-methyl-N-trimethylsilyl-trifluoroacetamide; NDP, nucleotide diphosphate; Neu, neuraminic acid; Neu5Ac, 5-N-acetyl-neuraminic acid; Neu5Gc, 5-N-glycolyl-neuraminic acid; NeuNAc, N-acetyl-neuraminic acid; NIST, National Institute of Standards and Technology; NMR, nuclear magnetic resonance; NT, nucleotide; NulO, nonulosonic acids; PAD, pulsed amperometric detection; PBA, phenylboronic acid; PFBO, pentafluorobenzyloxime; PGC, porous graphitic carbon; PMP, 1-phenyl-3-methyl-5-pyrazolone; PPMP, 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone; Pse, pseudaminic acid; Rha, rhamnose; RNA, ribonucleic acid; RP, reverse phase; SAX, strong anion exchange; SFC, supercritical fluid chromatography; SIA, sialic acid; SLIM, structures for lossless ion manipulations; SNFG, Symbol Nomenclature For Glycans; SPE, solid phase extraction; TFA, trifluoroacetic acid; TIMS, trapped ion mobility spectrometry; TMS, trimethylsilyl; TMT, tandem mass tag; TOF, time of flight; TWIMS, traveling wave ion mobility spectrometry; UDP, uridine diphosphate; UHPLC, ultrahigh-performance liquid chromatography; UTP, uridine triphosphate; UV, ultraviolet; Xyl, xylose.

1 | Introduction

While nucleic acids and proteins have a limited number of building blocks (8 and 22, respectively (Sanger 1981); Chaudhuri and Yeates 2005; Alberts et al. 2015) carbohydrates can compromise a highly diverse spectrum of monosaccharides (Herget et al. 2008; Cummings 2024; Harvey 1999; Pabst et al. 2022). Notably, monosaccharides and their derivatives could number in the hundreds or even thousands, with many yet to be discovered (Imperiali 2019). For example, the Kyoto Encyclopedia of Genes and Genome database (KEGG) lists ~100 monosaccharides that have been identified in pathways across all organisms mentioned in the database (Cummings 2024; Kanehisa n. d.). One of the most abundant monosaccharides in cells is glucose, which is metabolized to produce ATP, the energy currency of the cell (Chandel 2021). The metabolic intermediates are precursors for a spectrum of other monosaccharides. These other sugars are not directly involved in the energy metabolism, but are required to produce a large variety of other glycoconjugates, as well as free oligo- and polysaccharides (Harvey 1999; Varki et al. 2015a). Their analysis is challenged by the diversity of carbohydrate building blocks, including numerous connectivity possibilities and stereochemical configurations. Consequently, the study of oligo- and polysaccharides is still less developed than that of nucleic acids and proteins (Grabarics et al. 2021).

Carbohydrate oligo- and polymers are abundant and omnipresent, such as cellulose in plant tissues, chitin in the exoskeleton of invertebrates, lactose in milk, and raffinose in legumes (Tharanathan 2002; Bode 2015). Smaller, but structurally diverse oligomers can be attached to proteins, lipids, and, as recently discovered, to small RNAs forming the so called "glycoRNAs" (Varki et al. 2015a; Flynn et al. 2021). These glycans are involved in regulating protein folding and turnover, or are exposed on the cells exterior where they are involved in signaling and immune responses (Varki et al. 2015a). The extracellular space is also rich in glycosaminoglycans, large carbohydrate polymers carrying modifications such as phosphorylation and sulfation. Glycosaminoglycans provide structural support to cells and tissues, facilitate cell signaling, and contribute to the integrity of the extracellular matrix (Bülow and Hobert 2006; Ricard-Blum 2017; Zappe et al. 2022). In addition, free oligosaccharides, such as those found in milk, show probiotic effects, defend against pathogens and support the immune system (Grabarics et al. 2021; Bode 2015). In microbes, they are also involved in host/pathogen interactions, protection, and contribute to adaptability and survival in competitive environments (Hooper and Gordon 2001; Lee et al. 2022; Nothaft and Szymanski 2010).

The biocatalytic incorporation of the individual monosaccharides into oligosaccharides and conjugates requires prior activation of the sugars. This is commonly achieved by linking them to nucleotides—forming the so-called nucleotide-sugars (glycosyl esters of nucleoside mono- or pyrophosphates)—and in some rare cases to dolichol phosphate (e.g. dol-Man and dol-Glc precursors in the endoplasmic reticulum) (Varki et al. 2015a).

Since monosaccharides, their derivatives and activated forms are crucial building blocks in many biological processes, the identification and quantification of these molecules are essential in both research and industry. Quantitative metabolomics studies and 13C flux analysis have emerged as powerful techniques, such as for fundamental studies on model organisms, and for evaluating metabolic engineering efforts in cell factories (Van Gulik et al. 2000; Hackett et al. 2016). Cell factories are promising alternatives to chemical synthesis, in producing precursors for pharmaceuticals and biopolymers (Koster et al. 2022; Ko et al. 2020). In the pharmaceutical industry, monitoring the monosaccharide composition of recombinant therapeutic glycoproteins is crucial for ensuring their efficacy and safety (Edwards et al. 2022). In addition, in medical applications, monosaccharide analysis aids in diagnosing metabolic disorders such as galactosemia and mucopolysaccharidoses (Gilbert-Barness and Farrell 2016). Furthermore, cellular metabolism is significantly altered in cancer cells compared to normal cells, enabling the former to sustain high rates of proliferation (Cantor and Sabatini 2012). Finally, in environmental studies, monosaccharide analysis helps in tracking organic matter sources and biodegradation processes in soil and water samples (Otto and Simpson 2007).

While routine methods have been established for many of the aforementioned applications over the past decades, expansions to the microbial world beyond a few well-studied model organisms remains a challenge. Especially for uncultured microbes such as those involved in global biogeochemical cycles, members of the human microbiome, and potential symbiotic or pathogenic microbes, the monosaccharide building blocks and their metabolic routes are, to date, poorly understood. Microbes typically produce many species-specific monosaccharide derivatives, likely influenced by their ecological niche and nutrient availability. Using these monosaccharides, glycoconjugates are produced and presented on their cell surface, often forming important virulence factors and components of the cell wall (Samuel and Reeves 2003). However, the composition and the specific activated substrates involved in the production of most microbial glycomolecules remain largely unexplored. The same holds true for plants, where the biosynthetic routes of essential sugars of the plant cell wall, such as 3-deoxy-lyxo-2-heptulosaric acid and aceric acid (3-carboxy-5-deoxy-xylofuranose), have not yet been identified (Seifert 2004; Mikkola 2020). Additionally, the role of activation with unusual nucleotides, such as thymidine diphosphate, is currently unknown (Mikkola 2020).

However, the uniqueness of microbial monosaccharide derivatives, makes their metabolic pathways promising drug targets, increasing the interest in these sugars. For example, the enzyme non-hydrolyzing C2 epimerase, which is involved in the production of UDP-Man2NAc and UDP-L-Fuc2NAc, is found in most archaea and bacteria, but is absent in human cells. Inhibitors of this enzyme have shown promising results against methicillin-resistant *Staphylococcus aureus* (Srivastava, Sunthar, and Balaji 2020). The identification of novel monosaccharides has also led to the discovery of new antibiotics. For example, the biosynthesis of the antibiotic erythromycin involves a series of glycosylation steps catalyzed by specific glycosyltransferases, a process that is difficult to replicate via chemical synthesis (Moncrieffe et al. 2012). In addition, the identification of rare sugars—such as D-tagatose, used as a pharmaceutical and low-calorie sweetener—has led to the discovery of specific enzymes to catalyze their synthesis (Parıldı et al. 2022). Generally, carbohydrate-active enzymes are of

significant interest in the development of biotechnological applications involving carbohydrate molecules (Paliya et al. 2023).

A substantial number of reviews have focused on the mass spectrometric characterization of various oligo- and polysaccharides, glycoproteins, glycolipids, and other glycomolecules (Grabarics et al. 2021; Harvey 2023a; Lee and Ni 2019; Girgis et al. 2024; Trbojević-Akmačić et al. 2022; de Haan et al. 2020; Gerwig 2021a; Kailemia et al. 2014; Harvey 2011a; Wang et al. 2023, 2021; Pabst and Altmann 2011; Sarbu and Zamfir 2018; Hykollari, Paschinger, and Wilson 2022; Manz and Pagel 2018; Wang, Wang, and Lageveen-Kammeijer 2022a; Lageveen-Kammeijer et al. 2022). Furthermore, although sample preparation methods are essential for the identification of these compounds, this topic warrants a review of its own, and we have only covered it by referencing relevant literature where applicable. Therefore, this review focuses on advanced mass spectrometric approaches facilitating the identification and quantification of the fundamental monosaccharide building blocks, their derivatives, and activated forms. Mass spectrometry enables not only untargeted analysis but also the detection of sub-stoichiometric quantities in highly complex matrices. However, the mass spectrometric analysis of sugar molecules is hampered by the presence of many stereoisomers and the extensive molecular diversification, which is particularly prevalent in microbes. This necessitates the integration of high-performance separation techniques such as liquid and gas chromatography or capillary electrophoresis alongside mass spectrometric detection for many applications. On the other hand, sophisticated fragmentation experiments, ion mobility measurements, and infrared spectroscopy can provide additional structural information. Stable isotope tracing and metabolic network analysis have also emerged as powerful tools for exploring the biosynthetic routes of sugars in novel species. Finally, this review highlights emerging medical, biotechnological, and industrial applications.

2 | Monosaccharide Chemistry and Diversity

Simple monosaccharides are typically chains of chiral hydroxymethylene units and follow the general empirical formula $C_n(H_2O)_n$, where n ranges from 3 to 9, or in rare cases, up to 10. They terminate with a hydroxymethyl group at one end and contain either an aldehyde group (aldoses) or an α-hydroxy ketone group (ketoses) at the other end (Harvey 1999; Varki et al. 2015a; Seeberger 2017). Accordingly, glyceraldehyde is the simplest aldose, and dihydroxyacetone is the simplest ketose. Apart from dihydroxyacetone, all monosaccharides possess a number of asymmetric carbon atoms equal to n–2 for aldoses and n–3 for ketoses, where n is the number of carbon atoms (Harvey 1999; Varki et al. 2015a; Seeberger 2017). Consequently, the number of stereoisomers for each monosaccharide is 2 to the power of the number of asymmetric carbon atoms. For example, an aldohexose (with a total of six carbons) has four asymmetric carbon atoms (n–2 for aldoses) and, therefore, it has 2^4 = 16 possible isomeric forms.

Sugars can form cyclic structures called furanose and pyranose rings. Furanose rings are five-membered structures composed of four carbon atoms and one oxygen atom, while pyranose rings are six-membered structures comprising five carbon atoms and one oxygen atom. Hexoses, such as glucose, predominantly form pyranose rings but can also adopt a furanose structure, especially in certain derivatives or under specific conditions. Similarly, heptoses, containing seven carbon atoms, can form both furanose and pyranose rings depending on environmental conditions. In aqueous solutions, sugars exist in equilibrium between their cyclic and open-chain forms. In the case of aldohexoses, the pyranose form is created when the hydroxyl group on C-5 attacks the carbonyl carbon (C-1), forming a hemiacetal linkage. This reaction can occur from either side of the carbonyl group, leading to the formation of either the α- or β-anomer (Figure 1).

The diversity of monosaccharides is even further enhanced via modifications of the hydroxyl or carboxyl groups. For example, sugar-specific amino- or amidotransferases introduce an amino functionality to sugar phosphates or sugar nucleotides, forming the so called aminosugars (Seeberger 2017). Free amine groups in sugars are rare (only found in some glycosaminoglycans), and are commonly acetylated to N-acetamido groups in, for example, the widely found GlcNAc, GalNAc, and NeuNAc monosaccharides. Other frequent modifications include the replacement of hydroxyl groups with hydrogen atoms to form deoxysugars (Seeberger 2017). Common deoxy sugars include 2-deoxyribose, which is part of the DNA, and 6-deoxy-galactose (fucose), a widely utilized monosaccharides in vertebrates (Varki et al. 2015a). In microbes, many dideoxy sugars are found, such as abequose, a 3,6-dideoxyhexose present in the lipopolysaccharides of bacteria including *Citrobacter* and *Pectobacterium* (Katzenellenbogen et al. 2009; Kowalczyk et al. 2022). Other enzymes esterify the hydroxyl groups to produce phosphate esters, esters with acetic acid or fatty acids, and sulfate esters (Seeberger 2017). The phosphorylation of sugars makes them generally more reactive. Moreover, the addition of phosphate to glucose traps this monosaccharide within the cell (Ahern 2021). Further alkylation reactions can occur including methylation through a range of dedicated methyltransferases (Seeberger 2017). This not only introduces hydrophobicity, but also neutralizes charges on amines and carboxylic acids (Pabst et al. 2013a). Finally, the oxidation of neutral sugars can form carboxylic acid groups. For instance, the oxidation of C6 yields uronic acids, the oxidation of the aldehyde at C1 produces aldonic acids, and the oxidation of both C6 and C1 results in aldaric acids (Varki et al. 2015a; Bhagavan 2002; Touster 1969). A noteworthy case are nonulosonic acids, a class of nine-carbon alpha-keto acids, where the C2 ketone group is oxidized to a carboxylic acid (Varki et al. 2015a; Schauer and Kamerling 2018). These sugars are produced in cells via a condensation reaction between specific hexosamine sugars and phosphoenolpyruvate, forming a nine-carbon backbone and a terminal alpha-keto acid moiety (Varki et al. 2015a; Schauer and Kamerling 2018; Rangarajan et al. 2009). Some microbes contain dedicated lyases known as sialic acid aldolases, which, for example, catalyze the reversible aldol cleavage of the nonulosonic acid neuraminic acid (Neu), from pyruvate and ManNAc (Romero-Rivera, Iglesias-Fernández, and Osuna 2018). The broader group of known nonulosonic acid derivatives contains some 100 members. Neuraminic acid (Neu) and its unmodified variant, keto-deoxy-neuraminic acid (Kdn), are commonly found in animal tissues.

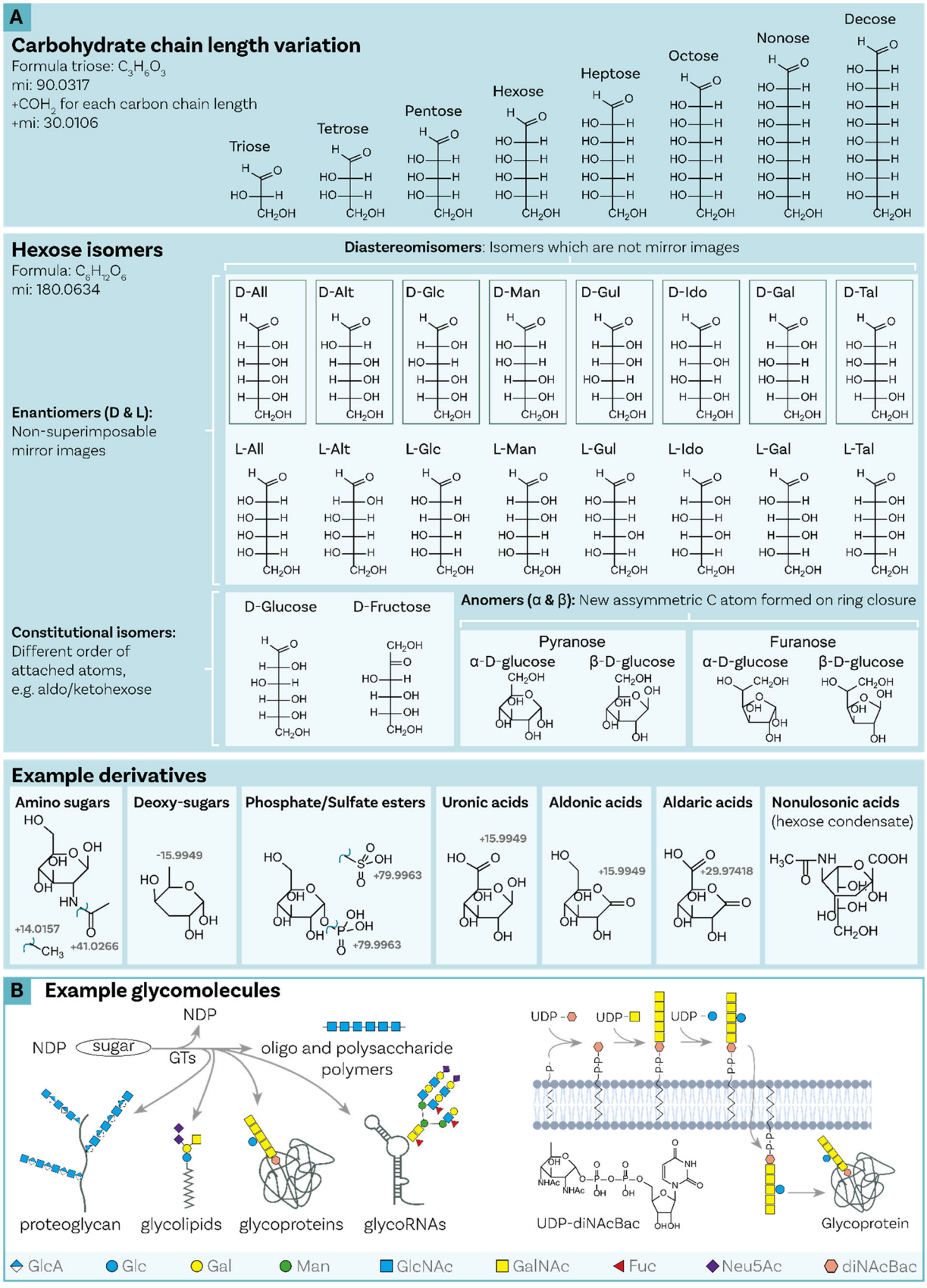


FIGURE 1 | Legend on next page.

Pseudaminic acid (Pse) and its stereoisomers, legionaminic acid (Leg), acinetaminic acid (Aci), and fusaminic acid (Fus), have been exclusively identified in prokaryotes (Schauer and Kamerling 2018; Lewis et al. 2009). Prokaryotic nonulosonic acids are often modified with, for example, formyl, glyceryl, hydroxybutyryl, lactoyl, and glutamyl groups, among many others (Kleikamp et al. 2020; Angata and Varki 2002). Neuraminic acids play a role in processes such as cell–cell interactions, signaling, adhesion, and immune responses. Similarly, Pse/Leg derivatives are found on the bacterial cell surface, including lipopolysaccharides, adhesins, pili, and flagella (Varki et al. 2015a; Schauer and Kamerling 2018).

Highly modified and rare monosaccharides are particularly frequent in microbes, where they often play crucial roles in determining their biological activity (He and Liu 2002). However, explanations for the development of modifications and rare monosaccharides are only speculative. For example, while the derivative Bac2Ac4Ac is present in the glycoproteins of *Campylobacter jejuni*, it can also be replaced by other N-acetyl sugars, such as Glc2NAc or Fuc2NAc (Srivastava, Sunthar, and Balaji 2020), demonstrating that similar properties can arise from different monosaccharide building blocks. This enhances adaptability and may be particularly crucial for the survival of microbes in competitive environments with fluctuating nutrient availability. The spectrum of monosaccharide building blocks is not dependent on the proteome size and differs greatly between species. Although a common group of monosaccharides is found across all three domains of life, most organisms do not use the exact same set of monosaccharides (Srivastava, Sunthar, and Balaji 2020). The development of more specific monosaccharides likely emerged from the neo- or sub-functionalization of enzymes at later stages of evolution (Srivastava, Sunthar, and Balaji 2020).

Richard D. Cummings recently introduced the "periodic table of monosaccharides" (Figure 2), analogous to the periodic table of elements, which illustrates the diversity and similarities of monosaccharides via their systematic arrangement (Cummings 2024). Currently, the table contains the 103 monosaccharides listed in the KEGG (Kanehisa) database and on the Symbol Nomenclature for Glycans (SNFG) website (Varki et al. 2015b). The sugars are ordered by the number of carbon atoms in their backbone, and are classified into five major groups: aldoses, ketoses, aminosugars, uronic acids, and deoxysugars. These groups are further organized into periods, starting with 3-carbon trioses and extending to 10-carbon decoses.

The table also lists rare sugars, namely, branched-chain sugars (Shafizadeh 1956), apart from common monosaccharides. These sugars follow the general empirical formula of monosaccharides, with the exception that the backbone hydroxyl groups are replaced by another carbon bond, thereby forming a branch. Many of these sugars are biologically active and exhibit antimicrobial and antiviral activities (Shafizadeh 1956; Kim et al. 2009). Plants can also contain branched sugars, such as hamamelose (2-C-hydroxymethyl ribose). This sugar is involved in the regulation of ribulose bisphosphate carboxylase/oxygenase, an enzyme responsible for CO_2 assimilation in higher plants, and many photosynthetic bacteria (Kim et al. 2009; Pičmanová and Møller 2016). Another branched chain sugar widely found in plants is apiose, which bridges pectin molecules to regulate the stiffness of plant cell walls (Pabst et al. 2013a; O'Neill et al. 1996).

As briefly mentioned in the introduction, individual monosaccharides are activated before incorporation into oligosaccharides and conjugates, which is often performed by linking them to nucleotides. The first nucleotide-sugar (i.e., UDP-Glc) was discovered by Luis F. Leloir and his colleagues, for which he was awarded the Nobel Prize in Chemistry in 1970 (Cardini et al. 1950). UDP-Glc is synthesized from glucose 1-phosphate (Glc1P) and UTP in a reversible reaction catalyzed by UDP-Glc pyrophosphorylase. In eukaryotes, UDP-Glc is the starting point for the synthesis of many NDP-sugars (Mikkola 2020; Ashihara, Crozier, and Ludwig 2020). Exceptions include sugars such as GDP-mannose and GDP-fucose, which use mannose 1-phosphate as the starting point. Mannose-1P can be obtained from glucose-1-phosphate (Glc1P) through the actions of the glucose- and mannose-6-phosphate isomerases, and phosphomannomutase. UDP-Gal can be synthesized from UDP-Glc by UDP-Glc 4-epimerase as part of the Leloir pathway (Frey 1996). UDP-Glc is also the precursor for many other common nucleotide sugars, such as UDP-Rha, and UDP-GlcA (Ashihara, Crozier, and Ludwig 2020). UDP-GlcA is the starting point for the synthesis of UDP-Xyl and UDP-Api. UDP-Ara can be produced from UDP-Xyl by UDP-Xyl 4-epimerase. UDP-Ara is first generated in the pyranose form and then converted into the furanose form by the enzyme UDP-Ara mutase (Pabst et al. 2010; Konishi et al. 2007).

Alpha-keto-acid sugars such as octulosonic acid (e.g. KDO) and nonulosonic acids (NulOs) are special cases as these are exclusively activated by CMP (Mikkola 2020; Pabst et al. 2010). While CMP-Neu5Ac is synthesized from UDP-GlcNAc via ManNAc and other intermediates, the desamino analog KDN is produced from mannose-6-phosphate (Mikkola 2020). The bacterial analog pseudaminic acid (Pse) is generated from UDP-N-acetylglucosamine and an altrose intermediate

FIGURE 1 | Diversity of monosaccharide building blocks. (A) The diversity of monosaccharides, highlighting variations in carbon atom count, stereochemistry—including diastereomers, enantiomers, and constitutional isomers—the positioning of the carbonyl group, which differentiates aldoses from ketoses, and isomerization resulting from ring formation (α/β pyranose and furanose forms). (B) The biocatalytic incorporation of monosaccharides into various glycomolecules. e.g., into proteoglycans, glycolipids, glycoproteins, or glycoRNAs, or free poly and oligosaccharides, by glycosyltransferases (GTs) requires the prior activation of these sugars. This activation is typically achieved by converting them into nucleotide-activated sugars (e.g., UDP-diNAcBac), which are glycosyl esters of nucleoside mono- or pyrophosphates (NDPs). As an example, in *Campylobacter jejuni* a heptasaccharide is sequentially assembled on a polyisoprenyl diphosphate carrier from activated monosaccharides and then transferred to the asparagine side chain of acceptor proteins. All, allose; Alt, altrose; diNAcBac, di-N-acetyl-bacillosamine; Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; GTs, glycosyltransferases; Gul, gulose; Ido, idose; Man: mannose; Tal, talose; UDP, uridine diphosphate. [Color figure can be viewed at wileyonlinelibrary.com]

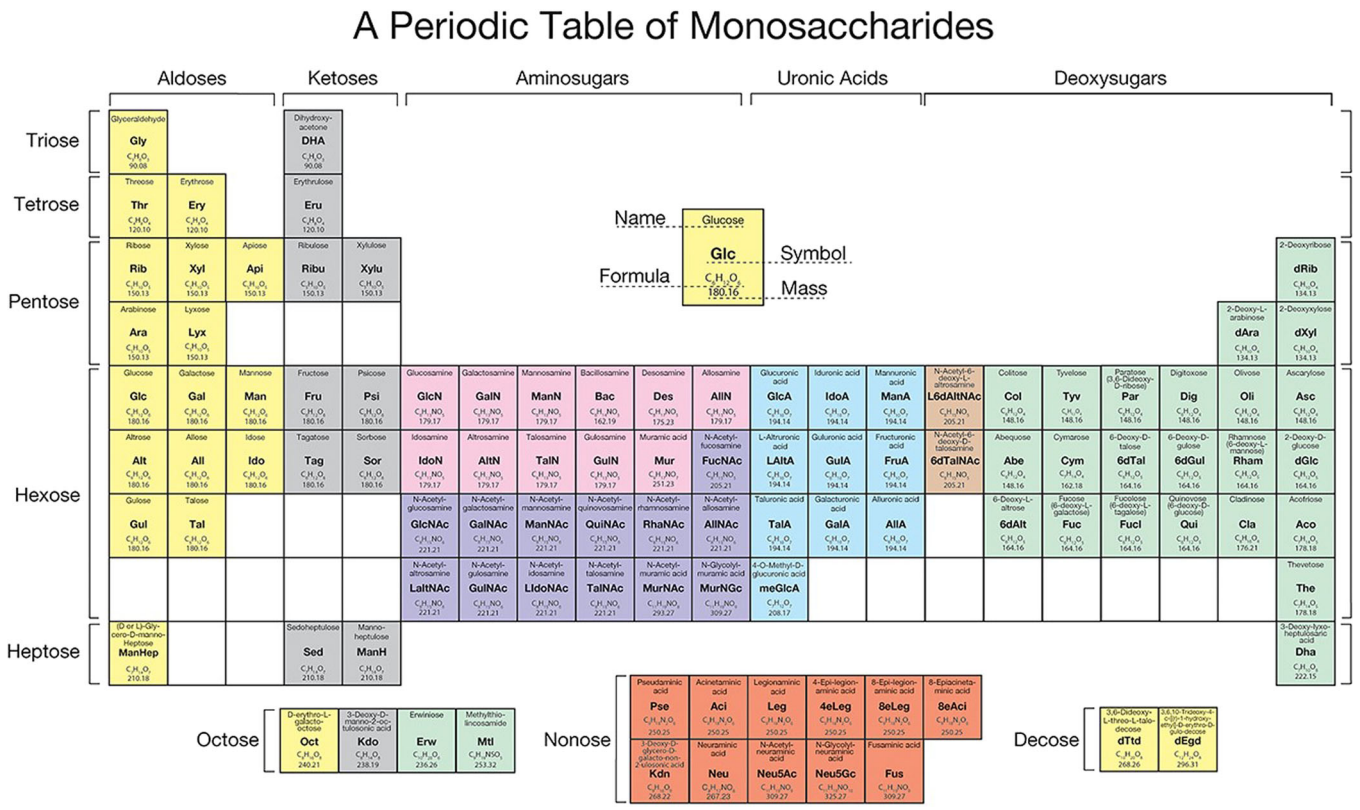


FIGURE 2 | The periodic table of monosaccharides, which was introduced by Richard D. Cummings, in 2024 (Cummings 2024). The monosaccharides listed on the KEGG and SNFG website were systematically organized into a table. The sugars are arranged by the number of carbon atoms and classified into major groups: aldoses, ketoses, amino sugars, uronic acids, and deoxy sugars. These groups are further organized into categories, ranging from 3-carbon trioses to 10-carbon decoses. Figure adapted from Cummings, Richard D. A periodic table of monosaccharides. Glycobiology, 202, 34.1, cwad088, by permission of Oxford University Press, Society for Glycobiology. [Color figure can be viewed at wileyonlinelibrary.com]

(6-deoxy-Altdi-NAc), while the legionaminic analog is produced from a bacillosamine (a glucosamine derivative) intermediate (Lewis et al. 2009; Zamora et al. 2017; Schoenhofen, Young, and Gilbert 2017). Interestingly, the known Pse biosynthesis pathway uses UDP precursors, while the known Leg biosynthesis pathway employs GDP-linked precursors (Ricaldi N et al. 2012). Overall, the nucleotide sugar metabolism involves a branched network of pathways with many interconversions and has only been well-established for a limited number of mammalian model cells, yeast, and a few bacterial pathogens such as *E. coli* and *M. tuberculosis*.

3 | Liquid Chromatography Coupled With Mass Spectrometry

One of the most widely employed separation techniques combined with mass spectrometry is liquid chromatography, mainly due to its suitability for polar and thermally labile molecules found in biological material that are challenging to analyze using historical gas chromatographic methods. Although the direct coupling of liquid chromatography with mass spectrometry was long considered difficult, the development of the electrospray ionization interface by Fenn et al. provided a solution that facilitated coupling and offered soft ionization of highly polar molecules—such as cellular metabolites, peptides, nucleotides, oligosaccharides, and even intact proteins (Fenn et al. 1989; Whitehouse et al. 1985). Various chromatographic separation phases have been employed for the separation of monosaccharide derivatives before mass spectrometric detection, each having their own advantages and disadvantages. In the following, we will discuss and review the most recent separation techniques which have been coupled to mass spectrometry to explore the fundamental sugar building blocks, including reverse phase chromatography, ion exchange chromatography, hydrophilic interaction chromatography, porous graphite carbon chromatography, mixed mode chromatography and supercritical fluid chromatography.

3.1 | Reverse-Phase Chromatography

Reverse phase (RP) chromatography employs a non-polar stationary phase, typically composed of long alkyl chains covalently attached to a solid support. The polar mobile phase consists of an aqueous-organic solvent, and organic compounds are separated based on hydrophobicity (Rassi 2021; Poole 2023). RP chromatography is appealing due to its ease of use and widespread application in analytical research laboratories. Moreover, a significant advantage is its robustness, and the possibility to employ volatile buffers that are compatible with the electrospray interface (Vreeker and Wuhrer 2016; Fan et al. 2019). Nevertheless, the polar and hydrophilic nature of monosaccharides and their derivatives results in poor retention

on conventional reverse-phase resins. Additionally, the ionization efficiency of sugars can be low (Fan et al. 2019; Wu et al. 2014). Therefore, adduct formation between uncharged sugars and ions, such as Cs^+, Na^+, and $NH4^+$ for positive ionization or Cl^- and CH_3CO^- for negative ionization, was introduced (Cai and Cole 2002; Hammad et al. 2009; Kohler and Leary 1995; Harvey 2005; Rogatsky et al. 2005) The ions can either be introduced post-column through a make-up flow or a triaxial electrospray probe (Kohler and Leary 1995; Rogatsky et al. 2005), or derived directly from the solvent buffer (Hammad et al. 2009). However, in these methods mostly underivatized carbohydrates were analyzed, therefore non-reverse phase separation phases were typically used. While adduct formation improved detection, it did not enhance the separation of sugar stereoisomers.

To address these challenges, various derivatization methods have been developed to decrease the polarity and increase ionization efficiency of sugars. Historically, different pre- and post-column derivatization methods were developed to increase retention or to introduce UV-absorbing groups to monosaccharides (Simatupang and Dietrichs 1978; White, Kennedy, and Golding 1979; Hase 1995; Gao et al. 2003). However, many of these early methods, such as benzoylation of hydroxyl groups, provided anomeric mixtures from reducing carbohydrates and provided poor derivatization yields (White, Kennedy, and Golding 1979). Dansylhydrazine was introduced which reacts specifically with aldehydes and ketones under mild conditions, introducing a fluorescent tag, which enabled reverse phase separation and fluorescence detection (Alpenfels 1981). Later, Han et al. employed another hydrazine label, 3-nitrophenylhydrazine, for small sugars. These were analyzed on a pentafluorophenyl-bonded phase column coupled with electrospray ionization mass spectrometry (ESI-MS) detection (Han et al. 2016a). Using a 13C-labeled internal standard, this method demonstrated excellent separation and quantitative properties. Alternatively, reductive amination of sugars was introduced for oligosaccharides by Hase et al. which provided a single derivative from reducing carbohydrates. Pyridylamination not only improved chromatographic separation but also facilitated sensitive analysis through fluorescence detection (Hase, Ikenaka, and Matsushima 1981). However, reductive amination occurs under acidic conditions, which can lead to the loss of acid-labile groups such as O-sulfate groups and sialic acid residues on oligosaccharides (Zhang et al. 2010). Honda et al. developed derivatization through condensation with an active methylene group, exemplified with the reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) for monosaccharides. Under basic conditions two PMP molecules were added to the reducing end of each monosaccharide (Honda et al. 1989). The hydrophobic PMP molecules enhanced monosaccharide separation on reverse-phase chromatography and enabled detection by UV. Since then, PMP has been widely used for monosaccharide analysis especially because of its quantitative conversion under mild conditions. However, the specificity of UV detection is generally low, therefore, separation conditions have been optimized to allow coupling to electrospray ionization mass spectrometry (Fan et al. 2019; Wu et al. 2014; Durán-Álvarez et al. 2021; Guo et al. 2019). Combined with product ion scanning, this provides high selectivity and accurate quantification of various monosaccharides from complex biomass samples (Fan et al. 2019). Additionally, micro-scale hydrolysis and PMP derivatization were performed in a 96-well plate without extraction or drying steps, followed by ESI-MS analysis. This method demonstrated increased throughput and efficiency (Rühmann, Schmid, and Sieber 2014).

Xu et al. recently reported an improved method that facilitated the simultaneous detection of PMP-labeled sugars, including underivatized sialic acids, by RP-MS in multiple reaction monitoring (MRM) mode. The authors optimized PMP concentrations to ensure complete derivatization while avoiding ion suppression caused by excess reagent. Separation was achieved in less than 10 min while detection limits reached attomole levels for some sugars, with linear ranges spanning up to six orders of magnitude (Xu et al. 2018).

Amicucci et al. developed a method for the absolute quantitation of 14 monosaccharides. This method involved a 96-well plate hydrolysis and PMP derivatization procedure, followed by a rapid and sensitive 10-min reverse-phase separation and triple quadrupole mass spectrometric analysis (Amicucci et al. 2019). Recently, Salas and colleagues developed an improved PMP derivatization approach followed by RP-ESI-Orbitrap MS analysis. This method enabled the identification and quantification of 18 monosaccharide derivatives, including neutral sugars, aminosugars, N-acetylamino sugars, and uronic acids. Notably, the analysis of acid-hydrolyzed samples from archaea, bacteria, fungi, and plants distinguished between these taxonomic groups based on their sugar markers (Salas et al. 2023) (Figure 3).

The combination with Orbitrap mass spectrometry demonstrated the potential to characterize rare and species-specific compounds commonly found in microbes. Zhang et al. introduced the alternative label 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone (PPMP), which produced mono-PPMP labeled monosaccharides under similarly mild conditions (Zhang et al. 2010). The mono-PPMP derivatives demonstrated better chemical stability than the bis-PMP derivatives. Moreover, excess label was more easily removed. The label was applied to determine the polysaccharide composition of *Spirulina*, dried biomass of the bacteria *Arthospira pletensis (*Zhang et al. 2010*)*. Anumula introduced reductive amination using the fluorescent label anthranilic acid (2-aminobenzoic acid) in the presence of sodium cyanoborohydride (Anumula 1994). Excess reagent removal was unnecessary because monosaccharide derivatives were separated from the reagent peak on a reverse-phase column. However, performing reductive amination in a methanol-acetate-borate buffer at elevated temperatures resulted in some epimerization of glucosamine to mannosamine. The separation of anthranilic acid and PMP-derivatized sugars has been optimized for small sample volumes and the separation of pentose and hexose sugars including methylated hexose variants (Stepan and Staudacher 2011). A spectrum of different tags has been developed by then (Gerwig 2021b; Harazono et al. 2011). For comprehensive comparative studies and reviews on different tags, we refer to earlier studies (Gerwig 2021b; Royle 2017; Pabst et al. 2009; Ruhaak et al. 2010a; Keser et al. 2018; Šoić et al. 2022). Many applications have relied solely on fluorescence or UV detection and direct comparison of retention times to standards (Stepan and Staudacher 2011; Gerwig 2021b; Dhume, Saddic, and Anumula 2008; Grass et al. 2011;

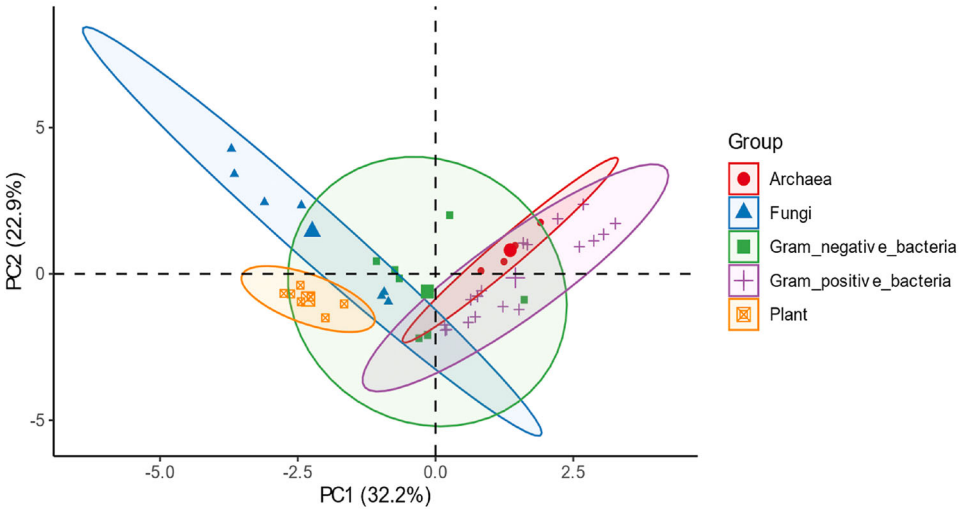


FIGURE 3 | A differentiation between archaea, bacteria, fungi, and plants based on their sugar markers (Salas et al. 2023). The monosaccharides were measured following PMP labeling of the acid-hydrolyzed samples. Reprinted with permission from Salas et al. A rapid and sensitive assay to quantify amino sugars, neutral sugars and uronic acid necromass biomarkers using pre-column derivatization, ultrahigh-performance liquid chromatography, and high-resolution mass spectrometry. Soil Biology and Biochemistry, 2023, 177: 108927. [Color figure can be viewed at wileyonlinelibrary.com]

Hamiot et al. 2023). However, complex sample matrices, unexpected sugars, derivatives or other conversion products take advantage from additional mass spectrometric detection (Wang et al. 2007; Windwarder et al. 2016). Zhu et al. utilized acid hydrolysis and PMP labeling, followed by reverse-phase high-performance liquid chromatography and off-line mass spectrometry, to analyze glycosaminoglycans (Zhu et al. 2014). The mass spectrometric detection enabled the unambiguous identification of acidolysis-resistant components, such as disaccharides, which can be easily misinterpreted when relying solely on fluorescence detection. Additionally, fragmentation provided tags to distinguish between heparin/heparan sulfate and chondroitin sulfate/dermatan sulfate (Zhu et al. 2014). Volatile buffers suitable for direct coupling to electrospray ionization interfaces often do not provide sufficient separation performance to distinguish all pentose and hexose diastereomers (Windwarder et al. 2016). Consequently, mass spectrometric coupling often comes at the cost of suboptimal separation. Windwarder and colleagues, however, developed a method based on permethylation of anthranilic acid-labeled monosaccharides, enabling the chromatographic separation of several pentose and hexose diastereomers (Windwarder et al. 2016). Additionally, their mass spectrometric approach allowed the determination of the degree of methylation of monosaccharides by using deuterated iodomethane in the permethylation step (Figure 4). Rath et al. investigated the *Selenomonas sputigena* flagellin O-glycan composition using the anthranilic acid-labeling/permethylation approach, which allowed to assign a methylated deoxysugar as methylated rhamnose (Rath et al. 2018).

Galermo and colleagues expanded the compositional analysis of oligosaccharides to include glycosidic linkage analysis (Galermo et al. 2018). In their method, oligosaccharides were first permethylated, hydrolyzed, and subsequently derivatized with PMP. The monosaccharide derivatives were then separated by reverse-phase chromatography and detected using MRM (Figure 5). This approach enabled the rapid determination of terminal, linear, bisecting, and trisecting monosaccharide linkages (Galermo et al. 2018). Recently, Wang et al. developed a "paired derivatization approach" employing the label O-(4-methoxybenzyl) hydroxylamine hydrochloride (4-MOBHA·HCl). Samples were labeled with the native form (4-MOBHA·HCl), while reference standards were labeled with a deuterated form (d3-4-MOBHA·HCl) (Wang et al. 2022b). This mass spectrometric targeting of both variants improved quantification accuracy and sensitivity in complex matrices such as plant and feces samples.

An alternative derivatization method, which also encompassed ketose sugars (e.g., fructose), was achieved through direct acetylation. This increased the hydrophobicity of sugars, enabling analysis by RP-ESI-MS. The method was applied to determine the monosaccharide composition of polysaccharides from various plants and fungi. Characteristic ion transitions were observed for aldopentoses, uronic acids, ketohexoses, aldohexoses, methyl-aldopentoses, and alditols (Gao et al. 2023). However, the aforementioned labeling approaches were primarily applied to pentose and hexose derivatives. In contrast, the larger nonulosonic acids (NulOs) were typically analyzed using more specific labeling techniques. Following early colorimetric assays, such as those using thiobarbituric acid (Aminoff 1961) and resorcinol (Uchida, Tsukada, and Sugimori 1977), more sensitive and reliable methods were developed based on fluorescence tags that specifically recognize the alpha-keto acid (Kitajima, Varki, and Sato 2015). In 1987, 1,2-diamino-4,5-methylenedioxybenzene (DMB) was first mentioned as reagent which specifically reacts with the alpha-keto acid of NulOs (Hara et al. 1987). Since then, DMB labeling followed by reverse-phase chromatography coupled with fluorescence detection or mass spectrometry has been widely employed, which has been extensively documented in the literature and several book chapters (Kitajima, Varki, and Sato 2015; Gerwig 2021c; Spichtig, Michaud, and Austin 2010; Zeleny et al. 2006; Zhang et al. 2019; Klein et al. 1997; Cheeseman et al. 2021). Furthermore, the mass spectrometric

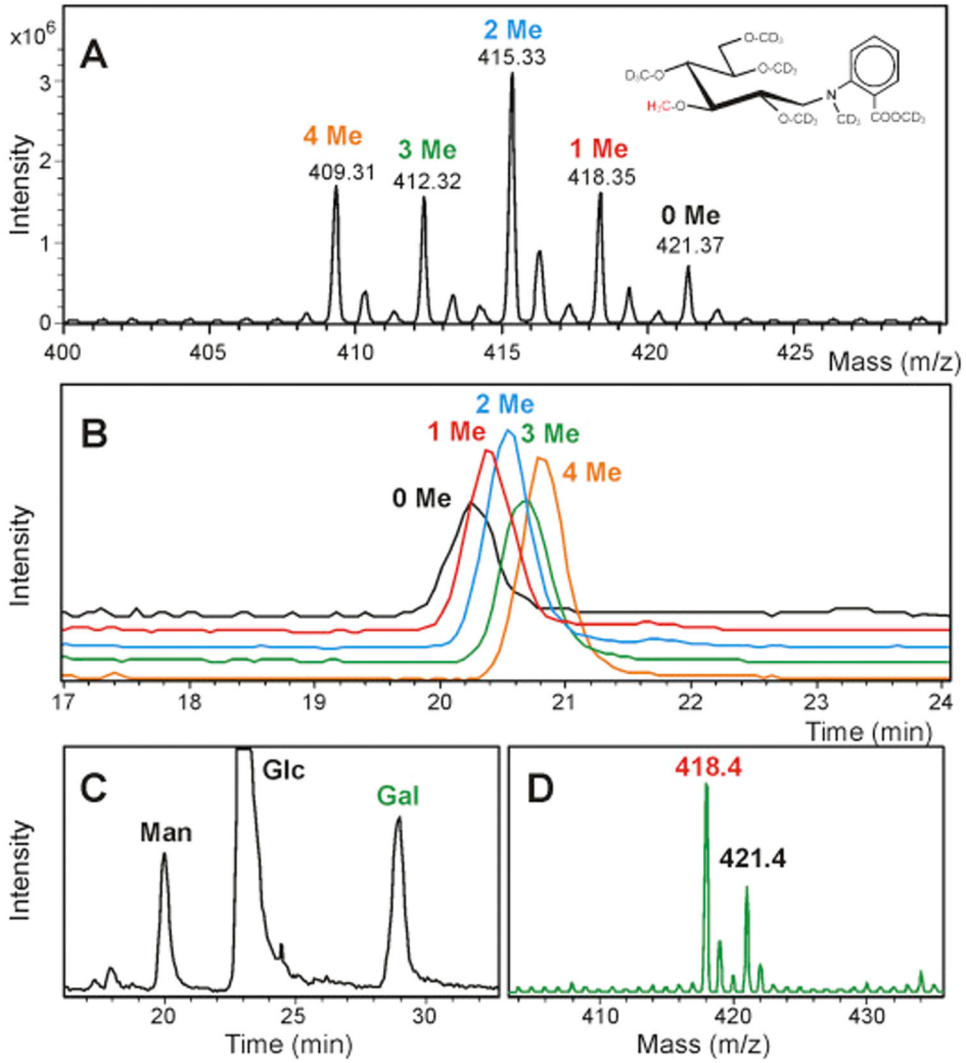


FIGURE 4 | Isomeric monosaccharides separation using reverse-phase chromatography followed by mass spectrometric detection of "hyper"-methylated anthranilic acid-labeled sugars (Windwarder et al. 2016). (A) The mass spectrum of a mannose sample with varying degrees of natural O-methylation, labeled with anthranilic acid (AA) and "hypermethylated" with deuteromethyl iodide. (B) Extracted ion chromatograms of the mannose species, illustrating how deuterium incorporation affects retention time. (C) Extracted ion chromatograms for different hexoses from a glycoprotein preparation obtained from *Achatina fulica*. (D) Mass spectrum of the galactose peak from panel C, showing varying degrees of methylation in the biological sample. Reprinted from Windwarder, Markus, et al. ""Hypermethylation" of anthranilic acid-labeled sugars confers the selectivity required for liquid chromatography-mass spectrometry." Analytical Biochemistry 514 (2016): 24–31. Copyright (2016), with permission from Elsevier. [Color figure can be viewed at wileyonlinelibrary.com]

detection allowed to identify NulOs in complex matrices. For example, Klein et al. were the first to demonstrate the separation and mass spectrometric analysis of DMB-labeled NulOs. The authors identified 28 different sialic acids in the glycolipids of *Lovenia cordiformis (*Klein et al. 1997*)*. Furthermore, Leg5Ac7AcAla was identified in *Vibrio vulnificus*, where the NulO was identified to contribute to membrane integrity (McDonald et al. 2018). NulO-deficient mutants exhibited also decreased virulence in the crustacean model *Artemia francisca*, emphasizing the importance of these sugars. By performing an additional reduction step before hydrolysis of CMP-activated NulOs, both free and CMP activated NulOs could be quantified (Galuska et al. 2010). However, this method required the removal of any NulO containing glycoconjugates before hydrolysis, to prevent interference of the quantification. Du et al. reported the use of 4,5-dimethylbenzene-1,2-diamine (DMBA) for analyzing NulOs via reverse-phase chromatography coupled with mass spectrometry (Cheeseman et al. 2021). DMBA demonstrated superior chromatographic separation efficiency and comparable mass spectrometric performance to DMB.

Finally, Kleikamp et al. conducted a fully untargeted mass spectrometric screening for nonulosonic acids (NulOs) in various prokaryotes. DMB labeling proved useful in providing the sensitivity and selectivity necessary for detecting novel NulO derivatives. This labeling introduced conserved ulosonic acid core fragment features distinct from the cell lysate background and altered the double bond equivalents and mass defect, enhancing sensitivity. Additionally, their automated filtering and scoring approach enabled the efficient screening of a large number of prokaryotic samples (Kleikamp et al. 2020). Applying this method, NulOs were detected in several environmental

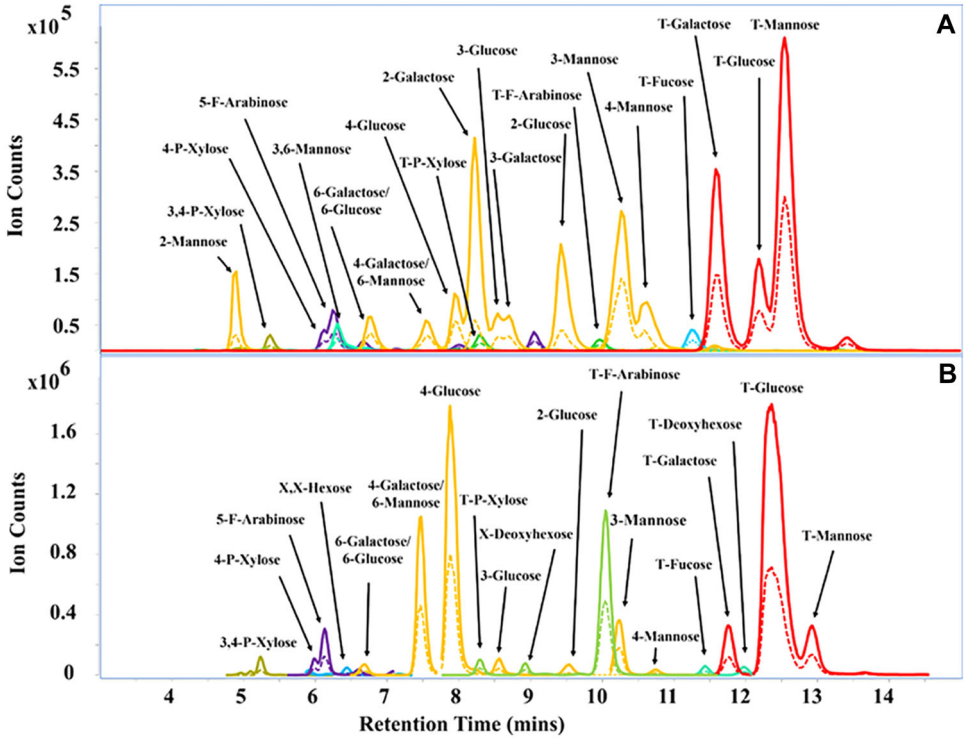


FIGURE 5 | Rapid determination of glycosidic linkages in complex biological samples (Galermo et al. 2018). The method involves permethylation, hydrolysis, and PMP derivatization of oligosaccharides, followed by reverse-phase chromatography and multiple reaction monitoring. (A) The MRM chromatogram of commercially available monosaccharide standards. (B) The MRM monosaccharide profile obtained from a plant biomass sample. Solid lines represent the quantifying transitions, while dashed lines indicate the qualifying transitions for each detected monosaccharide derivative. Reprinted with permission from Galermo, Ace G., et al. "Liquid chromatography–tandem mass spectrometry approach for determining glycosidic linkages." Analytical chemistry 90.21 (2018): 13073–13080. Copyright 2018 American Chemical Socie. [Color figure can be viewed at wileyonlinelibrary.com]

microbes, such as *Candidatus* Accumulibacter phosphatis enrichments, although its function remains to be elucidated (Kleikamp et al. 2020; Tomás-Martínez et al. 2021). However, DMB requires relatively high temperatures and acidic reaction conditions, which can lead to byproducts and degradation of sialic acids and unstable modifications. One such artifact is the spontaneous migration of acetyl groups between hydroxyl groups. Notably, the 8-O-acetyl ester is more labile than the 9-O-acetyl ester, with acetyl groups tending to migrate to the C-9 position even under neutral conditions at room temperature (Kamerling et al. 1987). To address this, Zhao et al. developed a method involving the sequential derivatization of the carbonyl and carboxyl groups using 3-NPH. This approach proved compatible with labile O-acetyl modifications, and the derivatives demonstrated good stability and chromatographic separation (Zhao et al. 2024).

3.2 | Ion-Pair Reverse-Phase Chromatography

The retention of highly polar monosaccharides, particularly for sugar phosphates and the nucleotide activated monosaccharides can be enhanced by the addition of an ion pair (IP) reagent to the mobile phase. Advantageously, compared to ion exchange chromatography, IP-RP allows for the separation of ionic analytes in parallel to neutral analytes (Sha et al. 2020; García-Alvarez-Coque et al. 2015). Initially, it was believed that the IP reagents form neutral ion-pairs with oppositely charged analytes, enabling their partition into the non-polar stationary phase due to their hydrophobic tails. This concept led to the term ion pair chromatography. However, the retention mechanism appeared to be more complex and still remains under debate (García-Alvarez-Coque et al. 2015; Ståhlberg 2000). In short, modern theories suggest that the IP reagent adsorbs to the surface of the stationary phase via hydrophobic interactions, thereby creating a charged ion layer. The retention of analytes is influenced via at least two factors, namely charge and hydrophobicity. Consequently, IP-RP chromatography allows for separation of isomeric nucleotide-sugars such as UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc (Kochanowski et al. 2006).

Commonly used IP reagents for phosphate and nucleotide activated sugars are tetra alkyl ammonium salts, like tetrabutylammonium bisulfate (Sha et al. 2020; Kochanowski et al. 2006). Separation can be enhanced by connecting multiple columns, although at the cost of the analysis time (Sha et al. 2020). Switching from HPLC to UHPLC mode can further improve separation (Buescher et al. 2010). Significant limitations of IP methods are, however, the high salt concentrations and the often non-volatile IP reagents, which challenge coupling to electrospray ionization mass spectrometry. Nevertheless, different approaches were developed to overcome this limitation, such as post-column removal of non-volatile IP reagents or by replacing them with volatile IP reagents, such as tributylamine, triethylamine, hexylamine and triethylammonium acetate (Buescher et al. 2010; Van Scherpenzeel et al. 2022; Coulier et al. 2006; Räbinä et al. 2001; Forngren et al. 1999). Various experimental parameters were shown to influence the retention and selectivity,

such as the type and concentration of the IP reagent, pH of the mobile phase, type and concentration of the organic modifier and the temperature (García-Alvarez-Coque et al. 2015). Although structural isomers often have a similar fragmentation pattern, the fragment ion intensity ratios can differ significantly. In combination with IP chromatographic separation, different hexose monophosphates could be differentiated and quantified based on their fragmentation pattern (Buescher et al. 2010). Van Scherpenzeel et al. optimized the application of IP-RP coupled to mass spectrometry for sugar-phosphates and nucleotide-sugars found in the central metabolic pathways. The authors applied this approach to a range of model organisms including HEK cells and microbes. The authors also traced sugar analogs on sugar metabolism within a cellular context (Van Scherpenzeel et al. 2022). The authors could detect UDP-arabinose as additional low abundant nucleotide--sugar, albeit its involvement in glycoconjugate biosynthesis remains unsolved to date. Furthermore, the authors explored the effect of the antitumor compound $3F_{ax}$-NeuNAc on sialic acid metabolism. Nevertheless, it was emphasized that IP-RP-ESI-MS is not ideal for multi-purpose instruments due to persistent contamination by IP reagents, necessitating extensive cleaning when switching applications or modes (Buescher et al. 2010; Van Scherpenzeel et al. 2022; Coulier et al. 2006).

3.3 | Ion Exchange Chromatography

Ion exchange chromatography (IEC) relies on the interaction of charged analytes with an oppositely charged stationary phase, a development which dates back to the 1940s (Cummins, Rochfort, and O'Connor 2017). Originally, the only types of monosaccharides that could be separated by anion-exchange chromatography were acidic monosaccharides, which are negatively charged at physiological pH. Later, high performance (also referred to as 'high-pH') anion exchange (HPAE) chromatography was developed which allows to analyze neutral monosaccharides (Hardy, Townsend, and Lee 1988; Hardy and Townsend 1988). Thereby, the hydroxyl groups of monosaccharides are deprotonated in a highly basic solvent (e.g. 0.1 M NaOH). The monosaccharide anions then interact with a positively charged stationary phase, which are often quaternary ammonium ions. Differences in the number of hydroxyl groups and pK_a values ultimately facilitate the separation of different monosaccharides (Cummins, Rochfort, and O'Connor 2017; Zhang et al. 2012; Lee 1996). This method became employed for the label free analysis of oligo- and monosaccharides from different matrices (Zhang et al. 2012; Karlsson and Hansson 1995; Behan and Smith 2011; Townsend and Hardy 1991; Mechelke et al. 2017; Cataldi, Campa, and De Benedetto 2000; Li et al. 2024). Elution can be achieved by either a pH gradient or increasing salt concentration (Cummins, Rochfort, and O'Connor 2017). However, the high concentrations of non-volatile salts make this method incompatible for coupling to mass spectrometry. Therefore, high performance anion exchange chromatography is usually combined with pulsed amperometric detection, which, however, does not allow the application to complex samples. For example, organic acids in the matrix background can interfere with the accurate determination of sugars (Hardy, Townsend, and Lee 1988; Zhang et al. 2012). Mass spectrometric analysis is often only achieved off-line, after fractionation (Barr et al. 1991; Okinaga, Ohashi, and Hoshi 1992; Walters et al. 2006). HPAE chromatography often also shows poor long-term stability and requires lengthy separation times (Kochanowski et al. 2006; Ramm et al. 2004). Furthermore, hydroxyl modifications, such as acetylation, often found on NulOs, are base-labile. Therefore, these sugars have not been analyzed using standard HPAE techniques. However, substituted NulOs were successfully separated by anion exchange using solvents at neutral pH (Manzi, Diaz, and Varki 1990).

Coupling HPAEC with electrospray ionization mass spectrometry was finally achieved by implementing post-column ion suppressors that, for example, replace Na^+ ions with H^+ ions. Additionally, make-up solvents were introduced that include dopants such as LiCl to improve ionization of the monosaccharides (Bruggink et al. 2005a; Wunschel et al. 1997; Tedesco et al. 2020; Zhao et al. 2020; Bruggink et al. 2005b). Zhao et al. incorporated an online sample pretreatment using a column-switching technique. In short, the injected sample passed through a reverse-phase pretreatment column, where monosaccharides eluted immediately due to their polarity, eliminating the matrix. The collected sugars were then directed to an ion exchange analytical column for separation. A suppressor column functioned as a desalter, making it MS-compatible, and a T-junction introduced an additional make-up solution containing LiOH, which improved ionization efficiency. The method was then applied to analyze monosaccharides in activated sludge from wastewater treatment plants, crucial for understanding the water treatment mechanism (Zhao et al. 2020). Optimized methods also demonstrated the simultaneous separation of neutral, amino and acidic monosaccharides (Zhang et al. 2012; Wunschel et al. 1997). For example, Zhang et al. achieved the simultaneous detection of Fuc, Rha, GalN, Ara, GlcN, Gal, Glc, Man, Xyl, Neu5Ac, Neu5GC, GalA, GulA, GlcA, ManA, and IdoA (Zhang et al. 2012).

Some studies also showed the application of HPAE chromatography to analyze nucleotides and nucleotide activated sugars, although this approach has been less widely employed (Tomiya et al. 2001; Alonso et al. 2010). For example, Alonso and coworkers demonstrated the detection of 16 hexose phosphates and nucleotide activated sugars involved in cell wall biosynthesis, including the separation of isomers such as UDP-GalNAc/UDP-GlcNAc, Glc6P/Fru6P/Man6P, UDP-GalA/UDP-GlcA, UDP-Gal/UDP-Glc and UDP-Ara/UDP-Xyl. The application of 13C labeled fructose in growth medium allowed to track the incorporation pattern in cell wall precursors (Alonso et al. 2010).

3.4 | Porous Graphitic Carbon Chromatography

The production of porous graphitic carbon as chromatographic separation material was developed by Knox and Gilbert and became commercially available in 1988 under the trade name Hypercarb (Pereira 2008). PGC acts as a strongly retentive alkyl-bonded silica gel for non-polar analytes, yet its retention and selectivity behavior towards polar and structurally related compounds is markedly different (Pereira 2008). The PGC particles are spherical and fully porous, with a porosity of ~75% and a crystalline surface. Chemically, they are composed of sheets of hexagonally arranged carbon atoms connected by conjugated

1.5-order bonds, similar to those found in polynuclear aromatic hydrocarbons (Pereira 2008). Consequently, the PGC separation phase can be operated across a wide pH range (1–14) and at high temperatures (250°C) (Pereira 2008).

Multiple studies and reviews have been dedicated to the retention mechanism and applications of PGC (Pereira 2008; West, Elfakir, and Lafosse 2010; Russo et al. 2024; De Matteis et al. 2012; Lepont, Gunatillaka, and Poole 2001; Knox and Ross 1997; Ross and Knox 1997; Knox, Kaur, and Millward 1986; Pabst and Altmann 2008; Corman et al. 2021, 2023; Chaimbault, Elfakir, and Lafosse 1998; Wan et al. 1995), thus its mechanisms will be discussed only briefly in this review. As mentioned above, although PGC behaves like a strongly retentive alkyl-bonded silica gel for non-polar analytes, its retention and selectivity towards polar and structurally related compounds are markedly different. In conventional alkyl-bonded silicas, adding a polar group to a molecule usually decreases retention in reverse-phase mode. However, with PGC, this retention reduction is much smaller or may even increase. An important factor contributing to this so-called polar retention effect involves charge-induced interactions between the analyte and the graphite surface. In these interactions, lone-pair or aromatic ring electrons interact with the graphite through electron transfer to the graphite's electron cloud (Pereira 2008; Russo et al. 2024). Furthermore, due to the flat and highly adsorptive surface of graphite, there is enhanced selectivity for structurally related compounds, such as structural isomers and diastereoisomers. This makes PGC well suited to the separation of very polar and ionized molecules such as carbohydrates and other compounds with several hydroxyl, carboxyl, amino and other polar groups. Despite its excellent chromatographic performance, PGC chromatography can suffer from retention time instability. This instability has been primarily attributed to column contamination and redox reactions with buffer components or analyte molecules. For example, the graphite surface has been affected by redox reagents such as hydrogen peroxide or sulfite (Shibukawa et al. 2004; Törnkvist, Markides, and Nyholm 2003). To mitigate these issues, regular backflushing and washing procedures with strong acids, bases, and solvents, such as acetone, tetrahydrofuran, hydrochloric acid, sodium hydroxide, and trifluoroacetate, have been employed (Russo et al. 2024; Bapiro, Richards, and Jodrell 2016). Moreover, additives in the mobile phase can potentially adsorb onto the stationary phase, altering separation and retention capabilities. The mobile phase may also affect the ionization state of both the PGC surface as well as the analytes, impacting the retention (West, Elfakir, and Lafosse 2010; Bapiro, Richards, and Jodrell 2016). Therefore, Bapiro et al. optimized the application of different mobile phase solvents in a gradient program which preserved the retention capacity of PGC columns across multiple runs (Bapiro, Richards, and Jodrell 2016). The authors demonstrated promising results in analyzing gemcitabine (a cytidine derivative with fluorination at the 2-position of the deoxyribose sugar molecule) and its metabolites, achieving good peak shapes and retention without the need for backflushing. In conjunction with electrospray ionization mass spectrometry, separation using PGC has been shown to be influenced by the electrospray voltage, leading to column polarization (Pabst and Altmann 2008; Törnkvist et al. 2004). This issue can be resolved by introducing electrical grounding points close to the separation column.

The strength of interaction of an analyte with the PGC separation phase depends on both the surface area of the analyte in contact with the graphite and the nature and type of functional groups (Pereira 2008). As a result, the relatively small monosaccharide components exhibit a comparatively weak interaction with the PGC separation phase. Although to a lesser extent, PGC chromatography coupled to ESI-MS has been employed to measure monosaccharides and phosphorylated derivatives. For example, Hammad and colleagues demonstrated a MRM method for the quantification of free and glycoprotein monosaccharides following separation by PGC chromatography (Hammad et al. 2009). Neutral monosaccharides were detected as their alditol acetate anion adducts, while sialic acids were detected as deprotonated ions. This method exhibited high sensitivity, with detection limits as low as 1 pg for glucose, galactose, and mannose, and showed linearity over three orders of magnitude (Hammad et al. 2009). Antonio et al. developed a method to study metabolic sugars and sugar-phosphate intermediates in *A. thaliana*. Although complete separation of sugar isomers was not achieved, distinct fragmentation profiles allowed for differentiation between isomers (Antonio et al. 2007). Interestingly, Li et al. developed an improved workflow to quantify glycolytic pathway intermediates, including sugar phosphates. This workflow employed TiO_2 enrichment of phosphorylated metabolites, phosphate methylation to enhance chromatographic performance and incorporated stable isotopes, followed by porous graphitic carbon chromatography and selected reaction monitoring (Li et al. 2022). Additionally, a 13C-labeled yeast metabolite extract was used as an internal standard. The method achieved limits of quantification ranging between 0.25 and 0.54 pmol on column (Li et al. 2022).

PGC chromatography coupled with mass spectrometry has found greater application in the analysis of the larger and more polar activated monosaccharides. For example, Pabst et al. demonstrated the separation of nucleotides and nucleotide-sugars using PGC coupled to ESI-MS (Pabst et al. 2010; Behmüller et al. 2014). The PGC separation phase allowed the use of volatile buffer components, such as ammonium acetate, thereby eliminating the need for ion-pairing reagents typically required for separating sugar nucleotides in reverse-phase chromatography. Sensitive detection was achieved in negative ionization mode. Retention time and peak shape instabilities were addressed by applying a column regeneration procedure employing hydrochloric acid, achieving satisfactory performance. This method proved effective for separating a spectrum of isomeric sugar nucleotides from various sources, including animal and microbial model cells, as well as plants (Pabst et al. 2010). Remarkably, the PGC-ESI-MS approach enabled the separation of UDP-activated Xyl, Araf, and Arap variants, and allowed for the detection of the chemically labile CMP-Kdo. To tackle retention time variabilities, relative retention times (to UDP-Glc) were used (Figure 6). Furthermore, López-Gutiérrez and colleagues employed PGC-ESI-MS to study the nucleotide-sugar profile in the different life stages of *Plasmodium falciparum*, a malaria parasite (López-Gutiérrez, Dinglasan, and Izquierdo 2017). Behmüller et al. employed porous graphitic carbon chromatography to quantify UDP-sugars in *A. thaliana* extracts, thereby employing a column regeneration step using 80% acetonitrile in water, containing 0.1% trifluoroacetic acid, to limit retention time instability. Although effective, the 20-min column regeneration, the 90-min flush with starting

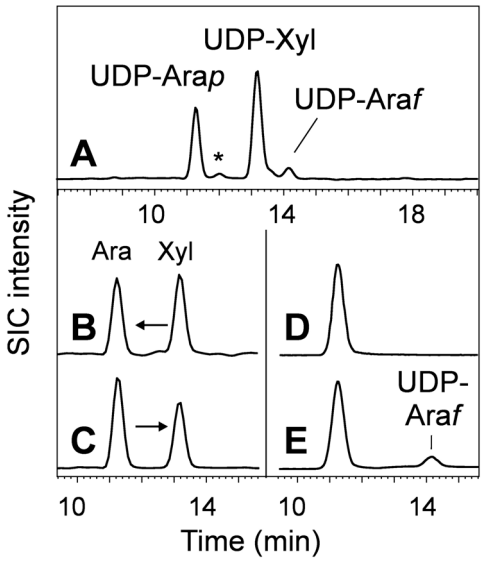


FIGURE 6 | Separation of UDP-pentose isomers using PGC-MS (Pabst et al. 2010). (A) Extracted ion chromatogram for UDP-pentose sugars (m/z 536.00-526.08) extracted from *Nicotiana benthamiana* leaves, identifying UDP-Arap (pyranose), UDP-Xyl, UDP-Araf, and an unidentified UDP-pentose sugar marked with an asterisk. (B) Extracted ion chromatogram illustrating the conversion of UDP-Xyl to UDP-Arap using UDP-xylose epimerase from mung bean sprout microsomes. (C) The same assay as in (B), but with UDP-Arap as the substrate. (D) and (E) display the conversion of UDP-Arap to UDP-Araf with recombinant UDP-arabinose mutase, where (D) shows the control without the recombinant enzyme. SIC: Selected ion chromatogram. Reprinted with permission from Pabst, et al. Nucleotide and nucleotide-sugar analysis by liquid chromatography-electrospray ionization-mass spectrometry on surface-conditioned porous graphitic carbon. Analytical chemistry 82.23 (2010): 9782–9788.

conditions, and the 30-min analysis time, added to a total runtime of 140 min per sample (Behmüller et al. 2014). Garcia et al. developed a rapid 6-min MRM method with PGC separation, applying it to various breast cancer cell lines (Garcia, Chavez, and Mechref 2013). This method enabled the identification and quantification of all seven targeted sugar nucleotides. Other recent applications of PGC chromatography coupled to ESI-MS include quantification of nucleotide-sugars in plasma and urine samples (Caron et al. 2022), studying the impact of antimicrobials on nucleotide sugar levels in biofilm models of the opportunistic pathogen *Candida albicans*(Wang et al. 2014), as well as demonstrating the in planta synthesis of CMP-Neu5Ac in transgenic *Arabidopsis thaliana* (Castilho et al. 2008). Profiling methods for sugar nucleotides, including the application of PGC, has also been reviewed by Rejzek and colleagues recently (Rejzek et al. 2017).

3.5 | Hydrophilic Interaction Liquid Chromatography

The term hydrophilic interaction chromatography (also referred to as hydrophilic interaction liquid chromatography, HILIC) was first introduced by Alpert in the 1990s (Alpert 1990). However, HILIC separations for monosaccharide analysis have a much longer history (Linden and Lawhead 1975). Because HILIC separates based on differences in polarity, this technique is particularly useful for the separation of the polar monosaccharides and their derivatives. Typical hydrophilic stationary phases consist of unbound or chemically modified silica (e.g., amino, amide, cyano) or polar polymers. The review by Inoue and Yamamoto summarizes the stationary phases that have been employed for the separation of sugars (Inoue and Yamamoto 2014). The mobile phase is usually an organic solvent, such as acetonitrile, with added water, providing the advantage of good compatibility with ESI-MS and low backpressure (Ito et al. 2014; McCalley 2010). The retention of analytes in HILIC occurs mainly via two mechanisms, partitioning and adsorption. Water molecules retained in the hydrophilic stationary phase facilitate the partitioning of polar analytes from the mobile phase to the stationary phase. Additionally, adsorption occurs through interactions, such as ionic interactions, hydrogen bonding, and dipole–dipole forces (McCalley 2013; Cubbon et al. 2010a). As a consequence of this complex retention mechanism, the retention of molecules can vary when different stationary phases are employed (McCalley 2010). For a deeper understanding of the retention mechanism of HILIC, we refer to earlier reviews (Guo 2015).

HILIC-MS has been extensively employed in both targeted and untargeted metabolomics studies, as well as an orthogonal approach to applications that employ reverse-phase separation, which have been thoroughly discussed in recent reviews (Tang et al. 2016; Cubbon et al. 2010b; Spagou et al. 2010; Kohler et al. 2022; Segers et al. 2019; Patti 2011; Kohler, Giera, and Derks 2016; Rojo, Barbas, and Rupérez 2012; Hosseinkhani et al. 2022; Kohler and Giera 2017; Wernisch and Pennathur 2016; Buszewski and Noga 2012). These also include applications to the analysis of monosaccharides and derivatives (Ikegami et al. 2008; Gao et al. 2011; Meyer et al. 2022; Mathon, Barding, and Larive 2017; Meriö-Talvio et al. 2021; Pismennõi et al. 2021; Yan 2014; Lowenthal, Kilpatrick, and Phinney 2015; Li et al. 2023). In the following section, we will cover only recent applications to monosaccharides and their activated sugars.

Su et al. applied HILIC-ESI$^-$-MS/MS to quantify phosphate-sugars of the glycolysis and pentose phosphate pathway. Thereby, two HILIC (Atlantis Premier BEH Z-HILIC and Acquity Premier BEH Amide) and a mixed-mode HILIC/strong anion-exchange (SAX) column were compared for its separation performance. Although unable to separate F6P from G1P, the BEH amide column provided the best separation on the tested phosphate sugars (Su et al. 2023). Furthermore, Hinterwirth and colleagues introduced a method utilizing a reverse-phase/weak anion-exchanger mixed mode column operated under HILIC conditions (Hinterwirth et al. 2010). This approach enabled the separation of sugar phosphates, including hexose and pentose phosphates, glucosamine 1- and 6-phosphate, and 2- and 3-phosphoglycerate. Hammad et al. demonstrated the simultaneous quantification of monosaccharides derived from glycoproteins and blood serum using an aminopropyl column followed by multiple-reaction monitoring (Hammad et al. 2010). Neutral monosaccharides were detected as $[M + CH_3CO_2]^-$ ions, while sialic acids were detected as $[M–H]^-$ ions. This method enabled the detection of picogram quantities of the sugars, with a linearity spanning over three orders of magnitude (Hammad et al. 2010). Matějíček and Vašíčková utilized HILIC with a zwitterionic ligand to quantify monosaccharide anhydrides, including galactosan, mannosan, and levoglucosan, from environmental samples such as aerosols.

The monosaccharide anhydrides were detected as $[M + HCOO]^-$ adduct ions (Matějíček and Vašíčková 2016). Furthermore, Yan et al. demonstrated a HILIC method using a BEH amide column, which allowed for the separation of all neutral monosaccharides commonly found in plant-derived oligo- and polysaccharides within 25 min (Yan et al. 2016). Although the authors used charged aerosol detector for detection, the method was also suitable for coupling with ESI-MS. The BEH amide column proved advantageous due to its high stability and longer column life.

The separation of isomeric sugars can be a shortcoming of HILIC, which is particularly important for the analysis of samples with unknown derivatives. This issue is critical for the study of rare sugars, which are monosaccharides with very low natural occurrence and abundance (Roca et al. 2015; Zhang et al. 2017). Nevertheless, they may have important functional roles or health effects when consumed (Ahmed et al. 2022). For instance, D-psicose and D-allulose have been researched for their effects on glucose tolerance and insulin sensitivity (Franchi et al. 2021; Hossain et al. 2011). Therefore, Chung and colleagues developed a HILIC method using a polyethyleneimine separation phase for both reducing and nonreducing monosaccharides, including rare sugars such as D-allose, D-psicose, and D-tagatose (Chung, Shimura, and Matsui 2018).

Fu and co-workers investigated the isomeric separation of hexoses, pentoses, and disaccharides using the HILIC poly-N-(1H-tetrazole-5-yl)-methacrylamide-bonded stationary phase (DCPak PTZ) combined with tandem mass spectrometry (Fu et al. 2020). Low temperatures (e.g. 10°C) and acidic conditions (e.g. 0.1% formic acid) provided selectivity for various isomeric sugars. Additionally, the interconversion between α and β anomers was studied at different temperatures and mobile phase pH values (Fu et al. 2020) (Figure 7).

Although one of the main advantages of HILIC is the avoidance of derivatization before chromatographic separation, post-column

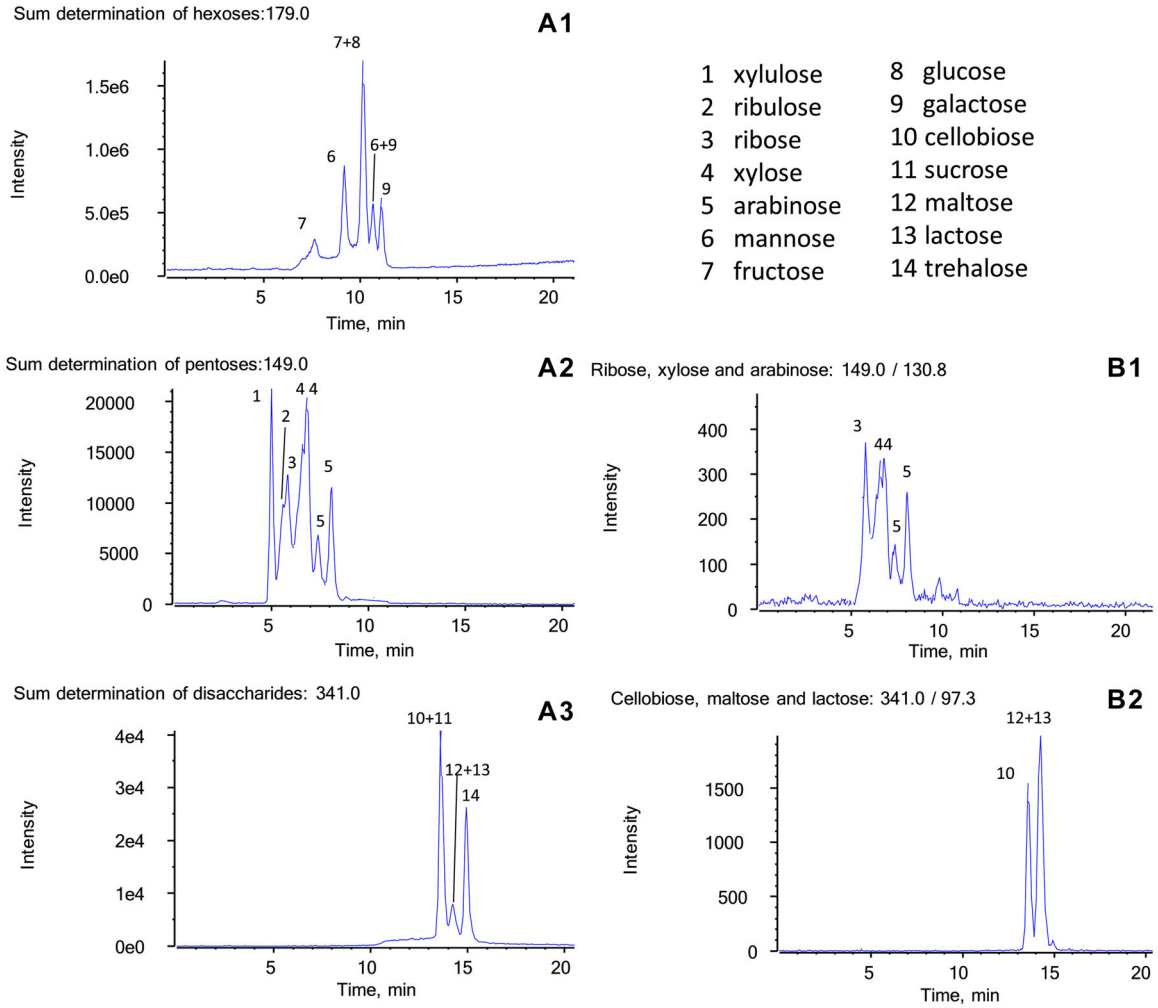


FIGURE 7 | Isomeric separation of hexoses, pentoses, and disaccharides using a HILIC DCPak PTZ stationary phase combined with charged aerosol or mass spectrometric detection (Fu et al. 2020). (A1) SIM chromatogram of a hexose mixture. (A2) SIM chromatogram of a pentose mixture. (A3) SIM chromatogram of a disaccharide mixture. (B1) SRM chromatogram for aldopentoses (ribose, xylose, and arabinose). (B2) SRM chromatogram for the disaccharides cellobiose, lactose, and maltose. Reprinted from Fu, Xiaoqing, et al. "Separation of carbohydrate isomers and anomers on poly-N-(1H-tetrazole-5-yl)-methacrylamide-bonded stationary phase by hydrophilic interaction chromatography as well as determination of anomer interconversion energy barriers." Journal of Chromatography A 1620 (2020): 460981. Copyright 2020, with permission from Elsevier. [Color figure can be viewed at wileyonlinelibrary.com]

derivatization methods have been developed to increase MS sensitivity, allowing to decrease the sample volume. Li et al. separated monosaccharides of plant samples by a SeQuant ZIC-HILIC column followed by an online chemical derivatization using 4-benzylamino-benzeneboronic acid (labeling cis-diols) and ESI^+-MS analysis (MRM mode). The observed increase in sensitivity ranged between a 36 to 42800 fold for the various analyzed monosaccharides (Li et al. 2020a). Heiss et al. eliminated the need of post-column derivatization hardware by developing a modified ESI interface, termed 'contained electrospray ionization'. This allowed for an in-source, droplet-based derivatization with phenylboronic acid (PBA) forming monosaccharide-bisPBA derivatives, resulting in enhanced sensitivity by MS analysis. Since the low flow rate required for contained-ESI and the large volume of HPLC pre-column components results in a significant gradient delay, either hardware modifications needed to be incorporated or an estimated injection delay has to be programmed (Heise et al. 2019).

Apart from the separation and detection of monosaccharides, HILIC has been applied to study nucleotide-sugars as well. Using a zwitterionic-silica based stationary phase (ZIC-HILIC, Merck SeQuant), 12 nucleotide-sugars could be detected and quantified in various plant cell wall extracts. Although different LC conditions were tested, the method was unfortunately insufficient to separate the structural isomers UDP-Gal/UDP-Glc and UDP-GalNAc/UDP-GlcNAc (Ito et al. 2014).

Ma et al. (2017) applied HILIC-ESI-IT-TOF (employing an Accucore 150-amide HILIC column) to detect NT-sugars in the fungus *Botrytis cinerea*. They observed an accumulation of UDP-KDG, an intermediate of the UDP-Rhamnose biosynthesis pathway, in a *Botrytis cinerea* mutant, which was associated with a reduced virulence of the fungi (Ma et al. 2017).

HILIC also made its appearance in supporting the discovery of novel biosynthesis pathways in bacteria. By using an Accucore 150-amid HILIC column, Li et al. looked into the intermediates of the CMP-Pse biosynthesis pathway of *Bacillus thuringiensis*. Where gram-negative bacteria, such as *C. jejuni* and *H. pylori* require six enzymes to convert UDP-GlcNAc into CMP-Pse, *B. thuringiensis* required seven enzymes. Using HILIC-ESI-MS complemented with NMR experiments, the enzymes Pen and Pal were identified, converting UDP-GlcNAc into UDP-2-acetamido-6-deoxy-D-xylo-hexopyranose-5,6-ene and UDP-2-acetamido-6-deoxy-D-xylo-hexopyranose-5,6-ene into UDP-2-acet-amido-2,6-dideoxy-L-arabino-hex-4-ulose (Li et al. 2015a). Finally, a rapid HILIC-ESI-MS/MS method using a ZIC-pHILIC stationary phase was developed for the quantification of UDP-glucose and UDP-glucuronic acid. The method was validated and applied to extracts from plant materials (Warth et al. 2015).

3.6 | Other Liquid Chromatography Separation Techniques

3.6.1 | Chiral Chromatography

Chiral chromatography performs the separation of chiral compounds, such as the D and L enantiomers which determine the absolute configuration in sugars. The chiral stationary phase usually employs a chiral ligand attached to the surface of an achiral support. For a comprehensive overview and mechanism about chiral stationary phases for liquid chromatography we refer to the review of Teixeira et al (Teixeira et al. 2019).

In sugars, D and L is determined based on the chiral carbon most remote from the carbonyl group, e.g. C-5 in glucose. The chromatographic determination of absolute configuration is particularly challenging. The enantiomeric separation of pentopyranoses and hexopyranoses was achieved after per-O-methylation and conversion into glycosyl chlorides followed by coupling with cesium salt of a fluorescent chiral derivatization reagent. Enantiomeric separation was achieved using a Develosil column (Bai et al. 1997). Recently, boric acid was used as an additive to enhance the separation of sugars by complexation to vicinal cis hydroxyl groups on the sugar molecules employing a cation-exchange column (De Muynck et al. 2006). However, D and L sugars did not differ in their complexation abilities, and consequently showed the same retention times. Lopes and Gaspar performed the simultaneous separation of enantiomers (D/L) and anomers (α/β) of several underivatized monosaccharides (arabinose, ribose, mannose, fucose, xylose, lyxose, glucose, and fructose) using the chiral separation phase Chiralpak AD-H (Lopes and Gaspar 2008). However, their mobile phase consisted of a mixture of hexane–ethanol–TFA and hence was not combined with mass spectrometric detection.

3.6.2 | Mixed Mode Chromatography

Mixed mode chromatography (MMC) combines multiple retention mechanisms within a single chromatographic separation phase (Wang et al. 2016). This provides flexibility and versatility for analyzing compounds with diverse physicochemical properties, e.g. ranging from polar to non-polar, making it particularly useful for complex samples (Wang et al. 2016; Sýkora et al. 2019; Ammann and Suter 2016). The concept of MMC was introduced in 1986, initially by simply combining two separate columns with different retention mechanisms. Later, stationary phases incorporating dual retention mechanisms were developed (Halfpenny and Brown 1986; Kennedy, Kopaciewicz, and Regnier 1986), which gained popularity, as evidenced by the increasing number of research groups developing MMC stationary phases. For a more comprehensive discussion on advances in mixed-mode chromatographic stationary phases, we refer to the review by Sýkora et al. (2019). The most common MMC columns integrate two or three retention mechanism, predominantly hydrophobic, hydrophilic and electrostatic interactions, although other mechanisms can also be included (Wang et al. 2016; Sýkora et al. 2019; Ammann and Suter 2016). Stationary phases can be categorized based on their structure, such as silica-based, polymer-based, hybrid, and monolithic (Sýkora et al. 2019). Despite the potential for combining multiple retention mechanisms, in practice, often only one mechanism is used at a time, dictated by the mobile phase conditions (Sýkora et al. 2019). For instance, IEX columns exhibit electrostatic interactions at low acetonitrile concentrations, while HILIC retention occurs at high acetonitrile concentrations (Wang et al. 2016). Another example includes the developed bistrimethoxysilylethane hydrolysis aminopropyltrimethoxysilane (BTME-H-APS) silica stationary, which can be used for normal phase chromatography, IEC, or MMC

combining HILIC and RP mechanisms. This column successfully separated different carbohydrates from biological samples, although it did not exploit the advantages of multiple retention mechanisms (Wang et al. 2016; Chen and Chen 2018). Eastwood et al. did exploit these advantages by using the commercial Primsep SB column which allowed them to detect and purify the nucleotide-sugar GDP-trifluoromethyl fucose in an enzymatic reaction mixture, relying on both electrostatic as well as hydrophobic interactions. An ammonium formate buffer was used to ensure compatibility with ESI-MS (Eastwood et al. 2015).

MMC shows a great potential for untargeted metabolomic studies, where the separation and identification of compounds with diverse characteristics is required. Ammann and co-workers managed by combining HILIC, anion exchange chromatography, cation exchange chromatography and RP to separate a variety of metabolite classes, such as amino acids, sugars, fatty acid derived compounds and antioxidants all within a single run of 50 min, using an ESI-MS compatible method. However, this method did not resolve sugar isomers (Ammann and Suter 2016). Xing et al. utilized a positively charged quaternary amine polyvinyl alcohol stationary phase coupled to ESI-MS, employing varying pH, salt concentrations and organic content to achieve both hydrophilic and electrostatic interactions. Their method covered 65.6% of a metabolite library comprising 607 metabolites within a 60-min run, missing only the compounds which e.g. required positive mode detection or were unstable in the applied pH range. Moreover, they demonstrated the separation of isomers such as glucose and fructose and four different hexose-monophosphates (Xing et al. 2021).

Another study demonstrated the potential for separating sugar isomers using a reverse-phase/weak anion exchanger operated under HILIC conditions. This method successfully separated six hexose monophosphates. By adjusting the temperature, the authors were able to fine-tune the conditions to achieve either the separation or co-elution of α- and β-anomers (Hinterwirth et al. 2010). However, the mobile phase used, included trifluoroacetic acid (TFA), rendering it incompatible with ESI-MS. The choice of the mobile phase is also critical for successful MMC (Wang et al. 2016). As mentioned before (HILIC section), Su et al. investigated the separation of isomeric glycolytic phosphorylated monosaccharides. While the HILIC BEH amide column outperformed their MMC HILIC/SAX column (HILICpak VT-50 2D), the latter enabled the separation of F6P from G1P, which the former could not achieve. Consequently, they combined the HILIC BEH amide and MMC HILIC/SAX columns for complete separation (Su et al. 2023). More recently, Rahm and colleagues developed a MMC-ESI-MS method to separate and quantify 17 nucleotide-sugars commonly found in human cells. They employed a mixed-mode separation phase (Atlantis Premier BEH C18 AX) that combined weak anion-exchange and reverse-phase chromatography. The mobile phase A consisted of water, while mobile phase B was an ammonium acetate buffer in 5% acetonitrile at pH 4.7 (Rahm et al. 2024). Interestingly, the authors not only demonstrated good separation (including UDP-GlcNAc and UDP-GalNAc) and high robustness of the method, but they also identified distinctive fragment ion patterns for various UDP-hexoses, including UDP-mannose, UDP-glucose, and UDP-galactose, supporting their identification (Rahm et al. 2024) (Figure 8).

3.6.3 | Supercritical Fluid Chromatography

A more recent approach for the analysis of monosaccharides is supercritical fluid chromatography (SFC) coupled to mass spectrometry (Pauk et al. 2017; Losacco et al. 2020). This

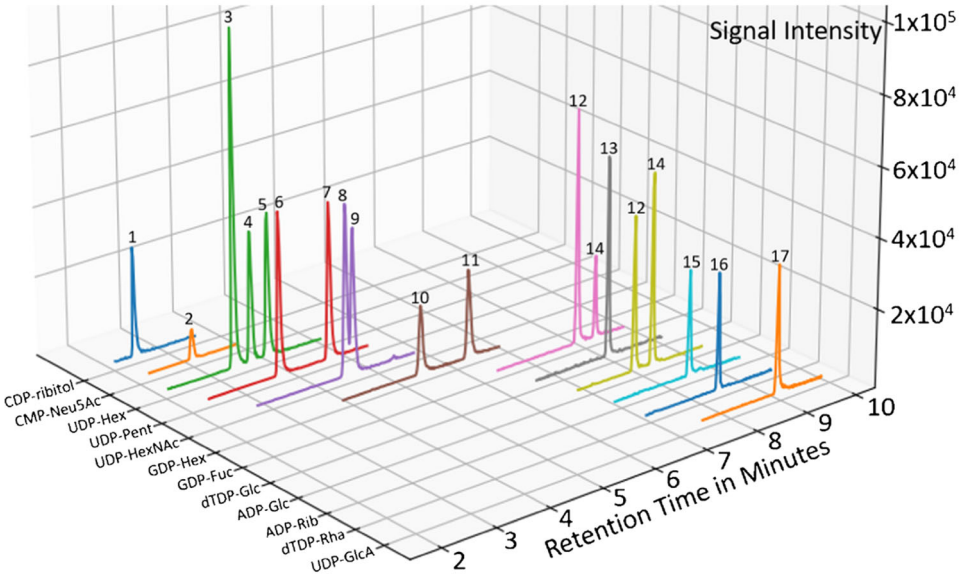


FIGURE 8 | The separation of 17 (isomeric) nucleotide-sugars commonly found in human cells, using mixed-mode chromatographic separation coupled to ESI-MS (Rahm et al. 2024). The graph shows the MRM transitions. for: 1. CDP-ribitol, 2. CMP-Neu5Ac, 3. UDP-Man, 4. UDP-Gal, 5. UDP-Glc, 6. UDP-Ara, 7. UDP-Xyl, 8. UDP-GalNAc, 9. UDP-GlcNAc, 10. GDP-Man, 11. GDP-Glc, 12. GDP-Fuc, 13. dTDP-Glc, 14. ADP-Glc, 15. ADP-Rib, 16. dTDP-Rha, 17. UDP-GlcA. Used with permission of Springer Nature BV, from Rahm, et al. Mixed-phase weak anion-exchange/reverse-phase LC–MS/MS for analysis of nucleotide-sugars in human fibroblasts. Analytical and bioanalytical chemistry (2024): 1–10; permission conveyed through Copyright Clearance Center Inc. [Color figure can be viewed at wileyonlinelibrary.com]

technique employs a supercritical mobile phase, which typically consists of CO_2 due to its low critical parameters ($T_c = 31°C$ and $P_c = 74$ bar). SFC combines the advantages of both a liquid (i.e. density) and a gas (i.e. viscosity and diffusivity) (van de Velde, Guillarme, and Kohler 2020; Losacco, Veuthey, and Guillarme 2019; Gazárková et al. 2022). The technique gained more attention when more robust instruments allowed an easy hyphenation to ESI-MS (van de Velde, Guillarme, and Kohler 2020; Losacco, Veuthey, and Guillarme 2019). Although SFC was originally used to analyze hydrophobic compounds due to the non-polar characteristics of CO_2, the addition of an organic modifier (frequently methanol), also allowed for the analysis of polar compounds (van de Velde, Guillarme, and Kohler 2020; Gazárková et al. 2022; Desfontaine et al. 2018). Highly polar compounds (such as monosaccharides and derivatives) can be analyzed by creating a gradient of 100% supercritical CO_2 to 100% organic modifier, thereby shifting the physical state by controlling pressure and temperature, known as unified chromatography (van de Velde, Guillarme, and Kohler 2020). Besides the advantage of the simultaneous analysis of both polar and non-polar compounds, the analysis time of SFC-MS is often much faster compared to traditional LC separation methods (Pauk et al. 2017; Desfontaine et al. 2018).

Desfontaine et al. evaluated SFC-MS for metabolomics. Their final optimized method employed a Poroshell HILIC (silica-based) column with a linear gradient starting from 98% supercritical CO_2 and 2% organic modifier to 100% organic modifier. The organic modifier consisted of 95/5 MeOH/H_2O (v/v) with 50 mM ammonium formate and 1 mM ammonium fluoride. A make-up solvent of pure MeOH was applied (Desfontaine et al. 2018). Using this method, they later showed a detectability of 66% from 597 compounds. Compounds with varying chemical characteristics were identified, including carbohydrates. Nevertheless, phosphate-containing analytes, including sugar phosphates and nucleotides were poorly detected (Losacco et al. 2020; Desfontaine et al. 2018). The potential of SFC-MS for the analysis of monosaccharides has been demonstrated for several applications. For example, various monosaccharides (including the isomers arabinose/xylose and glucose/galactose) could be separated from plant samples using a HSS C18SB column with an analysis time of only 4.5 min (Pauk et al. 2017). Yang and Zhengjin compared 10 different stationary phases for SFC to separate eight saccharides including fructose, glucose and sucrose (Huang and Jiang 2022). Overall, SFC-MS shows great potential for metabolomics because it allows the combined analysis of apolar and polar compounds (including monosaccharide derivatives). However, parameters such as type of stationary phase, type and concentration of organic modifier, temperature, additives etc., require optimization to obtain satisfactory performance.

4 | Gas Chromatography Coupled With Mass Spectrometry

Gas chromatographic separation operates by vaporizing the sample and passing it through a separation column with a stationary phase, separating compounds based on their volatility and interaction with the column material, providing high resolution and sensitivity. Gas chromatography (GC) can also be easily coupled with mass spectrometry, which makes it ideal for the analysis of complex mixtures (Gerwig 2021a; Ruiz-Matute et al. 2011). However, to make the non-volatile and thermally labile monosaccharide building blocks suitable for GC analysis these compounds require derivatization before analysis (Gerwig 2021a; Meyer et al. 2022). This makes the sample preparation more demanding compared to other methods. While monosaccharides are detectable with a simple flame ionization detector the combination with mass spectrometry opened the window to the analysis of unknown derivatives and complex samples (DeJongh et al. 1969). Nevertheless, because close isomers often show similar fragmentation patterns, the retention time obtained in gas chromatography remains crucial for identification (Ruiz-Matute et al. 2011). The first application of gas chromatography to volatile derivatives of monosaccharides was carried out in the early 60s, followed by coupling GC to electron ionization MS by DeJongh et al. in 1969 (DeJongh et al. 1969). Since then, GC-EI-MS has become widely employed for the analysis of various glycomolecules. For example, gas chromatography combined with methanolysis—a mild reaction which cleaves oligosaccharide chains into monosaccharide components—evolved as one of the most widely employed approaches for the compositional analysis of carbohydrate-containing molecules (Gerwig 2021a; Merkle and Poppe 1994; McInnes et al. 1958). This has led to a substantial number of scientific articles, book chapters, and reviews covering the fundamentals of GC-MS and its applications to carbohydrates over the past decades (Gerwig 2021a; Fox 2002; Jousse and Pujos-Guillot 2013; Obendorf, Horbowicz, and Lahuta 2012; Geyer and Geyer 1994; BeMiller 2017; Biermann 2021; Liu et al. 2021; Sweeley, Wells, and Bentley 1966). For example, the book "The Art of Carbohydrate Analysis" by Gerwig et al. provides a comprehensive introduction and detailed protocols for the application of GC to monosaccharide analysis (Gerwig 2021a). Therefore, this chapter only offers a concise overview of the basic concepts, recent advancements, and applications to the microbial world.

The preparation of volatile monosaccharide derivatives is challenged by the numerous functional groups, steric hindrance, presence of tautomeric forms, and molecular lability (Ruiz-Matute et al. 2011). Recently, the presence of other compounds such as amino acids has also been shown to alter the derivatization products of monosaccharides (Engel, Suralik, and Marchetti-Deschmann 2020). Common derivatization strategies for mono- and oligosaccharides have been comprehensively reviewed in earlier articles (Harvey 2011a; Meyer et al. 2022; Ruiz-Matute et al. 2011), which will therefore only be covered briefly in the following.

Classical derivatization approaches include the formation of methyl ethers, acetates, trifluoroacetates, and trimethylsilyl ethers. These derivatization products offer good volatility and stability. However, sugars with free carbonyl groups can be present as different tautomeric forms, which are stabilized through derivatization. This issue can be partially solved by performing a two-step derivatization of the reducing sugars, consisting of oximation followed by silylation to produce trimethylsilyl oximes. This derivatization procedure results in only two peaks, one for the syn (E) oxime form and one for the anti (Z) oxime form. Moreover, these forms are not observed

with nonreducing carbohydrates, because they do not form oximes. The peak splitting could be completely eliminated by reducing aldoses to alditol acetates, resulting in a single peak per sugar. However, different aldoses and ketoses can yield the same alditol upon reduction, making them indistinguishable through this procedure (Ruiz-Matute et al. 2011). Sugar acids, such as aldonic, uronic, and aldaric acids have been derivatized with methods like silylation or acetylation (Ruiz-Matute et al. 2011). The alpha-keto acid containing nonulosonic acids have been derivatized using a two-step process: first, methyl esterification of the alpha-keto acid using diazomethane, followed by derivatization of the hydroxyl groups with heptafluorobutyric anhydride (Kamerling and Gerwig 2006). Heptafluorobutyric anhydride derivatives are highly stable, exhibit excellent chromatographic properties on traditional methyl-siloxane columns, and are very volatile. This allowed the separation and identification of various nonulosonic acid derivatives, including O-acylated variants, methylated and sulfated derivatives, de-N-acetylated neuraminic acid, and sialic acid lactones. The unambiguous identification of nonulosonic acids from complex mixtures was based on distinct fragmentation patterns of the individual sialic acids (Pons et al. 2003; Zanetta et al. 2001; Kamerling and Gerwig 2006).

Phosphorylated sugars, such as glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate, are key intermediates in central metabolic pathways and are frequently studied in metabolic flux analysis of model cells including microbes. Their highly polar nature makes gas chromatographic analysis particularly challenging. However, their analysis has been achieved after derivatization with methoxyamine hydrochloride, which converts carbonyl groups into stable methoxime derivatives—which eliminates tautomeric forms—followed by derivatization using N-methyl-N-trimethylsilyl-trifluoroacetamide to silylate the hydroxyl groups of the sugar backbone and the phosphate groups (Roessner et al. 2000; Cipollina et al. 2009; Koek et al. 2006; de Jonge et al. 2012). Thereby, gas chromatography achieved baseline separation of sugar phosphate isomers, which allowed discrimination of positional phosphate isomers of metabolic intermediates (e.g. glucose-1P and glucose-6P). The typical ionization method for the GC-MS analysis of carbohydrates is electron ionization. However, when applied to sugar phosphates, this produces primarily fragment ions containing phosphate groups and only parts of the carbon backbone (Chu et al. 2015; Okahashi et al. 2019). This is disadvantageous for stable carbon isotope labeling experiments in metabolic flux analysis, as it results in a loss of labeling information. To prevent extensive fragmentation and consequently retain 13C labeling information, Okahashi et al. developed a GC-MS method using soft ionization by coupling gas chromatography to negative chemical ionization-mass spectrometry (Okahashi et al. 2019). This method involves derivatization with pentafluorobenzyloxime (PFBO) and trimethylsilyl. PFBO acts as an electron-capturing substituent of electrons generated by collision with the reagent gas, making this approach more sensitive and selective (Okahashi et al. 2019). Koubaa et al. also employed chemical ionization and reported improved isotopomer quantification in metabolic flux analysis using 13C-labeled sugars (Koubaa et al. 2012).

An alternative labeling of monosaccharides has been employed by Faraco and colleagues. They employed methylboronic derivatization followed by acetylation to analyze monosaccharides in plant gum extracts. Baseline separation of all monosaccharides was achieved employing a phosphonium-based ionic liquid capillary column. This method proved to be robust in the presence of inorganic substances and did not necessitate intermediate cleanup or evaporation steps (Faraco et al. 2016).

In addition to determining the monosaccharide composition, methylation analysis has been used to identify glycosidic linkages in glycomolecules. After permethylation of the intact glycomolecules, the linkages are hydrolyzed, and the resulting monosaccharides are reduced and peracetylated. The partially methylated alditol acetates are then separated and analyzed by GC-MS. This not only identifies the monosaccharide constituents but also provides unambiguous information on the individual sugar linkage positions (Geyer and Geyer 1994; Corey and Chaykovsky 1962; Hakomori 1964). The sample preparation has been further streamlined and miniaturized to make it suitable to small amounts of glycoproteins (Geyer and Geyer 1994). When performing linkage hydrolysis by methanolysis, the different cyclic tautomeric forms—in which monosaccharides exist in free solution—are locked as methyl glycosides. This increases the complexity of the data because it results in multiple peaks in the gas chromatogram (Ruiz-Matute et al. 2011). On the other hand, the peak pattern allows to identify monosaccharides according to their fixed ratio of the α/β furanose and α/β pyranose forms. As mentioned earlier, the peak splitting can be avoided by modification of the anomeric center (Ruiz-Matute et al. 2011).

Interestingly, a more recent study reevaluated derivatization and separation condition to propose an optimized GC-MS procedure for the analysis of complex monosaccharide mixtures (Haas, Lamour, and Trapp 2018). For example, the formose reaction—which is studied in the context of the origin of life—produces a remarkably complex mixture, ranging from the C1 building block formaldehyde to longer chain sugars hexoses and heptoses, which occur in different open-chain, furanose, and pyranose forms (Harsch et al. 1983; Pallmann et al. 2018). Their optimized method achieved complete separation of all oximated monosaccharides ranging from C2 to C7. However, while the method separated sugars by chain lengths into blocks and differentiated hexoses into aldoses and ketoses, it did not achieve baseline resolution of individual hexoses due to the large number of existing stereoisomers (Haas, Lamour, and Trapp 2018) (Figure 9).

Albeit these mixtures did not contain any derivatives, this clearly demonstrates that even methods with high resolving power face challenges in separating complex sugar mixtures. Therefore, a truly enantioselective separation of monosaccharides requires the application of either chiral stationary phases or reacting sugars with a chiral reagent to form diastereomers, which are then separated on an achiral column (König et al. 1988; Gerwig, Kamerling, and Vliegenthart 1978; Leontein, Lindberg, and Lönngren 1978). For example, there are four aldopentoses—lyxose, xylose, arabinose, and ribose—which in solution exist in equilibrium between the open-chain form, the α and β furanose

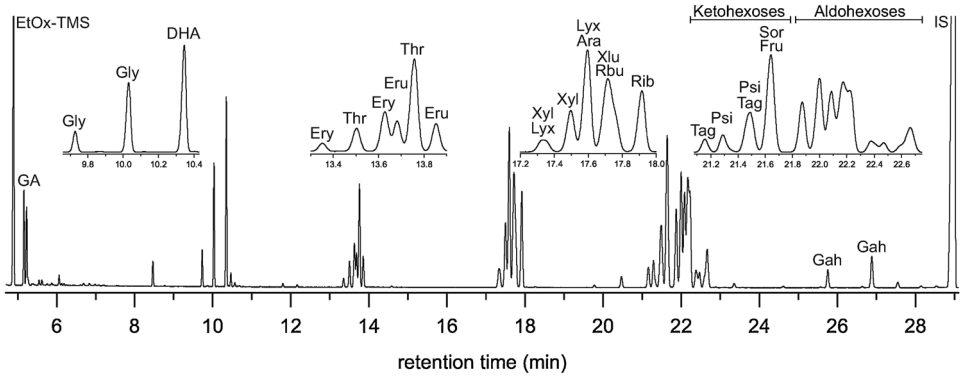


FIGURE 9 | An optimized gas chromatographic separation of monosaccharides, ranging from C2 to C7 units, using a SE-52 column (Haas, Lamour, and Trapp 2018). This was achieved following ethyloxime-trimethylsilyl derivatization and adjustment of the temperature program for monosaccharides. The shorter, unbranched compounds were individually separated, while the hexoses could only be partially separated due to the large number of stereoisomers. GA, glycolaldehyde; Gly, glyceraldehyde; DHA, dihydroxyacetone; Ery, erythrose; Thr, threose; Eru, erythrulose; Xyl, xylose; Lyx, lyxose; Ara, arabinose; Xlu, xylulose; Rbu, ribulose; Rib: ribose; Tag: tagatose; Psi: psicose; Sor: sorbose; Fru: fructose; Gah: galacto-heptose; IS, internal standard (phenyl-β-D-glucopyranoside). Reprinted from Haas, Maren, Saskia Lamour, and Oliver Trapp. Development of an advanced derivatization protocol for the unambiguous identification of monosaccharides in complex mixtures by gas and liquid chromatography. Journal of Chromatography A 1568 (2018): 160–167, Copyright 2018, with permission from Elsevier.

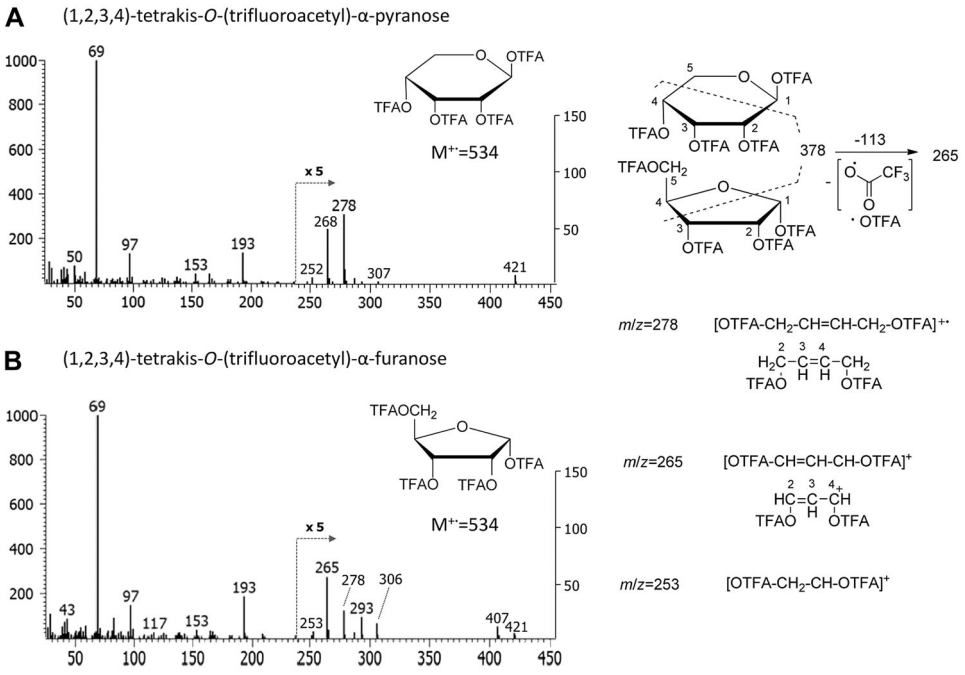


FIGURE 10 | Fragmentation analysis of ribopyranose and ribofuranose O-trifluoroacetyl derivatives using two-dimensional gas chromatography coupled to EI-MS (Myrgorodska et al. 2017). Distinct fragmentation patterns for the pyranose and furanose form were identified. (A) Fragmentation pattern of the pyranose form. (B) Fragmentation pattern of the furanose form. Structural diagrams in the graph illustrate the parent ions and the key fragment ions. Arrows indicate bond cleavages and rearrangements that led to the observed fragments. Reprinted from Myrgorodska et al. Enantioresolution and quantification of monosaccharides by comprehensive two-dimensional gas chromatography. Journal of Chromatography A 1487 (2017): 248-253, Copyright 2017, with permission from Elsevier.

forms and the α and β pyranose forms. Considering the two enantiomeric forms (D and L configurations), this accounts for 32 distinct aldopentose molecules. The demand for enantioselective analysis increases with the diversity of monosaccharide types. Myrgorodska et al. demonstrated the enantioresolution of a racemic mixture of aldopentoses following derivatization with trifluoroacetic anhydride and a two-dimensional gas chromatography including separation on a β-cyclodextrin column (Myrgorodska et al. 2017). Interestingly, the mass spectrometric fragmentation also showed distinct patterns for pyranose and furanose forms (Figure 10).

As mentioned above, carbohydrates are polar and thermally unstable, and their gas chromatographic analysis requires derivatization of the polar groups. Practically, this limits the approach to smaller sugars, such as mono-, di-, and

trisaccharides, and hinders the analysis of nucleotide-activated monosaccharides. The latter are thermally labile, which decompose at the high temperatures typically used in gas chromatography (Eisenberg and Bolden 1969).

Finally, apart from optimizing derivatization and column chemistry, advancements include coupling to high resolution mass spectrometers, such as GC-TOF-MS or GC-Orbitrap-MS. This enhances untargeted metabolomics work including the identification of novel monosaccharide derivatives (Duangkumpha et al. 2022). For advances in high-resolution GC-MS technologies we refer to the recent review by Misra Biswapriya (Misra 2021a).

The high performance and robustness of GC-MS methods have been utilized for the analysis of monosaccharides and their derivatives across various fields, including food analysis (Adebo et al. 2021; Wang et al. 2017), detection of specific sugars as surrogate indicators and biomarkers (De et al. 2015; Mairinger et al. 2020), for the compositional analysis of plant and fungal cell wall components (Pabst et al. 2013a; Millette et al. 2023; Zweckmair et al. 2017; Niemi et al. 2024), and for the characterization of different bacterial glycomolecules (Leker et al. 2017; Saad, Sidkey, and El-Fakharany 2023).

5 | Capillary Electrophoresis Coupled With Mass Spectrometry

Capillary electrophoresis is a highly effective method for separating polar and charged metabolites, particularly for substances like amino acids, nucleotides, small organic acids, sugars and sugar phosphates (Ramautar and Chen 2022; Ramautar, Somsen, and de Jong 2009). In metabolomics studies, capillary zone electrophoresis (CZE) has been the predominant separation mode in CE-MS (Ramautar, Somsen, and de Jong 2009; Zhang and Ramautar 2021; Zaia 2013; do Lago et al. 2021). Thereby, a narrow capillary is filled with an electrolyte solution, where metabolites are separated based on their charge-to-size ratio when an electric field is applied. The migration rate of molecules through the capillary depends on several factors, including the charge of the analyte, charge-to-hydrodynamic volume ratio, buffer pH and ionic strength, applied voltage, temperature, capillary length and diameter, and on the surface charge of the capillary wall (do Lago et al. 2021; Jorgenson and Lukacs 1983; Voeten et al. 2018; Monnig and Kennedy 1994; Harstad et al. 2016; Lindhardt and Pervin 1996; Mantovani et al. 2018). CE offers several advantages over liquid chromatography, including a higher resolution and faster analysis times. In addition, due to lower sample and reagent consumption, CE can be more environmental friendly. CE also requires only minimal sample preparation. However, CE has limitations such as challenges in separating neutral compounds and in regard to reproducibility of migration times, which can be affected by variations in the capillary surface and buffer conditions. Furthermore, in CE, the different charge states of metabolites necessitate multiple analytical runs, such as conducting separate runs for cationic and anionic analytes. The broader application of CE-MS to metabolomics studies, including separation modes, capillary coatings, and sample preparation techniques has been extensively reviewed by Ramautar and colleagues (Ramautar and Chen 2022; Ramautar, Somsen, and de Jong 2009; Zhang and Ramautar 2021; Ramautar, Somsen, and de Jong Somsen, and de Jong Somsen, and de Jong Somsen, and de Jong 2011, 2013, 2015, 2017, 2019), and will only be discussed briefly here in the context of monosaccharides and nucleotide activated sugars.

CE is often paired with UV, conventional fluorescence, and/or laser-induced fluorescence (LIF) detection (Zaia 2013; do Lago et al. 2021; Mantovani et al. 2018). For example, very sensitive oligosaccharide analysis with capillary gel electrophoresis (using conventional DNA sequences) has been performed after labeling with 9-aminopyrene-1,4,6-trisulfonic acid (APTS), and coupling to LIF for detection (Callewaert et al. 2001; Ruhaak et al. 2010b). While this allows for straightforward and reliable compound detection in many applications, additional mass spectrometric detection is often necessary for identifying compounds, especially in the analysis of sugar derivatives, unknown metabolites, and complex cellular extracts such as those from microbes.

Despite its high separation performance, CE remained underrepresented in metabolomic studies—including the analysis of monosaccharides—compared to more common LC and GC approaches. This is mainly because CE-MS is considered technically more challenging and less reproducible than other separation methods (Zhang and Ramautar 2021).

Moreover, the lack of convenient interfaces for connecting CE with mass spectrometers has challenged their application (Ramautar, Somsen, and de Jong 2017; Khatri et al. 2017). Generally, CE faces challenges with low liquid flow rates suboptimal for electrospray ionization. Sheath flow interfaces mix a sheath liquid with CE effluent to increase flow rates but may reduce sensitivity and may lead to peak distortion (Fang, Pan, and Fang 2018; Höcker, Montealegre, and Neusüß 2018; Sauer, Sydow, and Trapp 2020). Sheathless-flow interfaces struggle with spray stability and reproducibility (Höcker, Montealegre, and Neusüß 2018). Liquid-junction interfaces partially disconnect CE and ESI components and produce a stable electrospray (Khatri et al. 2017; Fanali et al. 2006).

In addition, bare fused-silica capillaries compromise separation efficiencies and migration time reproducibility due to potential analyte interactions. Surface coatings are commonly applied to prevent these interactions, stabilize the electroosmotic flow (EOF), and support MS coupling (Huhn et al. 2010). Moreover, commercially available microfluidic CE systems with integrated nanoelectrospray ionization interfaces have addressed this issue (Khatri et al. 2017; Mellors et al. 2008).

As mentioned earlier, CE relies on the charge of molecules for separation. Thus, CE has been frequently employed for analyzing charged metabolites, such as organic acids, sugar-phosphates and nucleotides. However, many monosaccharide building blocks are neutral, posing a challenge for their analysis by CE. Consequently, various carbohydrate labeling strategies have been developed to introduce a charge and overcome these issues. Reductive amination of the aldehyde group at the reducing end of sugars is one of the most commonly used derivatization methods (Mantovani et al. 2018). The applied labels often include a fluorophore and are therefore paired with sensitive fluorescence detection. For instance, monosaccharides have been

separated by CE following labeling with 1-phenyl-3-methyl-5-pyrazolone, 2-aminobenzoic acid, or 7-aminonaphthalene-1,3-disulfonic acid (Mantovani et al. 2018). The latter method facilitated the separation of ribose enantiomers (Sun et al. 2013). An updated protocol detailing the analysis of monosaccharides following 2-aminobenzoic acid has also been published recently (Abo et al. 2013). Another method was developed by Guttman, who performed CE analysis of APTS-labeled monosaccharides and 2-aminoacridone (AMAC) labeled sialic acids coupled with LIF detection (Guttman 1997). Szabo and colleagues used 2-AMAC to develop a rapid sialic acid speciation method. The classical reductive amination in the presence of sodium cyanoborohydride in DMSO/acetic acid caused the sialic acids to decarboxylate spontaneously. The resulting neutral sialic acids were analyzed using a neutral-coated capillary (to eliminate the EOF) in reversed polarity mode, with a background electrolyte containing 200 mM boric acid (Szabo et al. 2012). This achieved complete baseline separation between Neu5Gc and Neu5Ac. However, MS was only used to investigate the decarboxylation of sialic acids. Schmitt-Kopplin optimized borate concentration and buffer pH for the separation of acidic monosaccharides and their 1,4-lactones without the need for derivatization (Schmitt-Kopplin et al. 1998). In fact, many of these methods employed buffers which are incompatible with mass spectrometry (Khatri et al. 2017). Similarly, chiral ligand-exchange capillary electrophoresis successfully achieved the enantiomeric separation of monosaccharides, but it was only utilized in conjunction with fluorescence detection (Kodama et al. 2006, 2007). Furthermore, advancements in chiral capillary electrophoresis has been recently reviewed by Clark et al. (Clark, Somsen, and Kohler 2023).

Khatri et al. developed a CE-MS method for the analysis of monosaccharides, oligosaccharides and glycopeptides employing a microfluidic separation system with a nanoESI source as a detachable interface (Khatri et al. 2017). The interface minimized gaps, dead volumes, and sample dilution. Aminopropyl silane coating on the capillary surface reduced the EOF. Monosaccharides were labeled with aminoxy TMT reagent to introduce a positive charge, facilitating the electrophoretic migration of neutral saccharides and enabling multiplexing in tandem-MS experiments (Figure 11).

Additionally, a sialic acid derivatization method was applied to prevent interaction between negatively charged carboxyl groups and the positively charged capillary surface coating. Lageveen-Kammeijer and co-workers introduced a uniform positive charge to oligosaccharides by derivatizing them with linkage-specific sialic acid and labeling them with Girard's Reagent P hydrazide (Lageveen-Kammeijer et al. 2019). The authors enhanced labeling efficiency and devised a one-step, cleanup-free, rapid protocol, enabling direct injection of labeled sugars into the CE-MS system. This facilitated ultra-sensitive analysis of oligosaccharides. However, to the best of the authors' knowledge, this approach has not yet been applied to monosaccharide derivatives. Sugar acids including nonulosonic acids can be analyzed by CE without further derivatisation (Campa, Baiutti, and Flamigni 2008). Ortner and Buchberger demonstrated this by performing quantitative analysis of the underivatized sugars, extracted from glycoproteins after acid hydrolysis, to determine Neu5Ac/Neu5Gc ratios in various samples (Ortner and Buchberger 2008). The same group also investigated the possibility to analyze neutral carbohydrates by CE-MS without derivatization, using strongly alkaline (pH > 12) carrier electrolytes compatible with mass spectrometry (Klampfl and Buchberger 2001). The high pH favored detection in negative ionization mode (Klampfl and Buchberger 2001). Daniel and colleagues expanded this method to include tandem mass spectrometry, which facilitated the unambiguous identification of the analyzed monosaccharides (Daniel et al. 2018).

Furthermore, phosphate and nucleotide-activated sugars do not require derivatization or elevated buffer pH for their analysis with CE. A review with focus on anionic metabolites has been

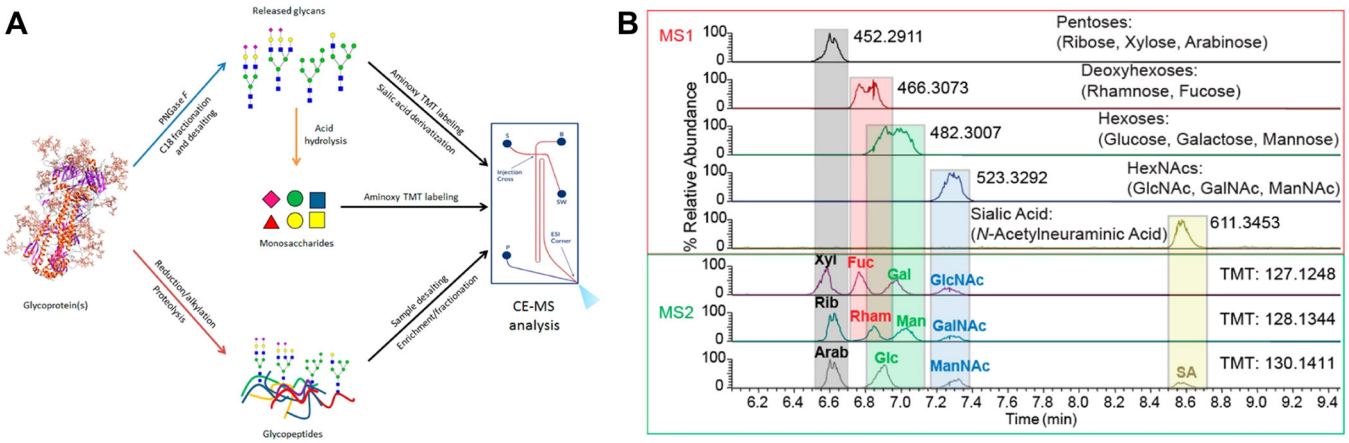


FIGURE 11 | Khatri et al. developed a microfluidic capillary electrophoresis–mass spectrometry approach for the analysis of monosaccharides, oligosaccharides, and glycopeptides (Khatri et al. 2017). (A) Workflow for the simultaneous analysis of monosaccharides, oligosaccharides, and glycopeptides using a microfluidic CE-ESI-MS approach, including the CE/ESI chip interface. Neutral monosaccharides are analyzed following aminoxy TMT labeling, while sialic acids are analyzed label-free. (B) The multiplexed analysis of common monosaccharides, utilizing different aminoxy TMT reagents to distinguish isomers based on migration time and fragment ion profiles. Reprinted with permission from Khatri, Kshitij, et al. Microfluidic capillary electrophoresis–mass spectrometry for analysis of monosaccharides, oligosaccharides, and glycopeptides." Analytical chemistry 89.12 (2017): 6645–6655. Copyright 2017, American Chemical Society. [Color figure can be viewed at wileyonlinelibrary.com]

conducted only recently (van Mever, Hankemeier, and Ramautar 2019). Soga et al. investigated the anionic metabolic profile of *B. subtilis* extracts using CE-MS. They employed cationic polymer-coated capillary and weakly alkaline ammonia buffers, utilizing reversed CE polarity and negative ionization mode, which allowed them to analyze sugar phosphates and nucleotides from the complete central carbon metabolism. Interestingly, to overcome corona discharge issues in negative mode—electrical discharge which results from the ionization of a fluid surrounding a conductor that is subjected to a high voltage—Zhang et al. introduced a sheathless CE-MS method for efficient and sensitive nucleotide analysis in positive ion mode (Zhang et al. 2020).

Capillary electrophoresis has also been combined with simple UV detection (260 nm), as demonstrated recently for the analysis of sugar nucleotides from mammalian cells (Bucsella et al. 2016; Lehmann et al. 2000; Feng et al. 2008). However, the identification of the peaks requires comparing migration times to reference sugar nucleotide samples. More sophisticated experiments, such as those involving stable isotope incorporation experiments require mass spectrometric detection. For example, the detection of sugar phosphates and their metabolic turnover rates in the Calvin cycle of tobacco leaves was measured (Hasunuma et al. 2010). The isotopomer abundance changes when switching to ^{13}C-labeled CO_2 were obtained by coupling capillary electrophoresis with tandem mass spectrometry, operating in multiple reaction monitoring mode. This provided accurate concentrations of the sugar phosphate intermediates and revealed significantly lower turnover of glucose-1-phosphate compared to glucose-6-phosphate.

Finally, Soo et al. developed and applied a novel CE-MS precursor ion scanning approach to detect nucleotide-activated sugars, involved in the glycoconjugate biosynthesis of *Campylobacter jejuni* strains (Soo et al. 2004). The authors employed N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) as a sample stacking buffer, achieving detection limits down to 0.2 pmol/mL and a good linear dynamic range for various nucleotide-sugar standards. Their parent ion scanning approach identified CMP-linked nonulosonic acids and potential UDP-linked precursor sugars. Although the authors did not provide a complete description of the sugar nucleotide profile in this article, the approach of comparing a parent strain with isogenic mutants shows great potential for delineating the biosynthesis routes of microbial glycoconjugates.

6 | Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

This chapter offers a concise overview of the applications of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to the analysis of monosaccharides, derivatives and activated forms. For a comprehensive introduction to the fundamentals of MALDI mass spectrometry, as well as general aspects related to matrices and sample preparation, we would like to refer to earlier reviews (Harvey 1999, 2023a; Karas and Krüger 2003; Knochenmuss and Zenobi 2003; Hillenkamp and Peter-Katalinic 2013; El-Aneed, Cohen, and Banoub 2009; Dreisewerd 2014; Zenobi and Knochenmuss 1998). In MALDI-MS the sample is co-crystallized with a matrix compound that absorbs laser energy, typically from UV lasers such as nitrogen lasers (337 nm) or Nd:YAG lasers (355 or 266 nm), resulting in the desorption and ionization of the analytes (Lu et al. 2015). MALDI typically generates molecular ions as adducts with cations such as protons or sodium ions, resulting in spectra with predominantly singly charged species such as $[M + H]^+$ (protonated) and $[M+Na]^+$ (sodiated), and minimal fragmentation (Harvey 1999, 2023a; Lu et al. 2015). The generally gentle ionization process, allows for the ionization and analysis of fragile and large biomolecules. For carbohydrates, positive-ion MALDI-MS predominantly generates sodiated (or potassiated) rather than protonated species, owing to their strong affinity for these alkali metal ions (Harvey 1999, 2006; Lee et al. 2016). However, the ionization of carbohydrates as sodiated species is less sensitive compared to the analysis of peptides, where protonated species dominate. As a result, higher laser power is typically required for carbohydrate analysis (Harvey 1999). A theoretical study on glucose also demonstrated that most protons produced by MALDI attach to matrices rather than to glucose, due to lower proton affinity of glucose and the higher concentration of matrix molecules. Even when protons do attach to glucose, protonated glucose rapidly dissociates into fragments because of the high temperatures during MALDI (Chen et al. 2016). For neutral carbohydrate oligomers, MALDI-TOF has demonstrated consistent ionization efficiency with increasing molecular size, in contrast to ESI, where efficiency declines with increasing molecular weight (Zaia 2004; Harvey 1993). Consequently, MALDI-MS is especially useful for profiling mixtures of neutral oligosaccharides. Furthermore, MALDI-MS generally requires minimal sample preparation, and through careful selection and preparation of the matrix it can analyze complex mixtures at high sensitivity (Harvey 1999, 2023a; Dreisewerd 2014; Batoy et al. 2008; Dreisewerd 2003). Another advantage is the possibility to provide spatial information of samples, through MALDI imaging mass spectrometry (MSI), which has been reviewed in several articles recently (McDonnell and Heeren 2007; Gessel, Norris, and Caprioli 2014; Amstalden van Hove, Smith, and Heeren 2010; Rzagalinski and Volmer 2017). The high spatial resolution enables detailed mapping of metabolites at cellular and even subcellular levels in tissues from clinical samples and microbial biofilm samples (Seeley and Caprioli 2011; Ucal et al. 2017; Cazares et al. 2011). However, MALDI-MS faces challenges in shot-to-shot reproducibility and sample heterogeneity due to inhomogeneous sample/matrix co-crystallization, which leads to "hot spots" with high matrix concentrations that significantly increase ionization efficiency compared to nearby areas (O'Rourke, Djordjevic, and Padula 2018). This impacts quantitative reproducibility and sample preparation automation. Nevertheless, the direct application of crude samples and the short analysis times enable high-throughput metabolic profiling, allowing for the analysis of hundreds to thousands of samples per day (Haslam et al. 2016; Urban et al. 2010; Pabst et al. 2013b; Yukihira et al. 2010). Excellent matrices are available for proteins, peptides, and polysaccharides, such as α-cyano-4-hydroxycinnamic acid (Beavis, Chaudhary, and Chait 1992) and 2,5-dihydroxybenzoic acid (Bourcier, Bouchonnet, and Hoppilliard 2001), including several other organic acids, ionic liquids or nanoparticles (Harvey 1999, 2023a; Abdelhamid 2017). Furthermore, MALDI-MS is also a powerful approach to study

and monitor glycomolecules for different fields of research. Applications to oligosaccharides and glycoconjugates have been extensively reviewed by Harvey and others (Harvey 1999, 2023a; Wang et al. 2023; Hykollari, Paschinger, and Wilson 2022; Harvey 2006, 2018a, 2021, 2023b, 2008, 2009, 2011b, 2012, 2015a, 2015b, 2018b).

However, the analysis of small mass compounds—such as monosaccharides and activated sugars—can be complicated by the spectral interference in the low m/z region, which is produced by conventional organic matrices (Harvey 2021; Qiao and Lissel 2021). Therefore, the selection of matrices that minimize interference in the low mass range is crucial for the analysis of monosaccharide building blocks. An overview of current matrices and strategies to design matrix systems for low molecular weight compounds has been provided in recent articles (Harvey 2021; Qiao and Lissel 2021; Calvano et al. 2018). Coupling MALDI to very high-resolution mass spectrometers, such as Fourier Transform Ion Cyclotron Resonance also enhances discrimination between matrix and analyte peaks in the low mass range (Pabst et al. 2014; Krismer et al. 2020; Wang et al. 2011). Furthermore, such very high resolution mass analyzers provide the sum formulae of unknown derivatives (and their fragments), which are commonly encountered in microbial samples.

Efforts have been made to adapt classical matrices for low molecular weight applications through structural modifications, such as polymerization or conversion into ionic liquids (Qiao and Lissel 2021; Armstrong et al. 2001). For example, Schmidt De León et al. employed norharmane containing ionic liquid matrices for the analysis of low molecular weight carbohydrates (Schmidt De León, Salum, and Erra-Balsells 2019). Additionally, new high molecular weight matrices, which are potentially "MALDI silent", meaning they do not result in matrix-related peaks in the spectrum, are under investigation. Horatz et al. tested different conjugated polymers which provided comparable profiles to 2, 5-dihydroxybenzoic acid (DHB) in positive mode and 9-aminoacridine in negative mode, making these suitable for metabolic studies (Horatz et al. 2018). Another higher molecular weight matrix was applied with addition of potassium hydroxide as dopant, which allowed direct analysis of fructose/glucose and sucrose from different beverages at high sensitivity (Ayorinde, Bezabeh, and Delves 2003). Finally, mesoporous silica SBA-15, modified with 1,8-naphthalimide, was developed as a matrix for small molecules such as monosaccharides and amino acids. This matrix exhibited minimal background interference in the low mass range (Dong, Sun, and Jin 2014).

Other strategies include reactive matrices that not only promote desorption and ionization of the analyte but also derivatize it to enhance detection and characterization (Calvano et al. 2018). For example, the aldehydes and ketones of carbohydrates react with amines to form Schiff bases. This reaction has been reported to improve the detection limit when using anthranilic acid or 2-Phenyl-3-(p-aminophenyl) acrylonitrile for various compounds, including oligosaccharides (Zhang and Gross 2002; Ling et al. 2019). Other reactive matrices include hydrazines, which condense with carbonyl groups to form hydrazones (Qiao and Lissel 2021; Calvano et al. 2018). While these matrices have rarely been employed for the analysis of monosaccharide components to the best of the authors' knowledge, these have the potential to facilitate the analysis in positive mode with good sensitivity. For example, Han et al. developed an on-tissue derivatization strategy for imaging and quantifying monosaccharides in biological tissues, employing the derivatization reagent 1-naphthaleneacethydrazide. The reactive matrix increased sensitivity and produced sugar-specific diagnostic ions during MALDI-MS/MS analysis (Han et al. 2022). This enabled the authors to distinguish between glucose and fructose.

MALDI matrices for positive ion mode typically have acidic functional groups, facilitating proton transfer. In contrast, matrices for negative ion mode need to be moderately strong bases to abstract protons from the analyte, as per the Brønsted-Lowry acid–base theory (Qiao and Lissel 2021; Calvano et al. 2018). Many matrices are designed for positive ion mode, but negative ion mode matrices often offer the advantage of fewer background signals (Qiao and Lissel 2021; Calvano et al. 2018). They are particularly useful for metabolomic studies, as many sugar intermediates in central metabolic pathways are negatively charged. Examples for negative mode matrices which were used for low mass applications are 9-Aminoacridine and 2-Aminoacridine (9AA and 2AA). The amine group in these matrices accepts protons from acidic analytes, resulting in the formation of deprotonated and negatively charged analyte species $[A\text{-}H]^-$ (Vermillion-Salsbury and Hercules 2002; Teearu et al. 2017). 9AA was successfully used in the analysis of low molecular weight metabolites including organic acids, sugar phosphates, and sugar nucleotides (Shroff, Muck, and Svatoš 2007; Ibáñez et al. 2013; Karst et al. 2017a). Advantageously, sugar nucleotides are negatively charged compounds. While their analysis using MALDI-MS has not been widely employed, Heinrich et al. demonstrated the possibility of analyzing activated sugars using negative MALDI-MS (Heinrich et al. 2008). Steinhoff et al. investigated a rapid MALDI-MS method in negative mode for determining the energy charge of mammalian cells (Steinhoff et al. 2014). The method provided insights not only into nucleotides but also into nucleotide-sugars, such as UDP-HexNAc and UDP-Hex. By spiking ^{13}C-labeled ATP into the cell lysates, the authors furthermore determined the degree of in-source decay for different negative mode matrices, specifically 9-aminoacridine (9-AA) and 2,4,6-trihydroxyacetophenone (2,4,6-THAP) (Figure 12). In-source decay was found to be strongly dependent on the applied laser power and the extraction pulse delay (Steinhoff et al. 2014). The same MALDI-MS approach was applied to rapid monitoring of metabolites from central metabolic pathways of Chinese hamster ovary cells, including sugar phosphate intermediates, nucleotides, and sugar nucleotides (Karst et al. 2017a, 2017b; Villiger et al. 2016).

MALDI-MS was also used to monitor the synthetic reactions of monosaccharides, as summarized in earlier articles and reviews (Harvey 2009, 2012, 2015b, 2018b; Sato et al. 2007). Additionally, advanced spotting and sample target devices enabled the detection of metabolites (e.g. sugar phosphates) from single mammalian and microbial cells (Ibáñez et al. 2013; Hu et al. 2023; Zenobi 2013; Guillaume-Gentil et al. 2017). Furthermore Gouw et al. introduced a quaternary ammonium center into sugars such as glucose, glucosamine, cellobiose, sucrose, raffinose, and

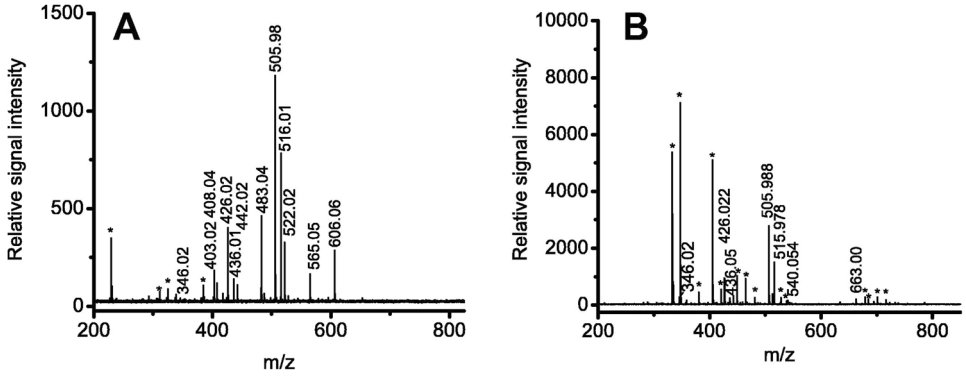


FIGURE 12 | Steinhoff et al. (2014) present a rapid negative mode MALDI-TOF MS method for analyzing central metabolic metabolites, including nucleotides and sugar nucleotides. The authors compared different matrices, specifically (A) 9-aminoacridine and (B) 2,4,6-trihydroxyacetophenone, and assessed the degree of in-source decay using 13C-labeled ATP (516.01 m/z). In addition to determining the energy charge, the method also provided strong signals for common nucleotide-sugars, e.g. UDP-Hex (566.05 m/z) and UDP-HexNAc (505.06 m/z). Reprinted from Steinhoff, Robert F., et al. "Rapid estimation of the energy charge from cell lysates using matrix-assisted laser desorption/ionization mass spectrometry: Role of in-source fragmentation." Analytical biochemistry 447 (2014): 107–113. Copyright (2014), with permission from Elsevier.

sorbitol using glycidyltrimethylammonium chloride or Girard's reagent T. The modified sugars, when analyzed with DHB, produced intense signals, while the matrix-related signals remained comparatively weak (Gouw et al. 2002). Another challenge when analyzing bacterial cells or biofilms directly is the efficient extraction of analytes from deeper within the bacterial biomass and from the cytoplasm of cells. To overcome this limitation, the authors employed a pulsed infrared laser to disrupt and ablate bacterial cells without matrix, followed by laser-induced post ionization (MALDI-2), facilitating the detection of multiple small molecules. This holds a great promise for the direct analysis of microbial samples.

Although not yet demonstrated for monosaccharides, innovative sample preparation techniques for MALDI-MS have significantly advanced carbohydrate analysis. For example, methods such as using frozen matrix/sample solutions or the Rapidly Freeze-Drying Droplet approach improve sample morphology homogeneity, lower the laser energy threshold, reduce fragmentation, and resolve the 'sweet spot' issue commonly encountered with traditional preparation methods (Wu et al. 2024; Liang et al. 2012). Finally, matrix-free strategies which are suitable for small-molecules such as monosaccharides, should also be mentioned. An example includes pulsed laser desorption-ionization from a porous silicon surface (Wei, Buriak, and Siuzdak 1999). Nevertheless, MALDI-MS has generally found limited applications in exploring monosaccharide components, primarily due to its inability to discriminate between structural and positional isomers—a critical factor when studying glycomolecules. Therefore, recent approaches have aimed to combine MALDI with ion mobility to provide an additional dimension of selectivity (Harvey 2023b; Djambazova et al. 2022). This demonstrated great potential for the untargeted identification of secondary metabolites, such as carbohydrates, in microbial cells and microbial biofilms (Kuik et al. 2024; Feucherolles and Frache 2022). The combined application of laser-based ionization with ion mobility spectrometry has been reviewed previously (Harvey 2023b; Kiss and Hopfgartner 2016). The application of ion mobility mass spectrometry to monosaccharide components is reviewed in Chapter 8.

7 | Flow Injection Mass Spectrometry

During flow injection mass spectrometry, the samples are injected into the mobile phase, which directly enters the mass spectrometer. This flow injection analysis of biological samples offers high-throughput profiling, making it ideal for studies with large sample numbers. Key advantages over chromatographic methods include extremely short analysis times (typically under 60 s) and simplicity. The increasing application of this approach has been driven by advancements in sensitivity and mass resolving power. This has led to its use in clinical diagnostics, metabolomics, and environmental sciences. Developments and applications of flow injection analysis have been described in recent reviews (Ruiz-Capillas, Herrero, and Jiménez-Colmenero 2018; Nanita and Kaldon 2016; Draper et al. 2013; González-Domínguez, Sayago, and Fernández-Recamales 2017). However, like direct MALDI-MS analysis, flow injection MS offers lower selectivity compared with chromatography-based approaches. Compounds such as structural isomers and enantiomers cannot be distinguished by mass alone. Some compounds may also undergo in-source ion fragmentation, which fragments may mimic ions of other analytes (Nanita and Kaldon 2016). Consequently, this approach is not particularly popular for studying monosaccharide components, except in the context of high-throughput monitoring of central metabolic pathways (Taki et al. 2020). However, Nagy and Pohl reported the application of a chiral mass spectrometry approach using a fixed ligand kinetic method, which allowed for the discrimination of sugar isomers by mass spectrometry. In this method, sugars form a trimeric ion complex with ligands in the gas phase via electrospray ionization, which is then subjected to collision-induced dissociation, resulting in two fragment ions. The relative intensities of these two fragments are characteristic of each sugar isomer. This approach successfully distinguished the complete set of pentose isomers, as well as 24 aldohexose and 2-ketohexose isomers (Nagy and Pohl 2015a) (Figures 13 and 14).

Later, Wooke and colleagues combined this method with prior chromatographic separation using ion-exclusion chromatography

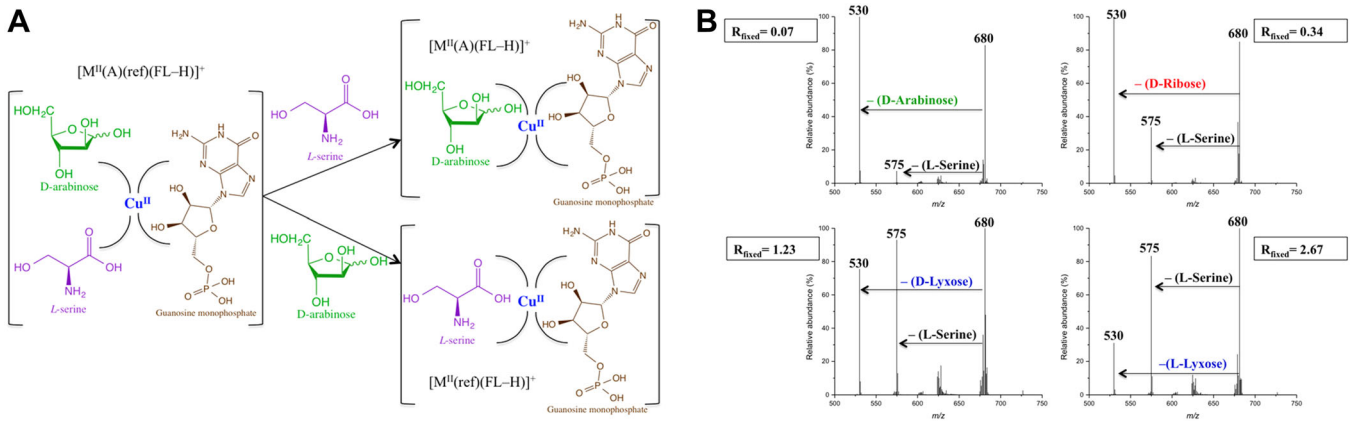


FIGURE 13 | Nagy and Pohl demonstrate a mass spectrometric method called fixed ligand kinetic method for distinguishing all 12 pentose isomers, including their absolute configuration. (A) Fixed ligand components: D-arabinose as the analyte (A), copper(II) as the metal cation (M^{II}), L-serine as the chiral reference (ref), and guanosine monophosphate as the fixed ligand (FL). These components form a trimeric complex $[M^{II}(A)(ref)(FL-H)]^{++}$ during electrospray ionization. The trimeric complex is then fragmented by collision-induced dissociation into the diastereomeric fragments $[M^{II}(A)(FL-H)]^{++}$ and $[M^{II}(ref)(FL-H)]^{++}$. (B) Fragmentation spectra of the trimeric complex with the fixed ligand combinations of Cu^{II}/L-Ser/5′ GMP, for the pentoses D-arabinose, D-ribose, as well as D/L-lyxose. Reprinted with permission from Nagy and Pohl. Monosaccharide identification as a first step toward de novo carbohydrate sequencing: mass spectrometry strategy for the identification and differentiation of diastereomeric and enantiomeric pentose isomers. Analytical chemistry 87.8 (2015): 4566-4571. Copyright 2015, American Chemical Society. [Color figure can be viewed at wileyonlinelibrary.com]

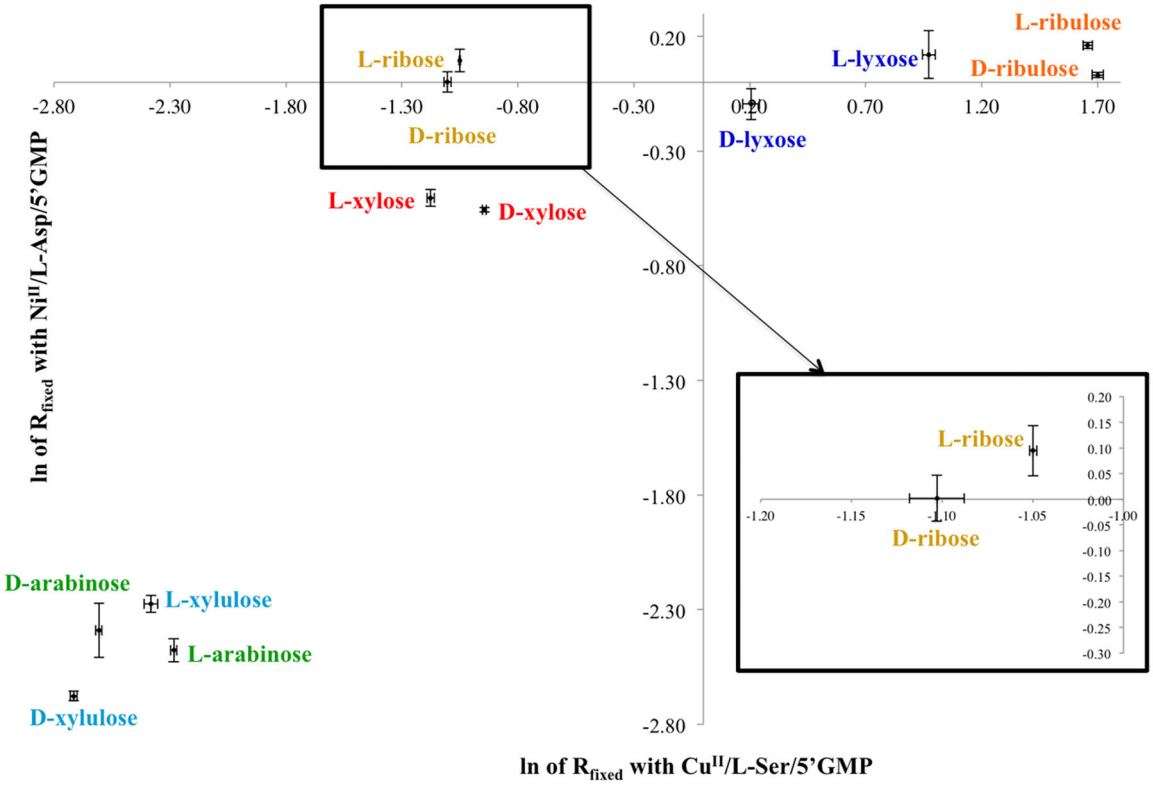


FIGURE 14 | Nagy and Pohl demonstrate a mass spectrometric approach "fixed ligand kinetic method" for distinguishing all 12 pentose isomers, including their absolute configuration. The plot shows the unique R_{fixed} values, with Cu^{II}/L-Ser/5′ GMP on the x-axis and Ni^{II}/L-Asp/5′ GMP on the y-axis, allowing to differentiate all pentose isomers. The error bars represent one standard deviation. Reprinted with permission from Nagy and Pohl. Monosaccharide identification as a first step toward de novo carbohydrate sequencing: mass spectrometry strategy for the identification and differentiation of diastereomeric and enantiomeric pentose isomers. Analytical chemistry 87.8 (2015): 4566-4571. Copyright 2015, American Chemical Society. [Color figure can be viewed at wileyonlinelibrary.com]

(Wooke et al. 2019). The eluents from the separation system were then mixed with a fixed ligand combination before performing mass spectrometric experiments to determine fragment ion ratios. Furthermore, Giorgi and Speranza demonstrated the isomeric discrimination of underivatized monosaccharides as their non-covalent hydrazine complexes based on their fragmentation pattern, after low-energy collision-induced fragmentation using an ion trap mass spectrometer (Giorgi and Speranza 2006). Interestingly, Madhusudanan and Srivastava demonstrated that oxocarbenium ions of aldohexoses and ketohexoses produce characteristic tandem mass spectra in the m/z 80 to 120 range (Madhusudanan and Srivastava 2008). This facilitated the differentiation of a range of diastereomers (Figure 15).

8 | Ion-Mobility Spectrometry

Mass spectrometry allows for the determination of the accurate mass and fragmentation profile of monosaccharides. This enables, to some extent, the differentiation of structural isomers, as described in chapter 7 (flow injection mass spectrometry). However, the highly sensitive analysis of complex isomeric mixtures requires an additional pre-separation step. Due to the narrow polarity range and similar size of monosaccharides and its derivatives, most chromatographic separation techniques do not ensure the complete separation of isomeric structures (Wooke et al. 2019; Burnum-Johnson et al. 2019). Because of their identical elemental composition and similar structures, isomeric monosaccharides tend to elute closely together even during long separation times, and their almost identical fragmentation profiles further complicate the analysis (Burnum-Johnson et al. 2019). To address these limitations, ion mobility spectrometry in combination with mass spectrometry is being increasingly utilized in various omics fields (Burnum-Johnson et al. 2019; Te Brinke, Arrizabalaga-Larrañaga, and Blokland 2022; Christofi and Barran 2023; Ibrahim et al. 2017; Ortmayr et al. 2016; Causon, Kurulugama, and Hann 2020). For reviews on the application of ion mobility spectrometry in carbohydrate research, we would like to refer to earlier articles (Zhu et al. 2009; Mu, Schulz, and Ferro 2018; Hofmann and Pagel 2017; Both et al. 2014; Gabryelski and Froese 2003).

Ion mobility spectrometry (IMS) is a gas-phase electrophoretic technique that enables rapid structural and conformational separations. In IMS, compounds with varying sizes, shapes, and charges are separated as they travel through a gas-filled mobility cell. Under the influence of an electric field, ions collide with inert gas particles. Larger, more diffuse species experience a higher frequency of collisions, resulting in slower electrophoretic motion compared to more compact ions (Morris et al. 2020; Zhou, Tu, and Zhu 2018). The measured IMS drift time can then be converted into a rotationally averaged collision cross section (CCS), which describes the three-dimensional gas phase structure of ions (Morris et al. 2020; Zhou, Tu, and Zhu 2018).

In addition to providing an orthogonal separation dimension, IMS can effectively improve the signal-to-noise ratio by reducing chemical noise and enhancing peak capacities when coupled with other separation techniques (Morris et al. 2020; Zhou, Tu, and Zhu 2018). Following the emergence of the first commercially available IMS-MS instrument, the Synapt HDMS, which utilized traveling wave IMS (TWIMS), several other IMS-MS devices were introduced. These instruments employed IMS devices including drift tube IMS (DTIMS), high-resolution cyclic IMS (cIMS), trapped IMS (TIMS), high-field asymmetric waveform IMS (FAIMS), differential mobility spectrometry (DMS), differential ion mobility spectrometry (DIMS), differential mobility analyzers (DMA), and structures for lossless ion manipulations (SLIM) (Ibrahim et al. 2017; Pringle et al. 2007; May et al. 2014; Groessl, Graf, and Knochenmuss 2015; Michelmann et al. 2014; Guevremont 2004; Kolakowski and Mester 2007). FAIMS, DMS, and DIMS are often discussed together, since they all operate similarly and only differ in the geometry of their electrodes. The differences between all the mentioned techniques are in the nature of the electric field used to propel the ions through the IM cell, variations in gas flow, ion packet distribution, and the ability to measure CCS (Dodds and Baker 2019). FAIMS, DMS, and DIMS are typically placed directly behind the ion source, serving as filtering devices. By scanning, only those analytes that respond to the changing electric field and match the applied compensation voltage can pass through and exit the drift region (Dodds and Baker 2019). For detailed information on various ion mobility devices and recent advancements, we would like to refer to earlier reviews (Christofi and Barran 2023; Dodds and Baker 2019; Cumeras et al. 2015; Olajide, Kartowikromo, and Hamid 2023; Naylor and Nagy 2024). The flagship instruments from different vendors have each established their own niche advantages, use cases, and drawbacks, as detailed by Delafield and co-workers (Delafield et al. 2022).

Although ion mobility was first paired with mass spectrometry in the 1960s, its application to carbohydrate research emerged much later, in 1997, when Liu and Clemmer demonstrated that different oligosaccharide isomers can be characterized based on their drift times (Liu and Clemmer 1997). In 2005, Clowers et al. demonstrated that IMS-MS can be used for unambiguous separation of isobaric carbohydrate mixtures (Clowers et al. 2005). Historically, ion mobility separations preceded tandem MS. However, recent advancements allowed for fragmentation to occur both before and after mobility separation, providing valuable insights into individual monosaccharide components of oligo- and polysaccharides, thereby facilitating the use of ion mobility for carbohydrate sequencing (Morrison and Clowers 2018). In that regard, most of the applications of IMS-MS focused on the analysis of carbohydrate oligo and polysaccharides (Both et al. 2014; Li et al. 2013; Harvey 2020; Seo, Andaya, and Leary 2012; Manabe et al. 2022; Bohrer and Clemmer 2011; Huang and Dodds 2013; Fenn and McLean 2011; Fasciotti et al. 2012; Li et al. 2015b; Hofmann et al. 2015; Williams et al. 2010; Sastre Toraño et al. 2021; Miller et al. 2020), with only a few studies focusing on the fundamental monosaccharide building blocks. Typically, isobaric monosaccharides and small oligosaccharides were considered to lack sufficiently distinct CCS values to be separated by commercially available IMS systems. To mitigate this issue, various shift reagents have been utilized to amplify the CCS differences between isobaric sugars, making it easier to obtain specific IMS profiles. These reagents, which can bind either covalently or non-covalently increase the mass of monosaccharides up to several times.

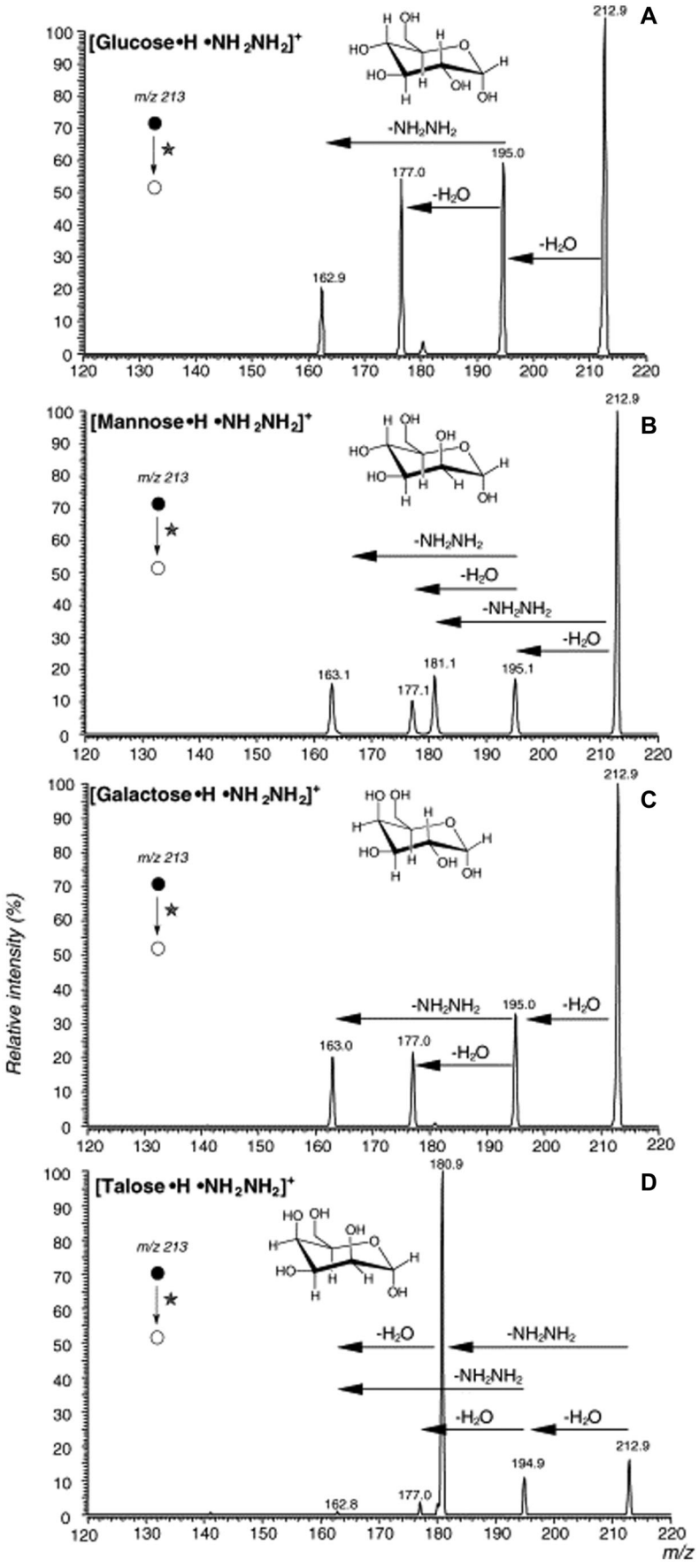


FIGURE 15 | Legend on next page.

Reagents include alkali and alkaline earth metal cations, 1-phenyl-3-methyl-5-pyrazolone (PMP), 3-carboxy-5-nitrophenyl boronic acid, or cyclodextrins, among others (Huang and Dodds 2013; Yang et al. 2016; Huang and Dodds 2015; Williamson and Nagy 2022; McKenna et al. 2019).

In 2007, one of the first studies which utilized IMS-MS for carbohydrate monomer research showed efficient separation of metal adducts of anomeric methyl glycoside isomers and anomeric forms of reducing monosaccharides (Dwivedi et al. 2007). Nevertheless, the extent of separation was significantly affected by the nature of the drift gas and by the nature of an adducting metal ion. Depending on the spatial orientations of the available electron donor groups, different isomers coordinate the metal ions differently, resulting in different overall shapes and compactness of the complex ion. Utilizing different drift gases and metal ions, complex monosaccharide mixtures may be resolved (Dwivedi et al. 2007; Li et al. 2012). Several other studies found distinguishable drift times for many, although not all, monosaccharide isomers (Both et al. 2014; Yang et al. 2016; Li et al. 2012; Gaye et al. Kurulugama, and Clemmer 2015, 2016). Interestingly, a recent study by Winkler and co-workers focused on the conformational isomers of acetylated sialic acids (Winkler et al. 2011). However, these isomers could not be completely resolved based solely on their mobilities. More recent high-resolution IMS (HR-IMS) setups, such as the cyclic IMS instruments, advanced the possibilities of separating isomeric monosaccharide mixtures. The IMS resolution scales approximately with the square root of the separation path length. Therefore, extended distances for ion mobility measurements, such as those used in cyclic IMS, provide advantages where complex isomer mixtures need to be analyzed (Delafield et al. 2022).

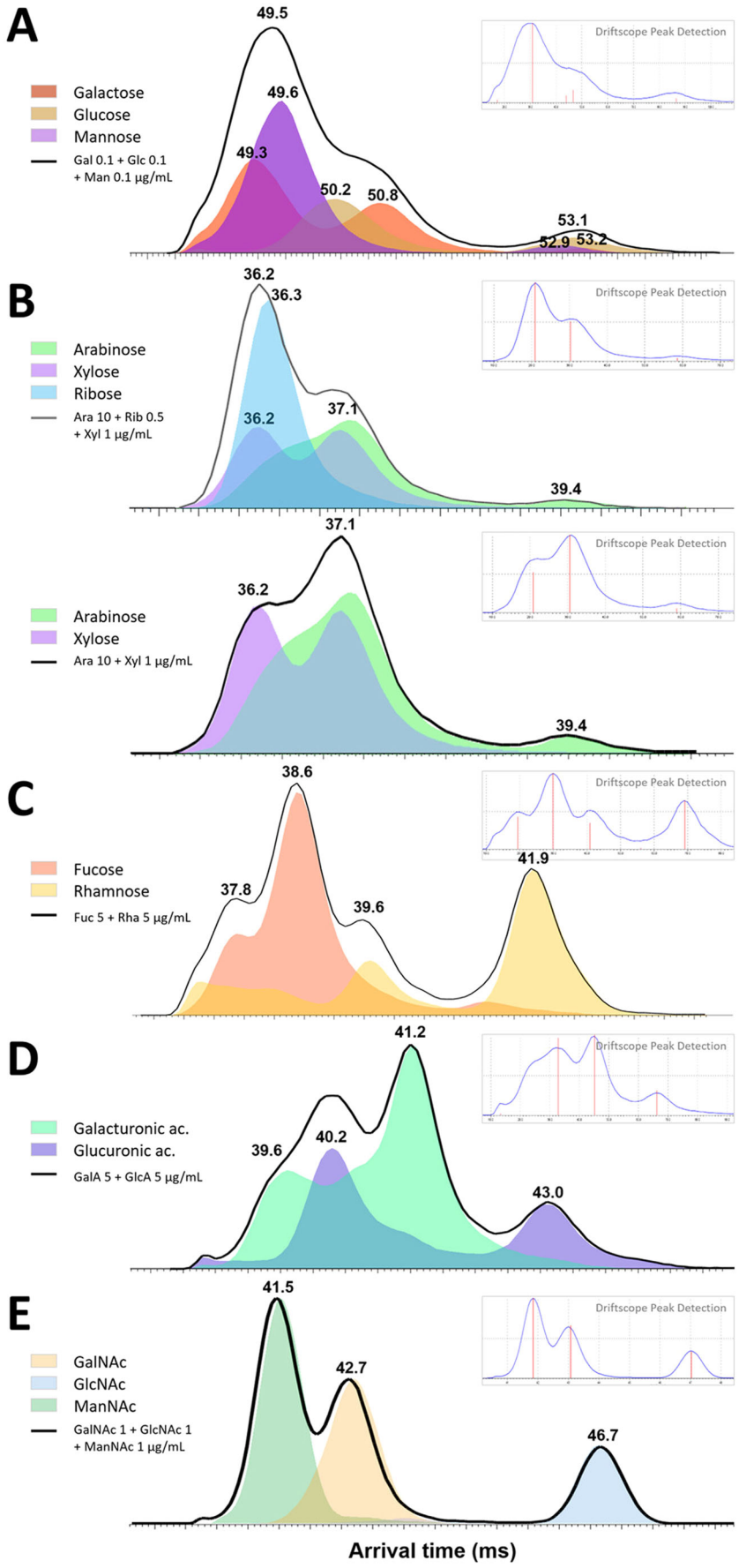
Earlier IMS-MS setups could not resolve some monosaccharide isomers (e.g., galactose and mannose), even after derivatization (Yang et al. 2016; Li et al. 2012). However, using cIMS, it became possible to resolve these diastereomers. Initially, this was done with the aid of shift reagents (Williamson and Nagy 2022; McKenna et al. 2019), but a recent study by Ollivier and co-workers, achieved separation even with underivatized monosaccharides (Ollivier et al. 2023) (Figure 16). The study successfully generated distinctive fingerprints of underivatized monosaccharides for some of the most common monomers composing plant or bacterial polysaccharides. These fingerprints could be used to identify monomer fragments produced by fragmentation of larger oligo- and polysaccharides, forming the basis for de novo sequencing of carbohydrates using IMS-MS (Ollivier et al. 2023; van de Put, de Bruijn, and Schols 2024). On top of that, the same platform was shown to be able to separate α/β anomers of common protected monosaccharide building blocks, paving the way for the use of cyclic ion mobility MS in high throughput screening platforms for synthetic carbohydrate reactions, along with screening for anomeric impurities (Peterson and Nagy 2021). In metabolomic studies, the application of DMS-MS has largely focused on targeted experiments and the removal of interferences. However, Wernisch and colleagues investigated DMS-MS for untargeted cellular metabolomics. They described the chemical selectivity of the DMS-MS platform for a variety of metabolite classes, including sugar phosphates and activated sugars (Wernisch et al. 2018). The general application of IMS to metabolomics has been reviewed and discussed in depth only recently (Paglia, Smith, and Astarita 2022; Delvaux, Rathahao-Paris, and Alves 2022; Levy et al. 2019; Mairinger, Causon, and Hann 2018; Zhang et al. 2018).

9 | Infrared Ion Spectroscopy

Infrared ion spectroscopy (IRIS) overcomes the limitations of traditional mass spectrometry by delivering structural information in addition to mass (Cismesia et al. 2018). This technique involves laser irradiation of mass-selected ions to obtain structural information about gas-phase analytes within the ion trap of a mass spectrometer. Unlike classical infrared spectroscopy, which measures the attenuation of the IR beam after passing through a sample, IRIS cannot obtain these conventional transmission spectra due to the extremely low density of ions in the mass spectrometer. Instead, infrared multiple photon dissociation (IRMPD) is routinely employed, as it can easily be integrated with existing MS platforms. This is commonly performed at room temperature, albeit it has also been employed at cryogenic temperatures to achieve higher-resolution infrared spectra (Cismesia et al. 2018, 2016, 2017). IRMPD detects absorption as a function of wavelength by observing the photoinduced fragmentation of irradiated ions. Resonant absorption of multiple photons induces cleavage of covalent bonds, which is dependent on the molecular structure of the ion. By plotting the photodissociation yield as a function of the wavenumber, the IRMPD spectra are obtained. Therefore, IRIS has been regarded as an additional MSn stage (Ho et al. 2021; Martens et al. 2020; van Outersterp et al. 2021). Proof-of-concept studies date back to the early 1980s (Wight and Beauchamp 1981). For recent advancements and a detailed technical description of IRIS, we refer to recent articles and reviews (Cismesia et al. 2018, 2016; Martens et al. 2020; Braak et al. 2022; Maitre et al. 2019; Kranenburg et al. 2020; Stroganova and Rijs 2021; Gray, Compagnon, and Flitsch 2020; Greis et al. 2022; Rijs and Oomens 2015; Eyler 2009; van Outersterp et al. 2023a; Carlo and Patrick 2022; Martens et al. 2017).

Since stereoisomers may exhibit different absorption patterns across a range of wavelengths, IRIS can differentiate subtle structural differences, making it highly useful for studying monosaccharides and their derivatives (Ho et al. 2021). In addition, IR spectra of small molecules can be reliably predicted using computational approaches based on modern quantum-chemical

FIGURE 15 | Giorgi and Speranza demonstrate how complexes between underivatized monosaccharides and hydrazine exhibit distinct losses of hydrazine and multiple water molecules during collision-induced dissociation (Giorgi and Speranza 2006). The spectra, from top to bottom, display selected low-energy MS/MS spectra of noncovalent complexes of (A) glucose, (B) mannose, (C) galactose, and (D) talose, with a collision energy of 0.7 eV. Reprinted from Giorgi and Speranza. Stereoselective noncovalent interactions of monosaccharides with hydrazine. International Journal of Mass Spectrometry 249 (2006): 112–119, Copyright 2006, with permission from Elsevier.



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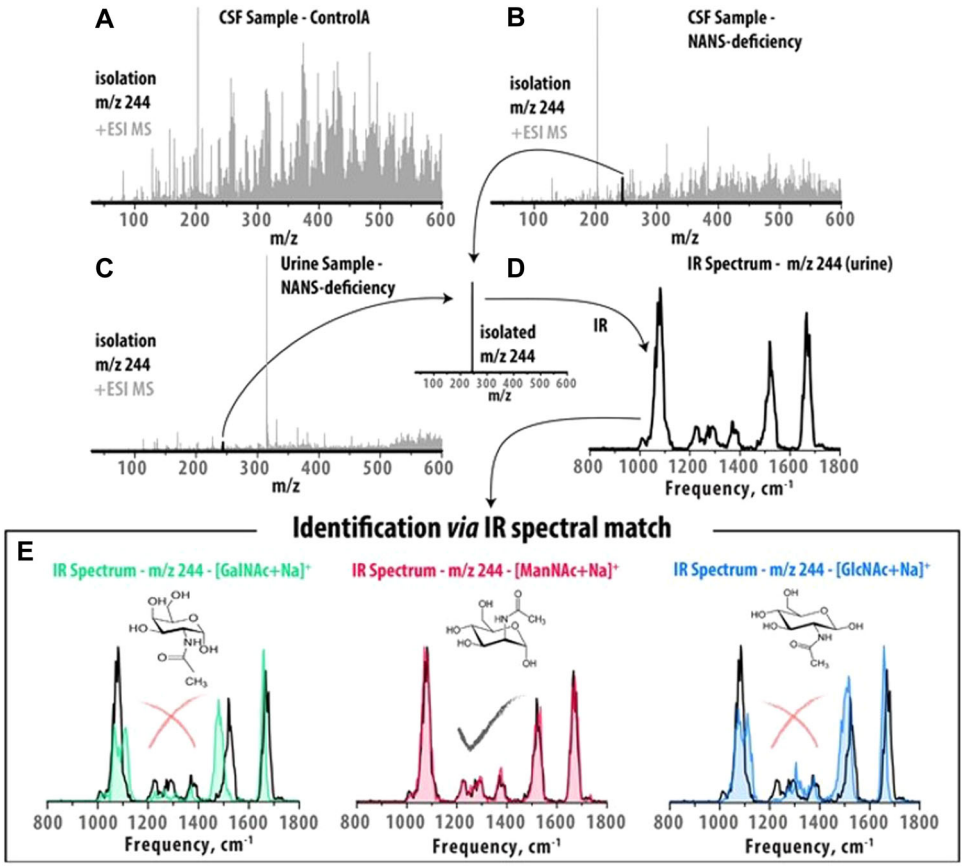

FIGURE 17 | Experimental workflow for combining mass spectrometry with infrared spectroscopy for metabolomics applications (Martens et al. 2017). (A) and (B) show the isolation of a metabolite ion peak at m/z 244 (HexNAc) from a CSF control and a patient with NANS deficiency. (C) A clear m/z 244 peak (ManNAc, [M + Na] m/z 244), a precursor for sialic acid (Neu5Ac), identified in the patient sample. (D) The isolation of ions at m/z 244, followed by subsequent IR analysis. (E) Measured IR spectrum of the isolated HexNAc ion from the patient sample is matched against reference spectra of various N-acetylhexosamines, confirming that the spectrum exclusively matches ManNAc. Reprinted with permission from Martens et al, J., Berden, G., van Outersterp, R.E. et al. Molecular identification in metabolomics using infrared ion spectroscopy. Sci Rep 7, 3363 (2017). [Color figure can be viewed at wileyonlinelibrary.com]

calculations. This allows for reference-free identification (Martens et al. 2020; Houthuijs et al. 2023). The differentiation of isomers and the reference free nature of this method is especially an exciting prospect for the characterization of monosaccharides derived from microbes, as their reference compounds are challenging to obtain. However, direct coupling of IRIS and LC separations, which is commonly necessary for analyzing complex metabolic samples, is complicated by the differing timescales of the two techniques. While an IRIS experiment usually takes minutes, LC peak widths are typically only seconds. To overcome this challenge, van Outersterp and colleagues used two switching valves and two sample loops to bypass direct online coupling. This method enabled them to record multiple IR spectra for two LC features from a single microliter injection (van Outersterp et al. 2023b).

After the first spectroscopic analysis and full structural assignment of a model glycoside in the gas phase by Talbot and Simons, in 2002 (Talbot and Simons 2002), IRIS was employed to investigate the existence of isomeric structures. For example, the IR fingerprint region (900–1800 cm^{-1} in most studies) was successfully used for distinguishing monosaccharides with different functional groups. The $O-H$ stretching region ($\sim 3000-3700\,cm^{-1}$) of the abundant hydroxyl groups showed great potential for monosaccharide isomer differentiation (Ho et al. 2021; Martens et al. 2017; Contreras et al. 2012; Martens et al. 2018; Pearson et al. 2015; Tan et al. 2017; Cagmat et al. 2010; van Outersterp et al. 2023a; Stefan and Eyler 2009) (Figure 17).

It was shown that also the formation of adducts reveal unambiguous structural information. For example, a study of

FIGURE 16 | Fingerprinting of isomeric, unlabeled monosaccharides using high-resolution ion mobility spectrometry (Ollivier et al. 2023). The graphs display the arrival time distributions for different monosaccharides: (A) hexoses; (B) pentoses; (C) deoxyhexoses; (D) hexuronic acids; (E) N-acetyl hexosamines. The arrival times of individual sugars are shown in color, while the arrival time distribution of mixtures within each isomeric family is represented by a solid black line. The insets show the results of the (Driftscope) peak detection for the mixtures. Reprinted from Ollivier et al. Fingerprinting of underivatized monosaccharide stereoisomers using high-resolution ion mobility spectrometry and its implications for carbohydrate sequencing. Analytical Chemistry 95.26 (2023): 10087-10095, Copyright 2023, with permission from Elsevier. [Color figure can be viewed at wileyonlinelibrary.com]

methyl galactosides concluded that ammonium adducts are diagnostic of the ring size (i.e. pyranose/furanose), while lithium adducts can differentiate stereochemistry (such as α and β anomers) (Ho et al. 2021). Identification of glucosamine epimers was achieved even without any sample preparation, from complex mixtures like urine and cerebrospinal fluid (Martens et al. 2017). Anomeric configuration and epimer discrimination was also demonstrated for protonated hexosamines (Barnes et al. 2017). Unlike the O − H region, the long-wavelength region of the IR spectrum (~300–1000 cm^{-1}), which includes more delocalized vibrations, has largely been neglected in saccharide analysis, but is now gaining momentum. In this longer wavelength IR range, spectra of many cation-saccharide complexes have shown distinct and highly characteristic vibrational features granting their straightforward identification. The enhanced IRIS spectral resolution when cooling the ions to cryogenic temperatures was used to study conformational heterogeneity of protonated glucosamine (Pellegrinelli et al. 2022; Scutelnic and Rizzo 2019; Bansal et al. 2020). In addition, the application of infrared ion spectroscopy enabled the elucidation of the glycosylation mechanism and the structure of short-lived glycosyl cations (Braak et al. 2022; Elferink et al. 2018) (Mucha et al. 2018; Elferink et al. 2019). Finally, Hernandez and coworkers demonstrated the combination of ion mobility separation with infrared ion spectroscopy (Hernandez et al. 2015). This allowed infrared characterization of both mobility- and mass-selected ions, facilitating carbohydrate isomer discrimination.

10 | Choice of Mass Analyzer Guided by the Analytical Goal

A wide variety of mass spectrometers exists, with the mass analyzer being one of the main components, responsible for determining the mass-to-charge ratio. A distinction is made between low- and high-resolution mass spectrometry (LRMS and HRMS, respectively). Li et al defined HRMS mass analyzers as those which provide a resolving power of 10,000 or higher (Xian, Hendrickson, and Marshall 2012). Among the five most commonly used mass analyzers, the quadrupole mass filters and ion traps are categorized as LRMS, while time-of-flight (TOF), Fourier-transform ion cyclotron resonance (FT-ICR), and Orbitrap analyzers are considered HRMS (Rathahao-Paris et al. 2016). The mechanisms of these mass analyzers are nicely explained by Haag (2016) (Haag 2016). Recent developments in HRMS are reviewed by Li et al. (2021) (Li et al. 2021).

When selecting an appropriate mass analyzer, several key factors should be considered, with the primary one being the analytical goal. HRMS is particularly well suited for detailed structural characterization and for the analysis of complex mixtures. Thanks to their high resolving power and mass accuracy, advanced HRMS instruments can distinguish molecules with nearly identical masses, such as those differing by a phosphate or a sulfate group. In addition, determining the exact mass enables the elucidation of the molecular formula (Xian, Hendrickson, and Marshall 2012). Although ideally the choice of mass spectrometer and analyzer solely depends on the analytical goal, practical considerations such as budget and laboratory space also play critical roles. HRMS is associated with higher costs compared to LRMS. Furthermore, significant variations in performance and acquisition costs between HRMS machines exist. For example, FT-ICR is superior in resolving power, mass accuracy and it offers the possibility to resolve isotopic fine structures, especially in the low molecular weight range. However, its reliance on a strong magnetic field necessitates a large superconducting magnet, posing two significant challenges: the need for substantial lab space and high operating costs due to the use of liquid helium as a coolant (Haag 2016). Consequently, while FT-ICR offers the highest resolving power, the Orbitrap represents a practical alternative, combining high resolving power with comparatively lower operating costs (Haag 2016). A more recently introduced high-resolution accurate mass (HRAM) analyzer, the Astral mass analyzer, shares operational principles with established instruments such as the Orbitrap, ion trap, and time-of-flight. Next to a high resolving power and mass accuracy, this analyzer also demonstrated a very good quantitative performance across a wide dynamic range (Stewart et al. 2023; Heil et al. 2023). As mentioned before, tandem mass spectrometry (MS/MS or MS^2) is essential for obtaining fragmentation profiles, which are valuable for tasks such as the structural characterization of saccharides. Multistage MS or MS^n in time can be achieved by mass spectrometers based on ion trapping (Grabarics et al. 2022). MS^n, using a linear ion trap, has been demonstrated advantageous in the structural analysis of oligosaccharides (Hsu et al. 2018).

For routine monitoring or quantification in simpler matrices, LRMS instruments are often sufficient. As an advantage, both the quadrupole and ion trap are compact in size and less expensive compared to HRMS systems (Haag 2016). A common low-resolution tandem MS instrument includes the triple quadrupole, in which three quadrupoles (Q1, Q2, and Q3, respectively) are connected in sequence, allowing various scan modes. Q1 and Q3 function as mass filter, while Q2 functions as collision cell. These instruments, when operated in MRM mode, are popular for the quantifications of compounds, including monosaccharides (Amicucci et al. 2019; Haag 2016).

In principle, all of the above-discussed MS-based methods and analyzers could also allow for the quantification of monosaccharides, provided that some additional requirements are met. Absolute quantification can be achieved through external or internal calibration (Salas et al. 2023; Han et al. 2016b; Zhang et al. 2022). Both approaches depend on pure standards to create calibration curves with high linearity (R^2 close to 1) within a specified concentration range. Internal calibration is typically more accurate due to the use of internal standards (IS), which are often isotopically labeled variants of the target analytes, which share similar chemical and physical properties. Spiking consistent amounts of IS into every sample, including calibration standards, accounts for differences in ionization efficiency, matrix effects, injection volume, and sample handling (Alseekh et al. 2021). When no internal standards are available, external calibration could be performed (Han et al. 2016b), although it requires additional experiments to evaluate potential matrix effects (Xu et al. 2018; Garcia, Chavez, and Mechref 2013; Alseekh et al. 2021). Wang et al. (2022) introduced a “paired derivatization” technique that involves labeling samples with O-(4-methoxybenzyl)hydroxylamine hydrochloride (4-MOBHA·HCl) and reference standards with

its deuterated form (d_3-4-MOBHA·HCl) (Wang et al. 2022b). This method allows the use of an internal standard even in the absence of commercially available isotopically labeled sugars. However, reference standards remain a prerequisite, and the derivatization step limits correction for sample handling effects, as spiking occurs after labeling. The availability of pure standards represents a major limitation for absolute quantification. While common monosaccharides such as glucose and galactose are readily available, rarer ones like 4-epi-legionaminic acid or acinetaminic acid, are not.

Although relative quantification does not require pure standards, the obtained information is limited to an analyte increase or decrease when comparing multiple samples. Moreover, as Millette et al. (2023) point out, such data could be misleading when expressed as a fraction of the total polysaccharide or sample amount, since a decrease in one monosaccharide will result in a proportional increase in others and vice versa (Millette et al. 2023). In glycomics, relative quantification has been achieved using isotopic or isobaric labeling. For a detailed overview of these techniques and recent advancements, readers are referred to Wang et al. (2024) (Wang, Zhang, and Li 2024).

Another key requirement for quantification is the baseline separation of isomers (Xu et al. 2018). Various approaches have been explored to address this challenge as discussed in this review, but complete separation of all isomers remains difficult. The limited availability of reference standards for uncommon monosaccharides and their derivatives further complicates the process. Consequently, most methods rely on a restricted set of monosaccharide reference standards, and comprehensive isomer separation has been investigated in only a few studies (Nagy and Pohl 2015b; Fang, Zirrolli, and Bendiak 2007).

Targeted quantification typically employs MRM or parallel reaction monitoring (PRM) modes. MRM targets specific transitions to ensure high specificity (Xu et al. 2018), while PRM, a targeted MS/MS approach, captures all fragment ions of a precursor, reducing interference and improving quantification accuracy. High-resolution mass spectrometry (HRMS) further enhances accuracy by providing superior mass precision, enabling the discrimination of closely related monosaccharides, and increasing confidence in results (Salas et al. 2023). These advances collectively support robust quantification of monosaccharides and nucleotide-sugars in complex biological samples.

11 | Databases and Bioinformatic Approaches

The unambiguous interpretation of chromatographic and mass spectrometric data commonly requires data from reference molecules. These enable identification through comparison of elution/migration times, fragmentation profiles or collision cross section values. However, especially for microbes, reference molecules for many monosaccharides and its derivatives are unavailable. Furthermore, sufficient sample quantities for NMR experiments are not always achievable, resulting in unassigned stereochemistry in many cases. Nevertheless, mass spectrometric experiments and additional spectroscopic and chemical analyses can help to classify these new compounds based on chain length, presence of an aldose/ketose, acidic/basic nature, oxidation state, and type of nucleotide activation. Comparative spectroscopic data for a variety of sugars and their derivatives can also be obtained from numerous metabolomics databases. This provides information on sugar conversion products, especially sugar phosphates, from major metabolic pathways. For example, a comprehensive summary of general metabolomics databases, repositories, and tools supporting the annotation of mass spectrometric data and related biological pathways has been made available via the Metabolomics Society website (Metabolomics-Society 2024). Comprehensive metabolomics databases supporting mass spectrometric identification include (among others) HMDB (Wishart et al. 2022), MiMeDB (Wishart et al. 2023), GMD (Hummel et al. 2013), MoNA (Fiehn 2024) and mzCloud (HighChemLLC 2024). MiMeDB (Microbial Metabolites Database) is dedicated to storing information about metabolites of the human microbiome. Another general compound information resource is the NIST Chemistry WebBook (http://webbook.nist.gov, 2024b), which is an extensive chemical and physical property database particularly useful for identifying compounds based on electron ionization (EI) mass spectrometric data (Linstrom and Mallard 2023). The Natural Products Atlas is another comprehensive resource containing information about more than 25,000 compounds (2024) found in microbes. Next to compound names, it provides structural data, source organisms, and synthesis information (Van Santen et al. 2019).

Li et al. have reviewed databases dedicated to glycans and monosaccharides, along with related bioinformatic tools (Li et al. 2020b). For example, UniCarb-DB is a LC-MS/MS library for N- and O-linked glycans including a data submission pipeline (Campbell et al. 2014). Another database dedicated to glycans is the Glycan Mass Spectral DataBase (GMDB) (Kameyama et al. 2005). The GMDB stores MS^n spectra of glycans released from proteins and lipids, providing structural information. Additionally, the Carbohydrate Structure Database (CSDB) is a repository of carbohydrate structures that includes detailed information about monosaccharide building blocks and derivatives from all domains of life, including bacteria and archaea (Toukach and Egorova 2016). The CSDB obtains data from scientific publications through automatic and manual curation. Furthermore, for ion mobility spectrometry, the METLIN-CCS database contains collision cross-section values derived from over 27,000 molecular standards (Baker et al. 2023). For infrared ion spectroscopy, Houthuijs and colleagues developed an in silico library of vibrational spectra. They created an automated workflow to generate IR spectra of molecular ions, resulting in over 75,000 calculated vibrational spectra for 4640 metabolites from the HMDB (Houthuijs et al. 2023). Moreover, the authors developed a scoring algorithm to identify molecular structures that closely match user-supplied experimental infrared ion spectra.

After identification, the biological interpretation requires integrating the data into metabolic networks. A useful resource to explore possible reaction pathways is the KEGG PATHWAY database which maps molecular interaction and reaction networks, including metabolic and regulatory pathways (Kanehisa). Additionally, KEGG GLYCAN collects experimentally determined glycan structures, including their biosynthesis pathways (Aoki-Kinoshita and Kanehisa 2015). MetaCyc is another comprehensive resource of metabolic pathways and

enzymes from all domains of life (Caspi et al. 2020). These databases are widely employed to facilitate pathway-based research. The CAZy database has been established to enable a categorization, for studying their structure, function, and evolutionary relationships (Cantarel et al. 2009). Nevertheless, the pathway and enzyme database are still incomplete in terms of the strains represented, as many microbial genomes and their associated pathways have yet to be fully mapped and included (Altman et al. 2013). This is particularly evident with the numerous enzymes involved in the production, conversion, and further modification of monosaccharides. Such limitations can bias the interpretation of biosynthetic routes and hinder understanding of metabolic capabilities. A more recent tool, MetaPathPredict, aims to address incomplete metabolic annotations by using deep neural networks, to predict the presence or absence of KEGG modules based on annotated features in the genome (Geller-McGrath et al. 2024).

The large-scale interpretation of metabolomics data also requires sophisticated bioinformatics tools, many of which have been developed in recent years. Specifically, such tools support the processing of mass spectrometric data, identification of metabolites, statistical analyses, and the correlation of metabolites with metabolic pathways. For a comprehensive discussion of bioinformatics tools dedicated to metabolomics studies, we refer to recently published reviews (Gardinassi et al. 2017; Misra 2021b; O'Shea and Misra 2020; Misra and Mohapatra 2019; Misra 2018; Misra, Fahrmann, and Grapov 2017; Banimfreg, Shamayleh, and Alshraideh 2022). In untargeted experiments thousands of features and molecular compounds are obtained, of which many may not provide library matches. Interestingly, Giera and colleagues reported that in source fragmentation is responsible for many of the ESI mass peaks instead of native molecules. The authors investigated ESI in source fragmentation across the METLIN MS/MS database of molecular standards (Giera et al. 2024; Smith et al. 2005). The authors concluded that a substantial fraction of the 'dark metabolome' may derive from in source fragmentation (Giera et al. 2024).

Over the past decades, collision induced dissociation (CID) has proven to be a valuable approach for obtaining structural insights into carbohydrates. However, the CID profiles of monosaccharides alone often fail to distinguish between both different anomeric forms and stereochemical variants. The CID profiles of glycopyranosyl-glycolaldehydes ions (m/z 221), generated using a Paul trap, did allow to differentiate between all 16 possible structural variants of unmodified hexoses. These glycopyranosyl-glycolaldehydes, produced through the fragmentation of disaccharides, consist of the intact nonreducing monosaccharide linked to a glycolaldehyde group (Fang and Bendiak 2007).

Despite these advancements, such methods still rely on chemical standards to construct CID profile databases. Recent progress has introduced promising database-independent approaches. In 2018, Huynh et al. investigated the CID behavior of sodiated glucose, galactose, and mannose to identify their anomeric configurations (α/β). Using a low-pressure linear ion trap combined with computational modeling, they demonstrated that the ratio of dehydration to cross-ring dissociation serves as a reliable indicator of anomeric configuration. Specifically, cis-anomers favored dehydration, while trans-anomers primarily underwent cross-ring cleavage. This rule-based approach has potential for broader application to other sugars, although it requires substantial computational effort (Huynh et al. 2018).

Building on these insights, a logically derived sequence (LODES) tandem mass spectrometry (MS^n) method was developed for the structural determination of underivatized oligosaccharides. This technique enables the assignment of both anomeric configuration, linkages as well as branch locations. A key advantage is that it eliminates the need for a complete oligosaccharide database; a disaccharide database suffices. The method was validated by commercial oligosaccharides, and recently has been shown effective for N-glycans (Hsu et al. 2018; Liew et al. 2021). LODES/MS^n shows potential for extension to oligosaccharides containing other monosaccharides, provided their dissociation mechanisms are elucidated and the corresponding disaccharide databases are available (Huang et al. 2021). Nevertheless, expanding this method to include all possible monosaccharides may introduce considerable complexity.

Alternatively, in silico-generated MS2 spectra may be used to infer molecular structures, which alone, however, are often insufficient to infer the true chemical structure (van Tetering et al. 2024). This is particularly true for compounds like monosaccharides which undergo extensive levels of molecular diversification. Kleikamp et al. developed a hybrid approach for nonulosonic acids, that involved fully untargeted screening of cell lysates using a data-independent fragmentation, combined with a carbohydrate chemistry-guided bioinformatics pipeline (Kleikamp et al. 2020). The pipeline identified compound peaks as ulosonic acids using an established fragmentation tree for labeled alpha-keto acids, and a theoretical sugar composition and modification database (Figure 18).

Recent advances in artificial intelligence (AI), particularly in machine learning (ML), offer promising opportunities to overcome the limitations of existing spectral libraries and reference databases discussed above. The remarkable success of AI in glycan analysis, demonstrated by tools like CandyCrunch which enable accurate prediction and classification of glycan structure from tandem mass spectra (Urban et al. 2024), suggests similar potential for characterization of monosaccharides and sugar metabolites. Machine learning algorithms now support various aspects of data pre-processing, including automated peak detection, spectral denoising, and retention time alignment, among others (Sirocchi et al. 2024). Moreover, neural networks, trained on existing spectral libraries, could help characterize structural features of atypical sugar metabolites by predicting fragmentation patterns when reference data is unavailable (Park, Jo, and Yoon 2024). These models could help recognize subtle spectral features that distinguish stereoisomeric monosaccharides, a task that traditionally requires extensive manual interpretation. Furthermore, deep learning has shown considerable promise in predicting liquid chromatography retention times of sugar metabolites, improving nontargeted identification. This information is particularly valuable for isobaric molecules, as it can be used to filter false positive hits and increase identification accuracy (Xue et al. 2024). In the realm of metabolic pathway elucidation, graph neural networks have

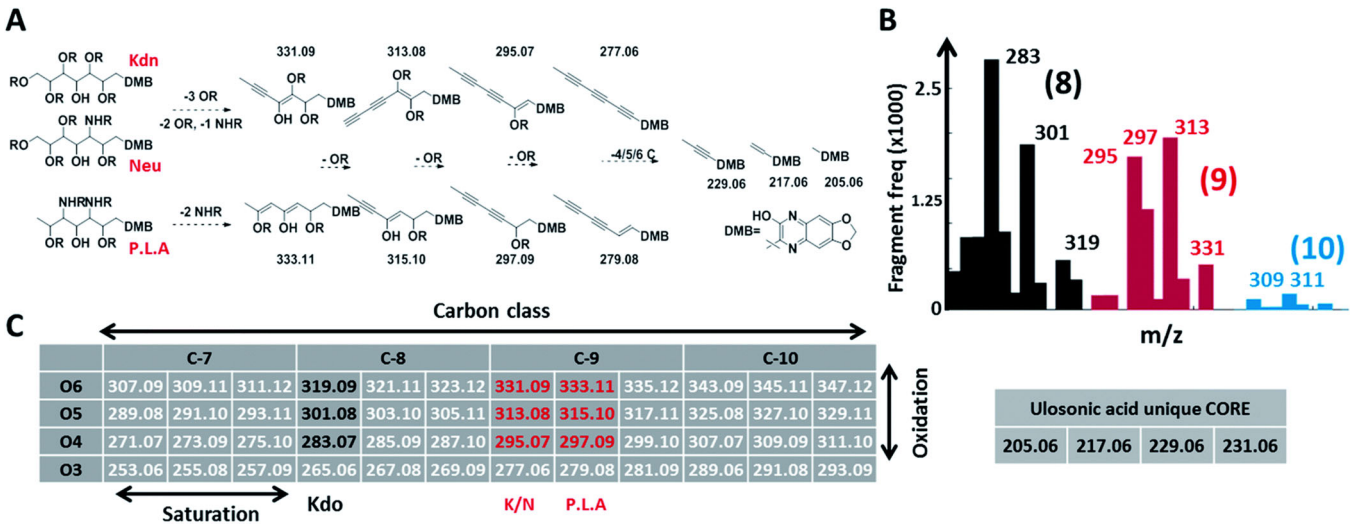

FIGURE 18 | Kleikamp et al. (2020) characterized the conserved fragment ion space for various nonulosonic acids (NulOs), taking into account different degrees of oxidation, saturation, and carbon chain lengths (Kleikamp et al. 2020). (A) The universal fragmentation routes for Kdn, Neu and Pse/Leg/Aci. (B) The frequency of ulosonic acid related compounds observed in a screening study, analyzing a large spectrum of microbial samples. (C) The alpha-keto acid specific fragment ions such as the quinoxaline-based ulosonic acid core fragments and carbon chain length markers. Reprinted with permission from Kleikamp et al. Tackling the chemical diversity of microbial nonulosonic acids–a universal large-scale survey approach. Chemical Science 11.11 (2020): 3074-3080. [Color figure can be viewed at wileyonlinelibrary.com]

emerged as powerful tools for predicting biosynthetic routes of novel monosaccharide derivatives based on known enzymatic functions and genomic data. For example, MetaPathPredict can reconstruct complete metabolic pathways in microbes, potentially aiding in the exploration of the functions of uncharacterized enzymes involved in monosaccharide modifications (Geller-McGrath et al. 2024). Additionally, machine learning has demonstrated the ability to utilize variation in monosaccharide composition among different tissue samples to accurately classify their tissue origins (Hsiung et al. 2023). Nevertheless, success of AI approaches depends not only on the quality and quantity of training data available but also on the careful validation of predictions against experimental results. The limited availability of validated training datasets for rare monosaccharides presents a substantial obstacle, particularly for microbial systems where novel derivatives are frequently encountered.

12 | Summary & Outlook

Carbohydrates not only constitute a source of energy but also mediate a wide range of functions essential to cellular processes across all domains of life. Furthermore, notably in microbes, highly modified and uncommon monosaccharides are prevalent, often playing crucial roles in determining biological activity. Despite many fundamental questions about the utilization and development of individual glycomolecules, numerous medical and industrial applications have emerged for these carbohydrates. For example, the unique metabolic pathways of microbial monosaccharide derivatives make them promising and highly specific drug targets, which are urgently needed as alternatives in the face of rising antimicrobial resistance. Furthermore, carbohydrate-active enzymes hold significant potential for biotechnological applications, including the production of carbohydrate molecules that are challenging to generate via chemical synthesis.

While the protein sequences can be implied from the genetic code alone, the sequence and isomeric composition of glycomolecules cannot, highlighting the importance of sensitive and selective methods. The mass spectrometric analysis of monosaccharide derivatives is complicated by the presence of stereoisomers and extensive molecular diversification. Therefore, while sensitive detection in complex matrices can be achieved, the primary challenge in analyzing monosaccharide building blocks is the identification of new derivatives and the discrimination of isomeric structures. This necessitates the application of high-performance separation techniques alongside mass spectrometric detection for most applications.

Among the diverse analytical approaches discussed, several methods have gained widespread adoption in monosaccharide analysis. Reverse-phase liquid chromatography coupled with mass spectrometry employing a derivatization step remains one of the most widely used approaches for routine analysis. Derivatization agents such as PMP increase ionization efficiency of sugars, offering both reliable separation and sensitive detection of common monosaccharides, including stereoisomers. Retention of highly polar sugars could be further enhanced by the addition of an ion-pairing reagent to the mobile phase. Hydrophilic interaction liquid chromatography (HILIC) has also gained prominence due to its natural affinity for polar molecules like monosaccharides and their activated sugars. For the analysis of nucleotide-activated sugars, porous graphitic carbon chromatography coupled to mass spectrometry has become the method of choice, as it provides excellent separation of isomers while avoiding the contamination issues associated with ion-pairing reagents. More recently, mixed-mode chromatography combining weak anion-exchange and reverse-phase separation has emerged as a promising alternative. In cases where high-throughput screening is required, such as in biotechnology applications, negative mode MALDI-MS has proven effective for rapid profiling of sugar nucleotides and

phosphorylated intermediates. While traditionally more suited for larger molecules, recent advances in matrix development and high-resolution mass analyzers have improved its application to smaller sugar molecules. Meanwhile, capillary electrophoresis-mass spectrometry has established itself as valuable for analyzing charged sugar species, particularly sugar phosphates and nucleotides, offering high separation efficiency with minimal sample requirements and short analysis times. Gas chromatography-mass spectrometry, a historically significant technique for sugar analysis, remains crucial for detailed stereochemical analysis of monosaccharides and their derivatives. Nevertheless, it requires extensive sample preparation as it is restricted to volatile and thermally stable compounds. For metabolic studies and pathway elucidation, approaches combining stable isotope incorporation and chemical labeling with advanced separation techniques have proven particularly powerful. These methods, especially when paired with high-resolution mass spectrometry, enable tracking of novel biosynthetic routes and verification of predicted pathways, proving indispensable when traditional genomic predictions may be insufficient. It is worth noting that the analytical approaches discussed often complement each other to provide comprehensive characterization of sugar compounds. For instance, high-throughput capabilities of MALDI-MS can be used for initial screening of samples, followed by more detailed analysis using chromatographic methods for structural confirmation. In addition, MALDI imaging can be used for the determination of spatial resolution in tissues and biofilms.

Looking toward emerging technologies, ion mobility spectrometry (IMS) and infrared ion spectroscopy show exceptional promise for resolving previously inseparable monosaccharide isomers without requiring derivatization, complementing cases where standard separation techniques fall short. The development of high-resolution IMS and its recent applications to the separation of isomeric monosaccharides have introduced a new dimension to the analysis of complex samples. Notably, platforms such as cyclic IMS enable the investigation of isomeric monosaccharides in glycoconjugates after the initial fragmentation of glycomolecules. Similarly, the recent integration of infrared ion spectroscopy with mass spectrometry offers a promising approach for the stereoselective analysis of carbohydrate molecules, including monosaccharides. An advantage of this methodology is that the infrared spectra of small molecules can be predicted using computational methods, making it independent of the availability of reference monosaccharides. Its ability to provide detailed structural information through computational prediction of vibrational spectra offers unique advantages for identification of unknown compounds.

The limited availability of reference standards for less common monosaccharides and their derivatives poses a significant challenge in the development and evaluation of novel analytical methods. As a result, most methods have been calibrated using only a limited set of monosaccharide reference standards, and the comprehensive separation of all possible isomers of a given monosaccharide has been explored in only a few cases. Additionally, the highly polar nature of activated monosaccharides—universal substrates for glycomolecule biosynthesis—further complicates the development of sensitive and selective analytical techniques. Recent developments include alternative approaches to traditional reverse-phase ion-pairing methods, such as porous graphitic carbon chromatography and mixed-mode separation coupled with tandem mass spectrometry. In this context, stable isotope incorporation and chemical labeling proved useful in investigating metabolic and biosynthetic pathways.

Finally, artificial intelligence is emerging as a promising player in addressing current analytical challenges. The integration of AI approaches with established analytical methods promises to accelerate the discovery and characterization of novel monosaccharide building blocks, particularly in understudied organisms. Machine learning approaches show particular potential for predicting chromatographic behavior of modified monosaccharides, identifying novel derivatives from complex mass spectra, and elucidating biosynthetic pathways. However, the success of these AI approaches heavily depends on the quality and quantity of available training data, which remains limited for rare and modified monosaccharides, particularly from microbial sources. While the past decades have seen the establishment of routine methods for the qualitative and quantitative analysis of common monosaccharides, the stereoselective resolution and fully untargeted identification of novel monosaccharide components remain ongoing challenges. The continued advancement of high-resolution mass spectrometers, coupled with sophisticated separation techniques and AI tools, promises to further expand our understanding of monosaccharide diversity and function across all domains of life.

Author Contributions

Jitske M. van Ede: conceptualization, data curation, formal analysis; investigation, methodology, visualization, writing–original draft, writing–review and editing. **Dinko Soic:** data curation, formal analysis, investigation, writing–original draft. **Martin Pabst:** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing–original draft, writing–review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

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