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Perspective

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Function without Structures: The Need for In-Depth Analysis of Dietary Carbohydrates

Matthew J. Amicucci,[†,‡] Eshani Nandita,[†,‡] and Carlito B. Lebrilla*[,†,‡]

[†]Department of Chemistry and [‡]Foods for Health Institute, University of California, Davis, 1 Shields Avenue, Davis, California 95616, United States

ABSTRACT: Carbohydrates make up the largest component of plant-based foods and have long been known to provide fuel. However, many carbohydrates possess intrinsic biological activities that are dictated by their structures. Carbohydrates are the most abundant biopolymers in nature and are also the most structurally complicated and diverse. Consequently, the structural analysis of carbohydrates remains severely limited. To further understand their biological activities, we need new analytical tools to analyze the different classes of carbohydrates that range in size from monosaccharides to polysaccharides. These tools must be capable of rapid throughput with highly sensitive quantitation for use in clinical studies that probe their fate in human and animal fluids and tissues.

KEYWORDS: *carbohydrate, fiber, oligosaccharide, polysaccharide, monosaccharide, mass spectrometry, human milk, microbiome, prebiotic*

INTRODUCTION

The importance of carbohydrates in the diet is well-established and has been extensively described.[1] Carbohydrates represent the largest component of plant-based foods and are recognized for their dietary importance. Their role in caloric transfer is critical to human health, but it belies their important intrinsic biological activities. Increased consumption of carbohydrates that resist digestion by the host, typically termed "dietary fiber", has been associated with a reduced risk of obesity, type 2 diabetes, certain gastrointestinal disorders, and coronary heart disease.[1] Even monosaccharides, the smallest carbohydrate unit, have their own inherent activity.[2] More recently, the ability of carbohydrates to modulate the gut microbiome has become of considerable interest.[3] While carbohydrates in food are undisputedly a necessary part of any healthy diet, the relative amounts, types of carbohydrates, and whether some foods can be called carbohydrates at all are the subject of considerable and even broad disagreements.[4] The conflicts regarding carbohydrates stem from our general ignorance of their chemical structures.

Little is known of the carbohydrate structures that make up even the most basic foods. In general, we know more about the micronutrients of foods (vitamins, minerals, and amino acids), which are important but generally minor components, than we know about the far more abundant carbohydrate components.[5] This contrast is illustrated by, for example, butternut squash, where there is abundant information regarding the micronutrient content, such as amino acids, fatty acids, and minerals, but little regarding the carbohydrates that make up over 80% of the dry weight (Figure 1). This lack of specific structural knowledge of food has obscured our understanding and affects the interpretation of numerous clinical data that probe the role of carbohydrates in the diet. This limitation even affects our discourse about diet because the meaning of carbohydrates is distorted.

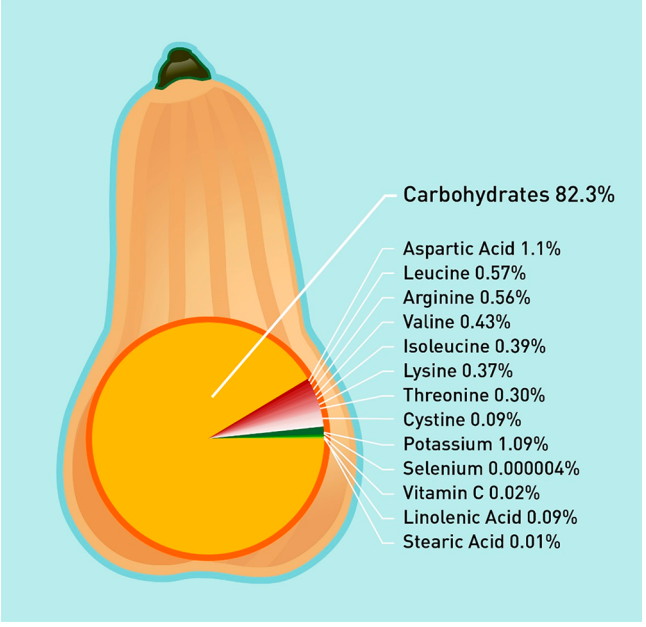

Figure 1. Known molecular composition of butternut squash contains in-depth details on the amino acids, minerals, vitamins, and lipids, despite their low abundances. However, little is known regarding the monosaccharide, oligosaccharide, or polysaccharide components that make up the majority of the dry mass.[5]

Even when precise labeling is intended, the description of carbohydrates remains vague. For example, the term "fiber", including dietary and indigestible, may denote some specific structural features but makes no distinction of the mono-

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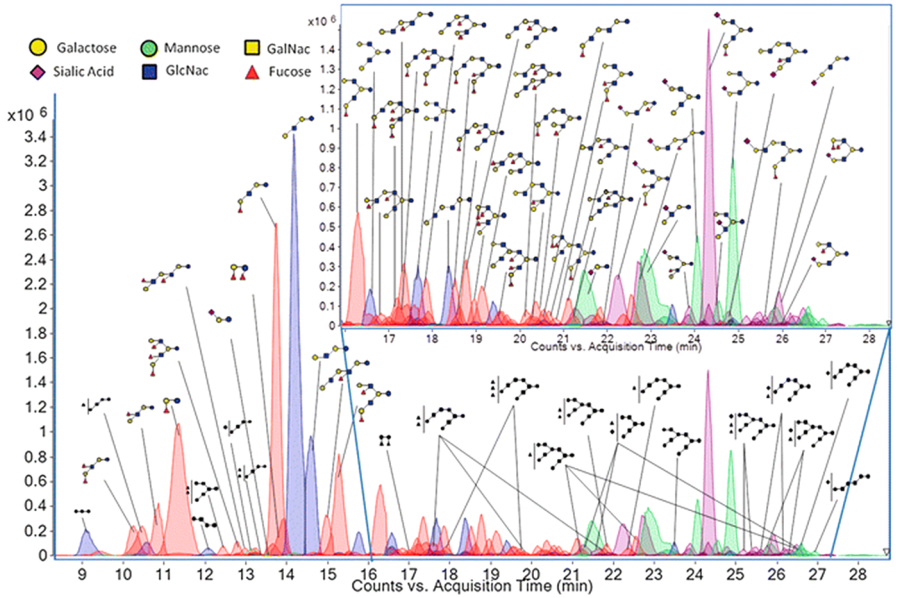

Figure 2. Nano-HPLC/Q-TOF MS was an enabling technology for the detection and characterization of several hundred human milk oligosaccharides.[13]

saccharide composition nor the primary structure of the molecule. The term "dietary fiber" is often used as a catch-all for a very large number of compounds, each with their unique structural variations and potentially specific activities.[4] Thus, the advice "eat more fiber", is rendered meaningless because fiber from two different sources can have completely different monosaccharide compositions, glycosidic bond linkages, degree of polymerization, and in turn, biological functions.

In this perspective, we call attention to the critical but unmet need of detailing carbohydrate structures in food. Carbohydrates, as a class of compounds, are highly structurally diverse and include subclasses, such as free monosaccharides, free disaccharides, oligosaccharides, and polysaccharides. In food, all subclasses may be simultaneously present and can be equally important. As a result of the presence of many isomeric monosaccharide building blocks, variable glycosidic linkages, branching, and large molecular weights, carbohydrates are much more complicated to analyze than other macromolecules. Additionally, unlike proteins and DNA, carbohydrates are not synthesized through templated biosynthetic pathways and are not genetically encoded. The current lack of deep structural analysis of carbohydrates is the result of analytical methods that are no longer sufficient. New tools are needed with high sensitivity, quantitation, and speed to address the large clinical studies that are necessary for understanding the role of carbohydrates in health.

ELUCIDATION OF HUMAN MILK OLIGOSACCHARIDE STRUCTURES SHOWED THE IMPORTANCE OF STRUCTURES TO UNDERSTAND FUNCTION

The best characterized food carbohydrates are those known as human milk oligosaccharides (HMOs). HMOs make up the third largest dry component of human milk after lactose and lipids. They comprise of 3−20 covalently linked monosaccharides consisting of glucose, galactose, *N*-acetylglucosamine, fucose, and *N*-acetyl neuraminic acid (sialic acid). The general structure of HMOs includes a lactose core extended by galactose and often branching GlcNAc. These structures can be further decorated by fucose and sialic acid. The important structural features of HMOs are their monosaccharide compositions and the glycosidic linkages that connect each monosaccharide unit. Despite the small number of monosaccharides and the relatively narrow size distribution, the combination of monosaccharides and linkages translates to over 100 structures for individual mothers (through 5 orders of magnitude in abundance) and over 200 unique structures when several milk samples are combined (Figure 2).[6]

HMOs were first described over 60 years ago,[7] however, only recently have their roles in infant nutrition been established. Humans do not possess the necessary enzymes required for HMO catabolism. Thus, they pass into the colon intact, where they selectively feed beneficial bacteria, specifically *Bifidobacterium*, which play important roles in infant immune development. Upon consumption of HMOs, *Bifidobacterium* produce short-chain fatty acids, which lower the pH of the gut and ward off pathogen colonization.[8] More recently, infants with higher levels of *Bifidobacterium* in their gut had reduced abundance and frequency of antimicrobial resistance genes.[9] HMO degradation by a suite of glycosyl hydrolases found in specific bacteria, especially *Bifidobacterium longhum* ssp. *infantis*, yielded the necessary mechanism for their function.[10]

The prebiotic role of HMOs was established only with the development of new analytical methods. In a sustained and systematic effort, HMOs were structurally elucidated using a combination of mass spectrometry (MS), tandem MS, and linkage-specific exoglycosidase digestion.[11] These tools involved advanced separation, including nanoflow high-performance liquid chromatography (nano-LC), and advanced MS, including quadrupole time-of-flight (Q-TOF) analyzers with collision-induced dissociation (CID), for structural analysis. Currently, nano-LC MS methods are routinely used to rapidly identify and quantify human milk oligosaccharides in hundreds if not thousands of samples. These compounds have been further observed in other bodily fluids, including the feces, urine, and plasma of the infant.[12,13]

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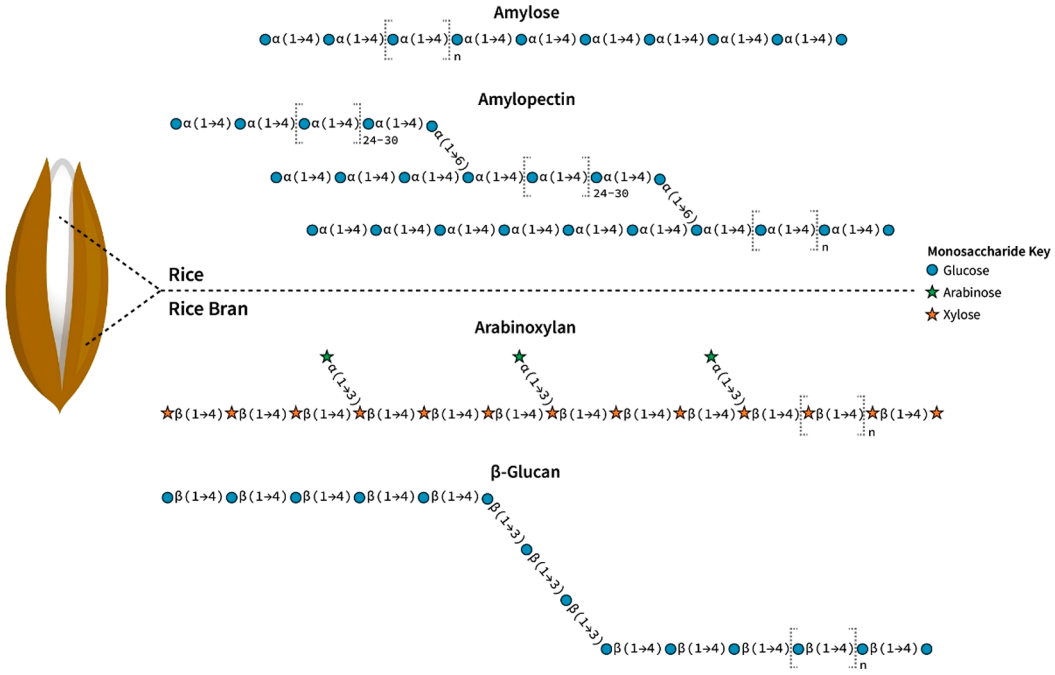

Figure 3. Polysaccharide components of even the most common foods can be highly diverse. Rice endosperm polysaccharides are primarily comprised of amylose and amylopectin, both components of starch, while the rice bran can contain many polysaccharides, including arabinoxylan, β-glucan, and cellulose.

The ability to profile HMO structures also yielded the classification of mothers milk into at least two phenotypes (from several genotypes).[14] Mothers whose milk contain oligosaccharides with $\alpha(1,2)$-fucose are referred to as secretors because they secrete their blood type, while mothers that do not are referred to as non-secretors. The ratio of secretors to non-secretors varies by ethnicity and geography. Secretor status is known to affect how the host interacts with various bacteria, including commensals, such as *Bifidobacterium infantis*, and those that are pathogenic, including *Campylobacter jejuni*, *Vibrio cholera*, and even viruses, such as HIV.[15]

Through weaning and into adulthood, the carbohydrates in human milk are replaced by plant-based carbohydrates. Indeed, the total carbohydrate content in human milk, including lactose and HMOs, is in the same general proportion as the carbohydrates in an adult meal. Similarly, carbohydrates that are indigestible to the host are transmitted to the distal gut, where they can act as prebiotics for saccharolytic bacteria and, thus, modulate the microbiome.[3] The interaction between carbohydrates and the microbiome must be understood at the structural level because the bacteria have enzymes, lectins, and transporters that act on highly specific carbohydrate structures.

CHALLENGES IN THE STRUCTURAL CHARACTERIZATION OF FOOD CARBOHYDRATES

Food carbohydrates can be classified on the basis of their size or degree of polymerization (DP). Mono- and disaccharides have a DP of 1 and 2, respectively. Oligosaccharides range between DP of 3 and 20, and polysaccharides have structures larger than DP of 20 and can reach over a million.[16] As carbohydrates become larger, their structures become significantly more complicated and their characterization becomes substantially more difficult. Additionally, carbohydrates in each of these groups require their own dedicated methods for analysis.

Monosaccharides are the building blocks of all carbohydrates and typically range from five to nine carbons in length. While the nucleic acid constituents of the genome and the amino acids of proteins are conserved throughout nearly all organisms, the monosaccharides that comprise carbohydrates are unique and vary depending upon the taxonomic kingdom. In plants, the primary building blocks of carbohydrates are glucose, galactose, mannose, fructose, xylose, arabinose, ribose, apiose, fucose, rhamnose, glucuronic acid, and galacturonic acid, although many other monosaccharides have also been reported. Additionally, many monosaccharides are isomeric and only differ by stereochemistry as a result of the inversion of distinct chiral centers.

The characterization of oligosaccharides is further complicated by the presence of several monosaccharides that are connected through numerous but distinct glycosidic linkages. Two hexoses (e.g., glucose, galactose, and mannose) can be linked by as many as 12 possible combinations. Oligosaccharides are also found with varying DP and often include several isomers. The analytical difficulty of oligosaccharides is illustrated by HMOs, as described above. Glycomic and glycoproteomic analyses have progressed substantially by employing advanced separation and MS methods, which may be applied to food oligosaccharides.[17] Beyond HMOs, however, there have been limited efforts to characterize food oligosaccharides.[18] For dietary oligosaccharides, each pool whether sourced from rice, cooked rice, or processed rice products, will need to be treated as a collection of structures that can include equivalent if not greater complexity than those observed in HMOs. Thus, fiber from different sources can be as unique as milk oligosaccharides from different animals. Rapid structural analysis of these compounds with quantitation will be needed for determining the functional aspects of oligosaccharide pools.

Polysaccharides are the major component of carbohydrates in most plant-based foods; however, their analysis is also the

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least advanced. Strategies that employ combinations of traditional gas chromatography (GC) and nuclear magnetic resonance (NMR) to analyze the monosaccharide constituents and linkage positions of polysaccharides have been used but are slow and suffer from low sensitivity.[19] The characterization of polysaccharides requires analysis of the monosaccharide components, their associated linkages, branching, and the nature of potential polymeric side chains.

Rice grain offers a simple example of the complicated nature of polysaccharides in food (Figure 3). It is perhaps the best structurally understood food as a result of its central importance in many diets. Rice contains primarily starch, which is comprised of two polymers, amylose and amylopectin. Amylose is a simple polysaccharide that contains linear $\alpha(1\rightarrow 4)$ glucose residues, while amylopectin contains the same backbone but with $\alpha(1\rightarrow 6)$-linked branches. In contrast, the major polysaccharide components of the rice bran layer are arabinoxylan, β-glucan, and cellulose. Arabinoxylan is a pentose polysaccharide consisting of a $\beta(1\rightarrow 4)$ xylose backbone with $\alpha(1\rightarrow 3)$ arabinofuranosyl decorations. β-Glucan is an unbranched polymer that contains a combination of $\beta(1\rightarrow 3)$- and $\beta(1\rightarrow 4)$-linked glucose monomers. While this level of detail is impressive, only few foods have been characterized in this manner. Furthermore, this knowledge is still limited because we know little about the relative amounts of these polymers and how changes as a result of variables, such as cultivar, ripening stages, and processing, including cooking and storage, may affect them. Furthermore, many details of the polysaccharide structures remain unknown, such as frequency of branching, chain length, and the presence or location of intramolecular cross-linking, all of which could potentially affect the bioactivity of the molecule.

BETTER ANALYTICAL TOOLS FOR CARBOHYDRATE ANALYSIS WILL ADVANCE OUR UNDERSTANDING OF FIBERS AND THEIR ROLE IN HEALTH

HMOs provide a template for exploring dietary activity; however, expanding this research to plant-based dietary polysaccharides has proven to be far more challenging. The major factor limiting our knowledge of carbohydrate bioactivity is our inability to characterize polysaccharide structures. The field of polysaccharide analysis has had few innovations over the last 50 years when compared to proteins and DNA. Consequently, most studies focusing on the structural characterization of polysaccharides still employ methods developed in the 1960s.[20,21] These traditional methods rely on gas chromatography−mass spectrometry (GC−MS) and suffer from poor levels of detection, limited dynamic range, tedious sample preparation, and extensive analytical run times that severely limit throughput.

To understand the function of carbohydrates, we need advances in monosaccharide, linkage, and oligosaccharide analyses. The fields of proteomics and genomics became possible with the discovery of enzymes, such as trypsin for proteomics, to yield structures that can be probed by liquid chromatography−mass spectrometry (LC−MS). Unfortunately, a universal enzyme may not exist for polysaccharides, given that there is no single common linkage that is found in all polysaccharides. Thus, there is a need for a universal cleaving reagent that can produce oligosaccharides from large polysaccharides.

The methods need to be also rapid, highly quantitative, and sensitive. In the past, these attributes have not been a problem because food samples are not limited. However, as clinical trials involving carbohydrates become more common and bodily fluids are monitored, including blood, feces, and urine, carbohydrate analysis needs to have high-throughput and able to quantitate micro- and nanogram amounts of material.[22]

RAPID-THROUGHPUT MONOSACCHARIDE AND LINKAGE ANALYSIS

Monosaccharide compositions and linkages are the major structural features of carbohydrates that define biological function. Enzymes produced by bacteria recognize specific monosaccharides with specified linkages. The current GC−MS platforms have limited scope and are neither rapid nor sufficiently sensitive. The standard methods include over 3 days of sample preparation and up to an hour of GC−MS analysis.[21] In this regard, LC−MS techniques may provide solutions, because their capabilities potentially exceed those currently available with GC−MS.[23] Liquid chromatography analysis of monosaccharides has been shown using 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization to allow for C_{18} chromatography to be employed. Further enhancements, including the use of MS, have resulted in LC platforms becoming more effective than GC with regard to the speed of analysis, sensitivity, and linear dynamic range. Recently, this methodology has been expanded and further developed in a monosaccharide analysis method that employs ultrahigh-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (UHPLC−QqQ MS) for the simultaneous analysis of 15 free monosaccharides in feces.[23] The method is fast, with a 10 min run, limits of detection approaching the attomole level and a linear dynamic range up to 6 orders of magnitude. This method has been applied to specifically analyze the monosaccharide compositions of food polysaccharides by optimizing acid depolymerization conditions and employing a 96-well plate workflow to create the first truly rapid-throughput monosaccharide analysis.[24]

The standard approach for linkage analysis also employs GC−MS.[20] However, as a result of long chromatographic run times (60−90 min) and extensive derivatization procedures, the GC-based platform is not well-suited for rapid-throughput analysis. As with monosaccharide analyses, LC−MS-based platforms may enable linkage analysis to be performed on large sample sets by shortening sample preparation and chromatographic separations while increasing sensitivity, linear dynamic range, and robustness. Recently, the first LC−MS method for characterizing the glycosidic linkages in polysaccharides was reported. The method combines permethylation, hydrolysis, and reducing end derivatization, followed by UHPLC−QqQ MS analysis to monitor 22 glycosidic linkages in a 15 min analytical run.[25] Other methods may also make this analysis faster. For instance, ion mobility MS is a promising new technique that may help rapidly identify individual linkages.[26]

Despite the recent advances in the permethylated approach, linkage analysis can still be improved in scope. The number of total potential linkage standards required for analysis of the most common monosaccharides may exceed 100, even while excluding the anomeric character. However, commercial standards with known structures yield only a fraction of the potential linkages. Synthetic approaches have overcome this limitation by partially permethylating native monosaccharides.[27] With the synthesization of currently unavailable

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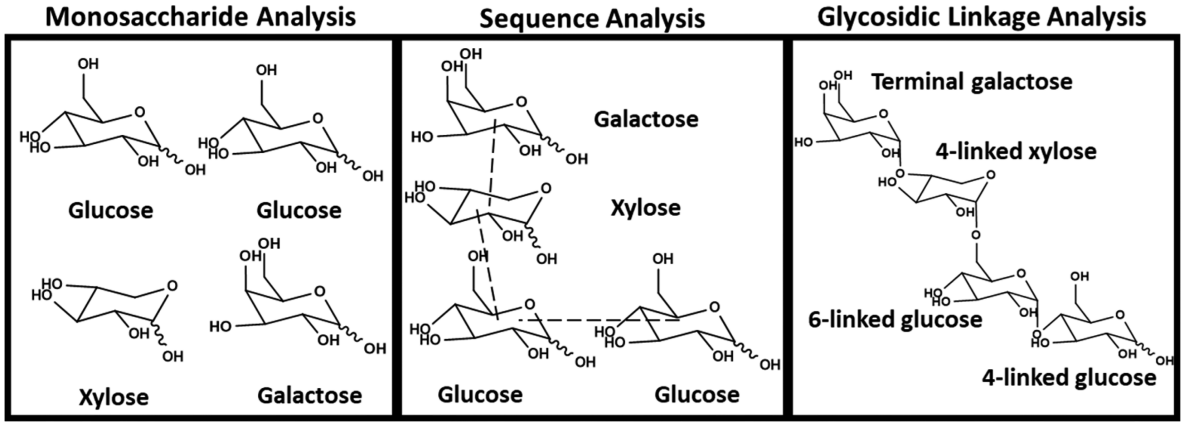

Figure 4. Carbohydrate elucidation requires three main components to be characterized: quantitation of the monosaccharide components, the spatial arrangement or sequence of the monosaccharides, and analysis of the glycosidic linkage positions and stereochemistry.

monosaccharide linkages, more comprehensive mass and retention time libraries have been developed.[27] However, without isolation of each linkage standard, the analysis remains a semi-quantitative approach. It is also worth noting that, for polysaccharides, the α/β stereochemistry of the glycosidic linkages cannot yet be elucidated by chemical approaches and must be elucidated enzymatically or by NMR.

GENERATING OLIGOSACCHARIDES FROM POLYSACCHARIDES FOR SEQUENCE ANALYSIS

Polysaccharide analysis requires monosaccharide profiling, linkage composition, and oligosaccharide analysis (Figure 4). Large polysaccharides cannot be analyzed directly with LC−MS because top-down analysis approaches are still not feasible. Additionally, a trypsin analogue for the bottom-up analysis of polysaccharides may not be possible as a result of the unique monosaccharide and linkage compositions of polysaccharides and the highly specific nature of glycosyl hydrolases.[28] Indeed, several notable efforts have probed this approach. However, only a few polysaccharides can be digested with commercially available enzymes. Recently, matrix-assisted laser desorption/ionization−mass spectrometry (MALDI−MS)-based platforms have been developed for profiling extracellular polysaccharides.[29] In this technique, glycosyl hydrolases are applied to plant cell wall material to generate oligosaccharides prior to MALDI analysis. While the technique may not provide absolute quantitation, it is useful for directly comparing samples. Furthermore, it has the potential for higher throughput. The technique can also be used for *in situ* analysis, where endoglycosidases are applied directly to plant material on a MALDI plate.[29] It was used to probe the diversity in polysaccharide structures according to plant biogeography.

Researchers have also focused on expanding the toolbox of glycosyl hydrolases available for polysaccharide degradation.[30] For example, the specificity of over 70 glycosyl hydrolases with potential for analytical applications has been expressed from several fungi.[30] Additionally, a new class of enzymes called lytic polysaccharide monooxygenases (LPMOs) has gathered substantial interest for its ability to catalyze the cleavage of recalcitrant polysaccharides using transition metals to induce oxidative cleavage.[31] LPMOs have not yet been applied for analytical applications; however, the utility is apparent.

Chemical approaches may also provide attractive solutions for controlled and unbiased polysaccharide degradation. Acid hydrolysis has long been used to degrade polysaccharides and overcomes the high specificity limitations of enzymatic methods.[32,33] However, acid can cleave in both endo and exo positions, which results in both the liberation of monosaccharides by trimming from the ends and the production of oligosaccharides by cleaving internal bonds. Thus, the resulting oligosaccharides may not wholly represent the parent structure. It also cleaves at the most acid-labile positions first and may be difficult to control. Notable examples of polysaccharide degradation to produce oligosaccharides include the partial hydrolysis of κ-carrageenan to yield repeating disaccharide units resulting in odd-numbered oligosaccharides between DP of 3 and 17.[32] Similarly, the degradation of olive xylans with acid treatment yielded oligosaccharides up to 2500 Da.[33]

Natural plant biology may also provide non-enzymatic degradation strategies. Early reports have shown the use of ascorbate and hydrogen peroxide, a cocktail used by plants to degrade polysaccharides during fruit ripening, for the *in vitro* breakdown of tamarind xyloglucans and several other polysaccharides.[34] This and other oxidative approaches may compliment acid hydrolysis if they can be controlled to yield primarily glycosidic bond cleavages and not overoxidized products.

CONCLUDING REMARKS

To understand the roles of the most abundant component in our diet, limitations in characterizing carbohydrate structures must be overcome. These carbohydrates are unlike other biopolymers because they can be composed of mono- and disaccharides, oligosaccharides, and polysaccharides simultaneously. The methods that are developed need to address the specific structural features of the various groups. They also need to be capable of rapid throughput to address the large number of foods in our diet while also being highly sensitive to deal with large clinical studies that include bodily fluids, such as blood, urine, saliva, and feces.

The role of the microbiome in health, where the primary structures of carbohydrates are known to modulate the gut microbiome more than any other dietary component, is currently the biggest impetus for characterizing carbohydrates in food.[35] Advances in determining the genome and transcriptome of bacteria have far surpassed the methods for analyzing carbohydrates. For this reason, carbohydrate−microbe interactions have focused primarily on profiling the

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microbial communities and their associated gene expression and much less on characterizing the carbohydrate structures being consumed. Deep transcriptome analysis further yields the specific gene loci responsible for growth on specific carbohydrate substrates.[36] Analysis of the degradation products would yield the enzyme specificity, while general knowledge of carbohydrate structures in different foods would add to the general scope of these studies.

Advanced analytical tools for carbohydrates could provide information regarding the carbohydrates present in all foods. A comprehensive database of food and their respective carbohydrates will be a valuable resource to the food industry and fundamental nutrition research. This resource would aid the numerous ongoing clinical studies, where diet is used as an intervention. In turn, this database would help provide effective and informative dietary guidelines.

AUTHOR INFORMATION

Corresponding Author

*E-mail: cblebrilla@ucdavis.edu.

ORCID

Matthew J. Amicucci: 0000-0002-1392-9252

Carlito B. Lebrilla: 0000-0001-7190-5323

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