

Development of an Extensive Linkage Library for Characterization of Carbohydrates

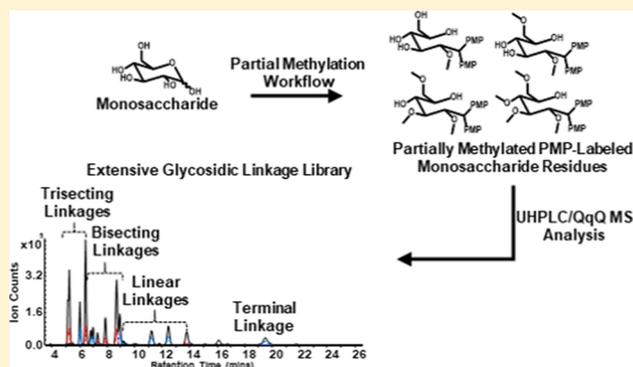
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Supporting Information

ABSTRACT: The extensive characterization of glycosidic linkages in carbohydrates remains a challenge because of the lack of known standards and limitations in current analytical techniques. This study encompasses the construction of an extensive glycosidic linkage library built from synthesized standards. It includes an improved liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitation of glycosidic linkages derived from disaccharides, oligosaccharides, and polysaccharides present in complicated matrices. We present a method capable of the simultaneous identification of over 90 unique glycosidic linkages using ultrahigh-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC/QqQ MS) operated in dynamic multiple reaction monitoring (dMRM) mode. To build the library, known monosaccharides commonly found in plants were subjected to partial methylation to yield partially derivatized species representing trisecting, bisecting, linear, and terminal structures. The library includes glycosidic linkage information for three hexoses (glucose, galactose, and mannose), three pentoses (xylose, arabinose, and ribose), two deoxyhexoses (fucose and rhamnose), and two hexuronic acids (glucuronic acid and galacturonic acid). The resulting partially methylated monosaccharides were then labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP) followed by separation and analysis by UHPLC/dMRM MS. Validation of the synthesized standards was performed using disaccharide, oligosaccharide, and polysaccharide standards. Accuracy, reproducibility, and robustness of the method was demonstrated by analysis of xyloglucan (tamarind) and whole carrot root. The synthesized standards represent the most comprehensive group of carbohydrate linkages to date.



Carbohydrates consist of monosaccharides, disaccharides, oligosaccharides, and polysaccharides, and are the most abundant biomolecules in nature. They are present in all living organisms and are known to have structural and functional biological properties that are associated with their specific compositions and structures. Carbohydrates are often found on the surface of cells as plant cell wall polysaccharides and as oligosaccharides that decorate the surfaces of animal cells that are involved in molecular trafficking, protein-folding, and biological recognition.^{1–3} Carbohydrates are common structural components that are degraded by enzymes in a host of biological reactions, one example being food for nutrition. The monosaccharide and glycosidic linkages of polysaccharides are specific substrates for glycosyl hydrolases present in the host or in their complement microbes.^{4–7} These glycosyl hydrolases can have broad or high specificity that depend on both the monosaccharide and linkage composition. For example, in human digestion, α -amylase is responsible for the hydrolysis of $\alpha(1 \rightarrow 4)$ glucose in amylose, the main component of starch. However, α -amylase is incapable of digesting $\alpha(1 \rightarrow$

4,6)glucose, which is the branching component of amylopectin.^{8–10} Thus, characterization of the monosaccharide composition and the associated glycosidic linkages are critical to fully understand the structural and functional attributes of carbohydrates.

The most commonly used method for linkage analysis employs the Hakomori approach using permethylation and acid hydrolysis followed by volatilization of the products for GC-MS analysis.^{11–16} Recently, we reported a method for linkage analysis based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) involving permethylation, acid hydrolysis, and labeling with 1-phenyl-3-methyl-5-pyrazolone (PMP) providing improvements in speed, throughput, and sensitivity over the standard GC-MS method.¹⁷ A library was also produced based on a limited set of available standards. The lack of highly pure and well-characterized commercial

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standards severely limited this approach. Thus, the earlier compilation was created with only a fraction of the possible linkages represented. Glycosidic linkages representing many branched monomers were most notably absent.

A solution to the lack of standards is to synthesize partially methylated species from monosaccharides representing all combinations of linkages including terminal, linear, bisecting and even trisecting species. This approach has been previously used to prepare linkage standards for GC-MS analysis.¹⁸ However, some important concerns have to be addressed in order to successfully adapt this approach from gas chromatography to liquid chromatography. One is the poor potential recovery of monosaccharides with higher numbers of unmethylated sites such as those representing bisecting and trisecting species during liquid–liquid extraction.^{19–22} To address this issue, unmethylated hydroxyls can be acetylated using acetic anhydride.^{18,23–25} A further concern with this method is that sodium hydroxide (NaOH), necessary for the methylation, will decompose acetic anhydride immediately after its addition to the solution. A more effective approach would be to use dimethyl sulfinyl anion rather than hydroxide during methylation.^{11,26–29} The extent of methylation is then controlled depending on the concentration of dimethyl sulfinyl anion.¹⁸ Recovery of the desired partially methylated structures is then obtained by enrichment using liquid–liquid extraction followed by deacetylation.^{18,20}

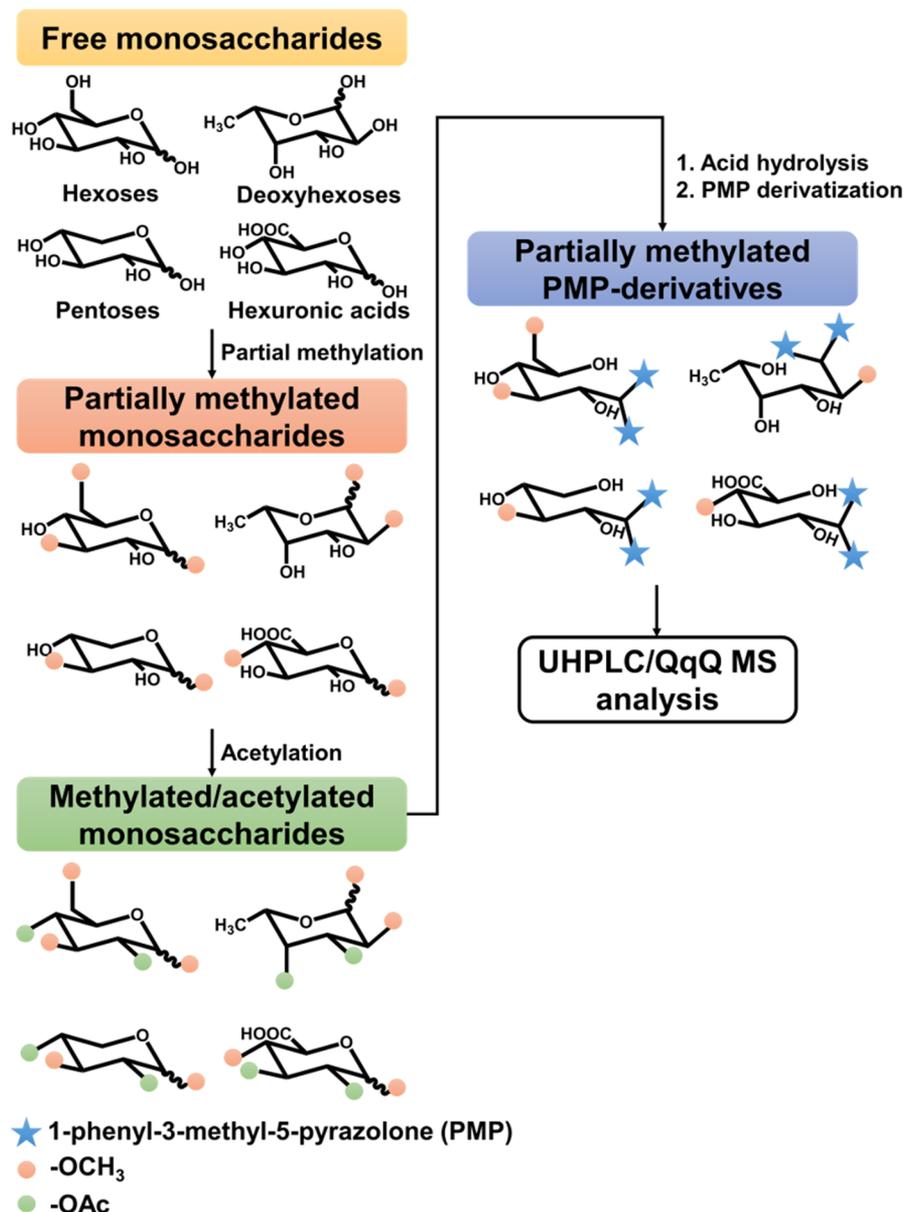
In this study, we constructed a considerably more comprehensive LC-MS/MS-based glycosidic linkage library by employing a synthetic approach involving the partial methylation of monosaccharides and reducing-end labeling in combination with ultrahigh-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC/QqQ MS). Tandem MS was performed in dynamic multiple reaction monitoring (dMRM) mode for the quantitation and detection of distinct glycosidic linkages derived from disaccharides, oligosaccharides, and polysaccharides present in complicated matrices. The UHPLC/dMRM MS method enabled the simultaneous analysis of an extensive group of over 90 glycosidic linkages representing terminal, linear, bisecting, and even trisecting monomers of glucose, galactose, mannose, xylose, arabinose, ribose, fucose, rhamnose, glucuronic acid, and galacturonic acid. The synthetic-based library improves over a previously developed linkage library built exclusively from commercial disaccharide, oligosaccharide, and polysaccharide standards.¹⁷ To build the extended library, partial methylation of monosaccharide standards was performed with iodomethane using various concentrations of dimethyl sulfinyl anion followed by acetylation using acetic anhydride, deacetylation using trifluoroacetic acid (TFA), and reducing-end labeling with PMP. Optimal chromatographic separation accompanied by optimized MS parameters allowed for separation and analysis in 30 min. Validation of the synthesized linkage standards was performed by analysis of disaccharide, oligosaccharide, and polysaccharide standards. The method was applied and further validated by the analysis of xyloglucan (tamarind) and whole carrot root. The accuracy, robustness, and reproducibility of this method makes it significantly useful for the elucidation of glycosidic linkages in disaccharides, oligosaccharides, and polysaccharides derived from foods and biological samples.

EXPERIMENTAL PROCEDURES

Samples and Materials. Iodomethane (contains copper stabilizer, 99.5%), TFA, α -D-glucose pentaacetate (99%), 1-methylimidazole (reagent grade, 99%), acetic anhydride (puriss, p.a., ACS reagent, reagent grade, ISO, reagent grade, Ph. Eur., $\geq 99\%$ (GC)), NaOH pellets (semiconductor grade, 99.99% trace metals basis), sodium hydride (NaH) (dry, 90%), hydrochloric acid (HCl) (ACS reagent, 37%), phenolphthalein (ACS reagent), ammonium acetate (NH₄Ac) (99.99% trace metals basis), ammonium hydroxide solution (NH₄OH) (28–30%, NH₃ basis), PMP, anhydrous dimethyl sulfoxide (DMSO), dichloromethane (DCM), methanol (MeOH) (HPLC grade), stachyose, D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, D-arabinose, D-xylose, D-ribose, D-glucuronic acid, and D-galacturonic acid were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) (LC-MS grade) was purchased from Honeywell (Muskegon, MI). 2-O-(α -D-Mannopyranosyl)-D-mannopyranose, 1,4-D-xylobiose, 1,5-a-L-arabinotriose, 1,3-a-1,6-a-D-mannotriose, isomaltotriose, 4-O-(β -D-galactopyranosyl)-D-galactopyranose, lactose, 2'-fucosyl-lactose (synthetic), nigerose, 3-O-(β -D-galactopyranosyl)-D-galactopyranose, 3-O-(α -D-mannopyranosyl)-D-mannopyranose, 1,4-b-D-mannotriose, maltohexaose, 1,6-a-D-mannotriose, and amylopectin were obtained from Carbosynth (Compton, U.K.). Xyloglucan (tamarind), 3³- α -L-arabinofuranosyl-xyloetraose, and sophorose were acquired from Megazyme (Chicago, IL). Whole carrots were purchased from Whole Foods (Davis, CA). Nanopure water was used for all experiments.

Optimization of Deacetylation Reaction. A stock solution of α -D-glucose pentaacetate was prepared at a concentration of 2 $\mu\text{g}/\mu\text{L}$ in MeOH. Aliquots containing 25 μL of this stock solution were transferred to microtubes with screw caps and dried by vacuum centrifugation. Next, the samples were hydrolyzed using 100 μL of 4 M TFA and by incubation at 100 °C for 0, 15, 30, 60, and 90 min, respectively. Three experimental replicates were performed for each time-point. After each time-point, 900 μL of ice-cold water was added to the samples followed by vortexing. An aliquot containing 50 μL of this solution was immediately subjected to PMP-labeling following a previously developed procedure.³⁰

Optimization of Acetylation Reaction. A stock solution of D-glucose was prepared in water at a concentration of 1 $\mu\text{g}/\mu\text{L}$. Aliquots containing 50 μL of this solution were transferred to cryovials (5 mL) and dried by vacuum centrifugation. Samples were purged with argon followed by the addition of 150 μL of anhydrous DMSO. Next, 75 μL of 1-methylimidazole was added followed by 750 μL of acetic anhydride. Samples were reacted on a shaker at room temperature for 0, 5, 10, and 20 min, respectively. Three experimental replicates were performed for each time-point. After each time point, the samples were placed in an ice-bath followed by the addition of 1.5 mL ice-cold water and vortexing to quench the excess acetic anhydride in each sample. After 10 min, the samples were removed from the ice-bath and enriched by liquid–liquid extraction using 500 μL of DCM and subsequent washes with 4 \times 750 μL ice-cold water. The aqueous top layer was discarded, and the bottom organic layer containing products was collected and dried by vacuum centrifugation. Samples were hydrolyzed using the optimized time of 30 min followed by PMP-labeling using the previously described method.

Scheme 1. Workflow for the Development of the Extended Linkage Library^a

^aThe library is built by sequential partial methylation, acetylation, hydrolysis, and PMP-labeling using monosaccharide standards as building blocks.

Preparation of Dimethyl Sulfinyl Anion. Anhydrous DMSO (20 mL, 280.0 mmol) preheated to 70 °C was added dropwise via addition funnel to solid NaH (1.5 g, 62.5 mmol) in a two-neck argon-purged round-bottomed flask. The round-bottomed flask was fitted with an entrance and exit gas port to allow fresh argon to enter and hydrogen gas byproduct to escape. The reaction mixture was vigorously stirred using a magnetic stir bar and reacted at 70 °C for 1 h in an oil bath. The solution was flushed with argon throughout the reaction. After 1 h, no further bubbling was observed, indicating completion of the reaction. The reaction solution was cooled to room temperature, and the resulting color of the final solution was dark green. The solution was centrifuged at 4k rpm for 30 min, and the supernatant was filtered using a 0.2 μm syringe filter and collected. The concentration of dimethyl sulfinyl anion in the filtrate was determined by titration with

0.1 M HCl using phenolphthalein as an indicator. Final concentration of the anion was determined to be 1.2 M.

Partial Methylation of Monosaccharides. Standard solutions of D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, D-arabinose, D-xylose, D-ribose, D-glucuronic acid, and D-galacturonic acid were prepared in water at a concentration of 10 μg/μL. Aliquots containing 5 μL of solution were transferred to cryovials (5 mL) and dried by vacuum centrifugation. Dimethyl sulfinyl anion was prepared at concentrations of 0.01, 0.1, and 1.0 M in anhydrous DMSO. Each of the monosaccharide samples were reconstituted in 150 μL of each dimethyl sulfinyl anion concentration, flushed with argon, and placed on a shaker at for 30 min at room temperature. Next, 40 μL of iodomethane was added followed by reacting on a shaker for 50 min. Residual iodomethane was then removed by vacuum centrifugation for 30 min. Acetylation of remaining free hydroxyl groups was carried

Table 1. Library Containing 92 Unique Glycosidic Linkages Obtained from the Partial Methylation and PMP-Labeling of Monosaccharide Standards

linkage residue	retention time (mins)	degree of permethylation (DoPe)	MRM transition pair	
			precursor ion <i>m/z</i>	product ions <i>m/z</i>
3,4-glucuronic acid	2.70	1	553.3	175.1, 231.2
2,X,X-mannose	2.80	1	525.2	175.1, 217.1
X-glucuronic acid	2.82	2	567.6	175.1, 231.2
2,X-glucuronic acid	2.90	1	553.3	175.1, 217.1
2,X-glucuronic acid	3.10	1	553.3	175.1, 217.1
2,X,X-mannose	3.16	1	525.2	175.1, 217.1
2,X-galacturonic acid	3.30	1	553.3	175.1, 217.1
2,X-mannose	3.30	2	539.2	175.1, 217.1
X-glucuronic acid	3.32	2	567.6	175.1, 231.2
3,4-galacturonic acid	3.72	1	553.3	175.1, 231.2
2,X,X-mannose	3.80	1	525.2	175.1, 217.1
X-galacturonic acid	3.84	2	567.6	175.1, 231.2
2,X-F-ribose	3.88	1	495.2	175.1, 217.1
X-galacturonic acid	4.04	2	567.6	175.1, 231.2
2,X-mannose	4.14	2	539.2	175.1, 217.1
2,X-rhamnose	4.16	1	509.2	175.1, 217.1
2,X,X-glucose	5.12	1	525.2	175.1, 217.1
2,X-mannose	5.15	2	539.2	175.1, 217.1
2,X-rhamnose	5.24	1	509.2	175.1, 217.1
2,X,X-glucose	5.24	1	525.2	175.1, 217.1
2-mannose ^a	5.25	3	553.3	175.1, 217.1
2-rhamnose	5.46	2	523.2	175.1, 217.1
2,X,X-galactose	5.66	1	525.2	175.1, 217.1
2,X-F-ribose	5.68	1	495.2	175.1, 217.1
2,X,X-galactose	5.88	1	525.2	175.1, 217.1
3,4,6-glucose	6.00	1	525.2	175.1, 231.2
2,X-P-xylose	6.02	1	495.2	175.1, 217.1
2,X,X-galactose	6.15	1	525.2	175.1, 217.1
3,4,6-galactose	6.40	1	525.2	175.1, 231.2
2,X,X-glucose	6.41	1	525.2	175.1, 217.1
2,X-galactose	6.54	2	539.2	175.1, 217.1
2,X-F-arabinose	6.55	1	495.2	175.1, 217.1
3,5-F-arabinose	6.78	1	495.2	175.1, 231.2
X,X-glucose	6.79	2	539.2	175.1, 231.2
3,4-P-xylose ^a	6.82	1	495.2	175.1, 231.2
2,X-F-arabinose	6.92	1	495.2	175.1, 217.1
4,6-glucose	6.98	2	539.2	175.1, 231.2
2,X-P-xylose	7.00	1	495.2	175.1, 217.1
3,4,6-mannose	7.15	1	525.2	175.1, 231.2
X,X-galactose	7.18	2	539.2	175.1, 231.2
2,X-glucose	7.30	2	539.2	175.1, 217.1
X,X-galactose	7.40	2	539.2	175.1, 231.2
3,4-fucose	7.44	1	509.2	175.1, 231.2
3,5-F-ribose	7.45	1	495.2	175.1, 231.2
2,X-glucose	7.85	2	539.2	175.1, 217.1
2,X-galactose	7.89	2	539.2	175.1, 217.1
4-P-xylose ^a	8.05	2	509.2	175.1, 231.2
5-F-arabinose ^a	8.18	2	509.2	175.1, 231.2
3,6-mannose ^a	8.20	2	539.2	175.1, 231.2
X,X-galactose	8.20	2	539.2	175.1, 231.2
2,X-fucose	8.25	1	509.2	175.1, 217.1
2-F-arabinose	8.32	2	509.2	175.1, 217.1
3-P-xylose	8.34	2	509.2	175.1, 231.2
2-P-xylose	8.52	2	509.2	175.1, 217.1
2,X-glucose	8.68	2	539.2	175.1, 217.1
2-F-ribose	8.70	2	509.2	175.1, 217.1
2,X-galactose	8.79	2	539.2	175.1, 217.1
X,X-glucose	8.92	2	539.2	175.1, 231.2

Table 1. continued

linkage residue	retention time (mins)	degree of permethylation (DoPe)	MRM transition pair	
			precursor ion <i>m/z</i>	product ions <i>m/z</i>
2,X-fucose	9.00	1	509.2	175.1, 217.1
X-F-arabinose	9.10	2	509.2	175.1, 231.2
3,4-rhamnose	9.10	1	509.2	175.1, 231.2
6-glucose ^a	9.14	3	553.3	175.1, 231.2
X-F-ribose	9.25	2	509.2	175.1, 231.2
6-galactose ^a	9.38	3	553.3	175.1, 231.2
X-fucose	9.48	2	523.2	175.1, 231.2
X,X-mannose	9.85	2	539.2	175.1, 231.2
X,X-mannose	10.02	2	539.2	175.1, 231.2
6-mannose ^a	10.10	3	553.3	175.1, 231.2
4-galactose ^a	10.26	3	553.3	175.1, 231.2
2-fucose	10.36	2	523.2	175.1, 217.1
X-F-ribose	10.42	2	509.2	175.1, 231.2
X-F-arabinose	10.65	2	509.2	175.1, 231.2
4-glucose ^a	11.20	3	553.3	175.1, 231.2
T-P-xylose ^a	11.25	3	523.2	175.1, 231.2
2-galactose ^a	11.28	3	553.3	175.1, 217.1
X-fucose	11.50	2	523.2	175.1, 231.2
3-galactose ^a	11.81	3	553.3	175.1, 231.2
X-rhamnose	12.35	2	523.2	175.1, 231.2
3-glucose ^a	12.45	3	553.3	175.1, 231.2
X-rhamnose	13.80	2	523.2	175.1, 231.2
2-glucose ^a	13.80	3	553.3	175.1, 217.1
T-F-arabinose ^a	13.92	3	523.2	175.1, 231.2
3-mannose ^a	14.70	3	553.3	175.1, 231.2
4-mannose ^a	15.10	3	553.3	175.1, 231.2
T-fucose ^a	16.55	3	537.2	175.1, 231.2
T-rhamnose	16.55	3	537.2	175.1, 231.2
T-galactose ^a	17.84	4	567.6	175.1, 231.2
T-F-ribose	18.40	3	523.2	175.1, 231.2
T-mannose ^a	19.00	4	567.6	175.1, 231.2
T-galacturonic acid	19.10	3	581.2	175.1, 231.2
T-glucose ^a	19.50	4	567.6	175.1, 231.2
T-glucuronic acid	23.10	3	581.2	175.1, 231.2

^aValidated with a commercial disaccharide, oligosaccharide, or polysaccharide standard.

out using the optimal reaction time of 20 min using the method previously described. Subsequent liquid–liquid extractions, hydrolysis, and PMP-labeling were then performed following the methods previously mentioned. The samples were reconstituted in 100 μL of 70% MeOH/water (v/v) and an aliquot containing 20 μL of partially methylated PMP-labeled monosaccharides produced by each concentration of dimethyl sulfanyl anion filtrate were pooled.

Preparation of Polysaccharide Standards and Whole Food Samples for Derivatization and Analysis. A stock solution of xyloglucan polysaccharide standard was prepared in water at a concentration of 10 $\mu\text{g}/\mu\text{L}$. Whole carrot root was chopped, lyophilized, and ground to a fine powder using a mortar and pestle; then a stock solution of this processed material was prepared at a concentration of 10 $\mu\text{g}/\mu\text{L}$ in water. Further homogenization of sample was accomplished by bead blasting for 3 min using 1.4 mm stainless steel magnetic beads and a Next Advance Bullet Blender Storm 24 (Next Advance, Troy, NY). The resulting sample solutions were incubated at 100 $^{\circ}\text{C}$ for 60 min followed by a second round of bead blasting.

UHPLC/QqQ MS Analysis. The partially methylated PMP-labeled monosaccharides were separated and analyzed on an

Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6495A triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA.). The analysis utilizes 1 μL of sample injected onto a Agilent Poroshell 120 EC-C18 column (2.1 mm \times 150 mm i.d., 1.9 μm particle size) equipped with an Agilent Poroshell 120 EC-C18 guard cartridge (2.1 mm \times 5 mm i.d., 1.9 μm particle size) and separated using a 26 min isocratic method followed by a 4 min wash and re-equilibration step at a constant flow rate of 0.35 mL/min: Mobile phase A: 25 mM NH_4Ac in 5% ACN/water (v/v), adjusted to pH 8.2 using NH_4OH ; Mobile phase B: 95% ACN/water (v/v). The following elution gradient was used: 0.00–26.00 min, 17.00% B; 26.00–26.01 min, 17.00–99.00% B; 26.01–28.00 min, 99.00% B; 28.00–28.01 min, 99.00–17.00% B; 28.01–30.00 min, 17.00% B.

An Agilent jet stream electrospray ionization (AJS-ESI) source operated in positive ion mode was used to introduce sample into the QqQ. Capillary and fragmentor voltages were set at 1800 and 380 V, respectively. Nebulizer pressure was set to 30 psi. Drying and sheath gas flow rates were set at 11 L/min and 12 L/min, respectively. Nitrogen drying and sheath gas temperatures were set at 290 and 300 $^{\circ}\text{C}$, respectively. Collision-induced dissociation (CID) was performed by setting

the collision energy to 35 V. Acquired UHPLC/QqQ MS data was retrieved using Agilent MassHunter Workstation Data Acquisition Version B.06.01 and analyzed with Agilent MassHunter Quantitative Analysis software Version B.06.00.

RESULTS AND DISCUSSION

A synthesis-based methodology was used to develop an extensive library for the analysis of linkage residues derived from disaccharides, oligosaccharides, and polysaccharides existing in complicated matrices. The method is composed of partial methylation, acetylation, deacetylation, and PMP-labeling followed by UHPLC/QqQ MS analysis (Scheme 1). First, partial methylation was performed using varying concentrations of prepared dimethyl sulfinyl anion. Remaining free hydroxyls were then acetylated to improve enrichment efficiency during liquid–liquid extraction following partial methylation. Next, deacetylation by acid was performed to reestablish the free hydroxyl groups that mimic positions of putative glycosidic bonds. The partially methylated monosaccharides were labeled further to improve chromatographic separation and ionization in the LC-MS/MS analysis.

Construction of the Synthetic Glycosidic Linkage Library. Glycosidic linkage reference compounds representing over 90 terminal, linear, bisecting, and trisecting neutral and acidic monosaccharide species were produced by the partial methylation and PMP-labeling of D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, D-xylose, D-arabinose, D-ribose, D-glucuronic acid, and D-galacturonic acid. Validation of the synthetic linkages was performed using commercial disaccharide, oligosaccharide, and polysaccharide standards prepared using a previously established procedure.¹⁷ Disaccharide, oligosaccharide, and polysaccharide standards used for the validation include stachyose, sophorose, isomaltotriose, lactose, nigerose, 2'-fucosyllactose, 1,5-a-L-arabinotriose, 1,4-D-xylobiose, 3³- α -L-arabinofuranosyl-xylohexaose, 1,6-a-D-mannotriose, 1,4-b-D-mannotriose, 1,3-a-1,6-a-D-mannotriose, 3-O-(a-D-mannopyranosyl)-D-mannopyranose, 2-O-(a-D-mannopyranosyl)-D-mannopyranose, 3-O-(b-D-galactopyranosyl)-D-galactopyranose, 4-O-(b-D-galactopyranosyl)-D-galactopyranose, and xyloglucan. Table 1 displays the synthesis-based LC-MS/MS linkage library that lists 92 neutral and acidic linkage residues with their corresponding retention times, degree of permethylation (DoPe), precursor ions, and product ions.

The retention time, DoPe value, and corresponding MRM transitions are used to identify each linkage.¹⁷ For MRM, the precursor ion mass is equal to the sum of the native monosaccharide mass, the mass of a methyl group multiplied by the DoPe value, the mass of two PMP molecules, and a proton ($[M + 14 * \text{DoPe value} + 330 + H]^+$). The DoPe value provides the number of methyl groups associated with each monosaccharide class, gives an indication of the linkages, and can be used to calculate the precursor ion mass. For example, terminal hexoses are represented by a DoPe value of 4, which corresponds to a precursor ion mass of m/z 567.6. Linear, bisecting, and trisecting hexoses are represented by DoPe values of 3, 2, or 1, respectively, which correspond to a precursor ion mass of m/z 553.3, m/z 539.2, or m/z 525.2, respectively. However, terminal pentoses, deoxyhexoses, and hexuronic acids are represented by a DoPe value of 3; thus, their respective precursor ion masses are m/z 523.2, m/z 537.2, or m/z 581.2, respectively. Linear pentoses, deoxyhexoses, and hexuronic acids are represented by a DoPe value

of 2, which corresponds to a precursor ion mass of m/z 509.2, m/z 523.2, or m/z 567.6, respectively. Bisecting species of pentoses, deoxyhexoses, and hexuronic acids are represented by a DoPe value of 1 and correspond to a precursor ion mass of m/z 495.2, m/z 509.2, or m/z 553.3, respectively. Because of their inherent structures, pentoses, deoxyhexoses, and hexuronic acids cannot possess a trisecting linkage.

The precursor ion is fragmented by CID to yield a fragment ion common to all, m/z 175.1, and is used as the quantifying ion. Some monosaccharides yield unique fragments that readily identify the linkage. For example, a 2-linked hexose (glucose, galactose, mannose) yields unique fragments corresponding to both m/z 217.1 and m/z 175.1. However, structures lacking a linkage at the 2-position such as a 3-, 4-, or 6-linked hexose yield unique fragments corresponding to both m/z 231.2 and m/z 175.1.

The synthesis produced a stochastic set of partially methylated species whose identity was assigned using a combination of known standards and a process of deduction. The possible combinations for each linkage species (terminal, linear, bisecting, and trisecting) must first be understood. For example, for hexoses such as glucose, galactose, and mannose, there are three possible combinations for trisecting species: (1 \rightarrow 2,3,6)-, (1 \rightarrow 2,4,6)-, or (1 \rightarrow 3,4,6)-positions. Bisecting hexose species have six possible combinations, which include linkages at the (1 \rightarrow 2,3)-, (1 \rightarrow 2,4)-, (1 \rightarrow 2,6)-, (1 \rightarrow 3,4)-, (1 \rightarrow 3,6)-, or (1 \rightarrow 4,6)-positions. Linear hexose species can exist in four possible combinations with linkages at the (1 \rightarrow 2)-, (1 \rightarrow 3)-, (1 \rightarrow 4)-, or (1 \rightarrow 6)-positions. The terminal position yields a single product and was readily identified.

Deoxyhexoses such as fucose and rhamnose lack trisecting species; however, they have three possible combinations for bisecting species with linkages at the (1 \rightarrow 2,3)-, (1 \rightarrow 2,4)-, or (1 \rightarrow 3,4)-positions. Linear deoxyhexose species possess three possible linkage combinations with linkages at the (1 \rightarrow 2)-, (1 \rightarrow 3)-, or (1 \rightarrow 4)-positions. With the addition of a terminal deoxyhexose species, a total of seven linkage combinations are possible for a single deoxyhexose monosaccharide. Pentoses such as xylose, which exists typically in the pyranose form in plant carbohydrates,³¹ share the same linkage combinations for bisecting, linear and terminal species.

Pentoses in the furanose form, such as arabinose and ribose, also lack a trisecting species. However, they have three possible combinations for bisecting species with linkages at the (1 \rightarrow 2,3)-, (1 \rightarrow 2,5)-, or (1 \rightarrow 3,5)-positions. The linear species of pentoses in the furanose form can have three possible combinations with linkages at the (1 \rightarrow 2)-, (1 \rightarrow 3)-, or (1 \rightarrow 5)-positions. Seven possible linkage combinations, including the terminal species, are possible for pentoses existing in the furanose form. For the remainder of this article, the short-hand notation described in previous work for the linkages was used.¹⁷ For example, the notation 2,3,6-glucose is used to represent glucose linked at the (1 \rightarrow 2,3,6)-positions, while 4-P-xylose is used to represent xylose in the pyranose form linked at the (1 \rightarrow 4)-positions.

To improve enrichment of partially methylated structures during liquid–liquid extraction, general conditions for complete deacetylation and acetylation were first optimized. After partial methylation, remaining hydroxyls are acetylated to maximize partitioning of the compounds into the organic layer during liquid–liquid extraction. The acetyl groups are then removed by acid hydrolysis to obtain the desired partially methylated structures. α -D-Glucose pentaacetate and D-glucose

were used as models for fully acetylated and minimally methylated monosaccharides, respectively. After each condition, the monosaccharides were derivatized with PMP, and their corresponding absolute ion peak area abundances were compared. Deacetylation was optimized by hydrolysis of α -D-glucose pentaacetate with TFA at varying time points (Figure S-1A). Next, acetylation was optimized with D-glucose at varying time points followed by complete deacetylation after enrichment by liquid–liquid extraction (Figure S-1B). Complete deacetylation and acetylation were observed at 30 and 20 min, respectively, and were applied to construct the library.

To ensure the generation of all linkage types possible for each monosaccharide, partial methylation was separately performed using three dimethyl sulfonyl anion concentrations spanning 3 orders of magnitude. Validation of the partial methylation workflow was demonstrated by analysis of linear and terminal hexose residues produced by partial methylation of glucose and by comparison with linkage residues derived from sophorose, nigerose, lactose, and isomaltotriose. MRM transitions developed in previous work were used to monitor linear hexose species (m/z 553.3 \rightarrow m/z 175.1, m/z 217.1, m/z 231.2) and terminal hexose species (m/z 567.6 \rightarrow m/z 175.1, m/z 231.2).¹⁷ Figure 1A–E displays MRM chromatograms for linear and terminal glucose residues obtained by analysis of sophorose, nigerose, lactose, isomaltotriose, and glucose linkages generated by the partial methylation workflow, respectively. As shown by Figure 1E, partially methylated glucose residues corresponding to 6-, 4-, 3-, 2-, and T-glucose

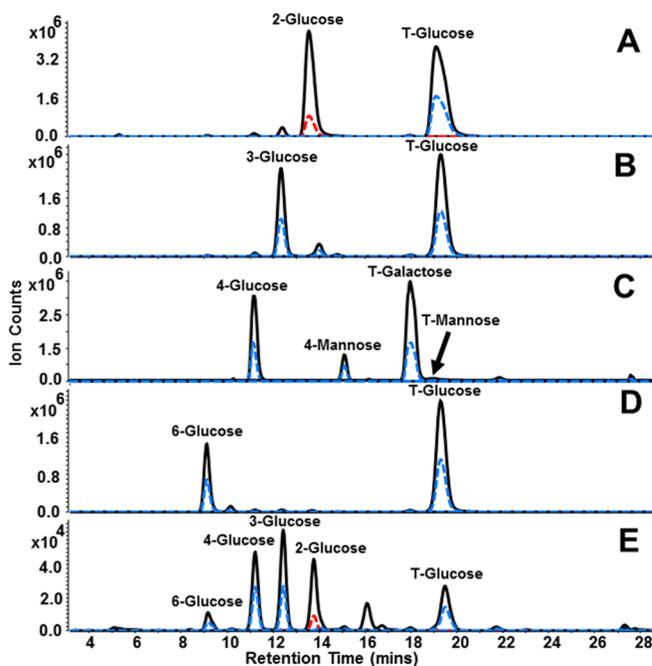


Figure 1. MRM chromatograms illustrating linkage linear and terminal residues obtained from (A) sophorose, (B) nigerose, (C) lactose, (D) isomaltotriose, and (E) glucose linkage standards generated using the partial methylation workflow. The solid black trace represents transitions including the quantifying ion m/z 175.1, while the dashed blue and red traces represent transitions including qualifying ions m/z 231.2 and m/z 217.1, respectively. Additional 4-mannose and T-mannose peaks were observed for lactose and are contaminants present in the stock reagent bottle. The contaminants were assigned by analysis of 1,4-b-D-mannotriose standard.

were produced and observed at 9.14, 11.20, 12.45, 13.80, and 19.50 min, respectively. These signals match the retention times and transitions observed by analysis of the standards (Figure 1A–D), thereby validating the utility of the partial methylation method. Additional signals for 4-mannose and T-mannose were observed for the lactose sample (Figure 1C) because of contamination of a mannose-containing disaccharide or oligosaccharide present in the stock reagent bottle. The contaminants were identified and confirmed by analysis of 1,4-b-D-mannotriose.

Additional peaks that were identified as linkage residues using both quantifying and qualifying transitions, but were not assignable using standards, were tentatively assigned into the linkage library for later evaluation. Examples include bisecting species obtained by partial methylation of arabinose, xylose, and ribose (Figure S-2). It was expected that three bisecting species each for arabinose, xylose, and ribose would be observed, respectively, when monitoring the transitions m/z 495.2 \rightarrow m/z 175.1, m/z 217.1, m/z 231.2. Because there can only be linkages at the 3,5-positions for arabinose and ribose in the furanose form and 3,4-positions for xylose in the pyranose form, peaks corresponding to transitions of m/z 495.2 \rightarrow m/z 175.1, m/z 231.2 were observed at retention times 6.78, 6.82, and 7.45 min for arabinose, xylose, and ribose, respectively. Additional bisecting species identified at retention times 6.55 and 6.92 min for arabinose, 6.02 and 7.00 min for xylose, and 3.88 and 5.68 min for ribose, were observed with transitions of m/z 495.2 \rightarrow m/z 175.1, m/z 217.1 indicating linkage at the 2-position. Because the two possible linkage combinations for the trisecting species containing a linkage at the 2-position are not distinguishable, these bisecting species were tentatively assigned as “2,X-” followed by the corresponding monosaccharide.

Linkage Analysis of Polysaccharides. The ability to perform linkage analysis on both soluble and insoluble branched polysaccharides is necessary to accurately characterize samples in complicated matrices such as whole food. Method validation and reproducibility were evaluated using xyloglucan (tamarind), a branched hemicellulose polysaccharide with low solubility. Using retention time information and transitions corresponding to the linkage residues listed in Table 1, the quadrupoles were operated in dMRM mode to improve signal intensities while lowering the duty cycle. A representative dMRM chromatogram for xyloglucan is shown in Figure 2A. The results obtained from the analysis qualitatively match the theoretical structure of xyloglucan.³² A total of 20 linkage residues were identified, and a list containing linkages with relative peak area abundances $\geq \sim 0.1\%$ are shown in Table 2. Standard deviation values of $\leq 0.8\%$ were determined for each linkage residue, indicating robustness and reproducibility of the method for analysis of branched insoluble polysaccharides. Abundant signals for T-P-xylose, 2-P-xylose, and T-galactose were expected and observed with relative abundances of $45.8 \pm 0.6\%$, $26.8 \pm 0.5\%$, and $12.6 \pm 0.1\%$, respectively. A signal corresponding to the coeluting T-F-arabinose/X-rhamnose peak with a relative abundance of $4.9 \pm 0.3\%$ was additionally observed. On the basis of a previous monosaccharide analysis, the composition of T-F-arabinose/X-rhamnose is solely due to the presence of T-F-arabinose monosaccharide rather than X-rhamnose.³³ Representing the backbone and branched points, 4-glucose and 4,6-glucose were observed with relative abundances of $3.5 \pm 0.8\%$ and $4.6 \pm 0.4\%$, respectively.

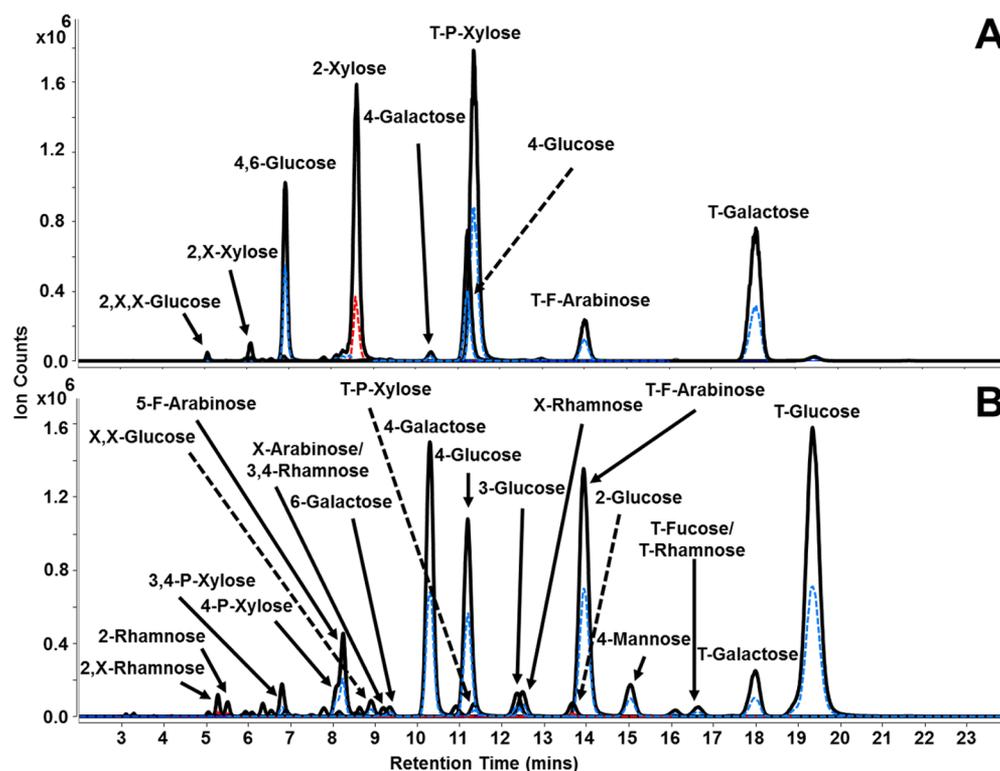


Figure 2. Representative dynamic MRM chromatograms for the linkage analysis of (A) xyloglucan and (B) whole carrot root. The solid black trace represents transitions with the quantifying ion m/z 175.1, while the dashed blue and red traces represent transitions of qualifying ions m/z 231.2 and m/z 217.1, respectively.

Table 2. Relative Peak Area Linkage Composition of Xyloglucan (Tamarind) ($n = 3$)

linkage residue	RT (mins)	relative abundance (%)
T-P-xylose	11.25	45.8 ± 0.6
2-P-xylose	8.52	26.8 ± 0.5
T-galactose	17.84	12.6 ± 0.1
T-F-arabinose	13.92	4.9 ± 0.3
4,6-glucose	6.98	4.6 ± 0.4
4-glucose	11.20	3.5 ± 0.8
2,X-P-xylose	6.02	0.4 ± 0.1
4-galactose	10.26	0.3 ± 0.0
3,4-P-xylose	6.82	0.1 ± 0.0
2,X,X-glucose	5.12	0.1 ± 0.0
2,X,X-galactose	6.41	0.1 ± 0.0
X,X-glucose	8.92	0.1 ± 0.0
2,X-F-arabinose	6.55	0.1 ± 0.0
2,X-mannose	5.15	0.1 ± 0.0
2,X-glucose/2,X-galactose	7.85	0.1 ± 0.0
2,X-glucose	8.68	0.1 ± 0.0

The overall method was used to analyze carbohydrates in a complicated matrix such as whole carrot root. In a previous GC-MS study, a total of 13 linkages were determined.¹⁷ In that study, we also described a more limited LC-MS/MS workflow which identified 24 linkages with relative abundances of $\geq 0.1\%$. The presented workflow in this study is an improvement over the previous methods facilitated by improved LC-MS/MS conditions and the use of a synthetic-based library. Figure 2B displays a representative annotated dMRM chromatogram for whole carrot root with over 39 linkages having relative abundances of $\geq 0.1\%$ (Table 3). The relative standard deviations were determined to be $\leq 3.9\%$, demon-

strating the reproducibility of the method for analysis of linkage residues derived from disaccharides, oligosaccharides, and polysaccharides existing in complicated matrices. Slightly lower relative T-glucose ($28.1 \pm 2.4\%$) and 4-glucose ($9.4 \pm 2.0\%$) abundances were observed and expected in comparison to the more limited LC-MS/MS study and is a result of the increase in the number of linkages that can be monitored.¹⁷

The linkage composition further pointed to the presence of polysaccharides other than starch and cellulose, both of which are known to be significant carbohydrate components in carrots.³⁴ For example, the identification of T-F-arabinose ($28.4 \pm 3.9\%$), 4-galactose ($12.5 \pm 0.8\%$), and T-galactose ($3.0 \pm 0.1\%$) suggested the presence of type I arabinogalactans, which typically possess a $\beta(1 \rightarrow 4)$ -galactose backbone with T-F-arabinose capping, rather than type II arabinogalactans, which consist of repeating $\beta(1 \rightarrow 3)$ -galactose monosaccharides.³⁵ Other pentoses such as 4-P-xylose ($1.5 \pm 0.8\%$), 3,4-P-xylose ($1.0 \pm 0.1\%$), and 5-F-arabinose ($5.6 \pm 0.1\%$) were also identified and suggested the additional presence of xylans and arabinans.^{36,37}

Quantitative information was obtained using this method; however, there are certain caveats that should be considered in the LC-MS platform which are also common for GC-MS. Although the PMP labels produced equal ionization between the different linkage representations of a given species, changes in the effluent compositions and/or the simultaneous elution of two or more species could have yielded competitive conditions where the signals of the different species were attenuated relative to the single eluting species. Because of the vast number of linkage possibilities, it is difficult to achieve complete isomeric separation. For example, there were three pairs of coeluting isomeric compounds unable to be chromato-

Table 3. Relative Peak Area Linkage Composition of Whole Carrot Root ($n = 3$)

linkage residue	RT (mins)	relative abundance (%)
T-F-arabinose	13.92	28.4 ± 3.9
T-glucose	19.50	28.1 ± 2.4
4-galactose	10.26	12.5 ± 0.8
4-glucose	11.20	9.4 ± 2.0
5-F-arabinose	8.18	5.6 ± 0.1
T-galactose	17.84	3.0 ± 0.1
4-mannose	15.10	1.8 ± 0.2
4-P-xylose	8.05	1.5 ± 0.8
3-glucose	12.45	1.4 ± 0.3
3,4-P-xylose	6.82	1.0 ± 0.1
T-P-xylose	11.25	0.9 ± 0.1
T-fucose/T-rhamnose	16.55	0.7 ± 0.1
X,X-glucose	8.92	0.6 ± 0.0
2-glucose	13.80	0.6 ± 0.0
X-F-arabinose/3,4-rhamnose	9.10	0.6 ± 0.1
2,X-rhamnose	5.24	0.4 ± 0.1
6-galactose	9.38	0.4 ± 0.0
2-rhamnose	5.46	0.4 ± 0.1
2,X-glucose	8.68	0.3 ± 0.0
2,X-glucose	7.85	0.2 ± 0.1
3,4,6-galactose	6.40	0.2 ± 0.1
2,X,X-glucose	6.41	0.2 ± 0.1
4,6-glucose	6.98	0.2 ± 0.0
2,X-F-arabinose	6.55	0.2 ± 0.0
X,X-galactose/X,X-mannose	8.20	0.1 ± 0.0
2-xylose	8.52	0.1 ± 0.2
6-glucose	9.14	0.1 ± 0.0
2,X,X-galactose	6.15	0.1 ± 0.0
3,4-fucose	7.44	0.1 ± 0.0
3,4,6-glucose	6.00	0.1 ± 0.1
2,X,X-glucose	5.12	0.1 ± 0.1

graphically resolved by the method: 3,6-mannose/X,X-galactose (8.20 min), X-F-arabinose/3,4-rhamnose (9.10 min), and T-fucose/T-rhamnose (16.55 min). However, a complementary monosaccharide analysis may provide additional information to accurately identify the correct associated monosaccharide. Additionally, the permethylation reaction could be affected by the availability of the polymer to the reaction. Similarly, acid hydrolysis could be affected by the secondary and tertiary structures of the polymer in the matrix. Lastly, in-depth characterization of individual carbohydrate structures existing in a complicated mixture would require isolation of the components prior to application of this analytical method.

CONCLUSIONS

In this study, a synthetic-based linkage library in addition to an improved UHPLC/QqQ MS method employing dynamic multiple reaction monitoring was developed for the accurate simultaneous profiling of 92 glycosidic linkages derived from disaccharides, oligosaccharides, and polysaccharides existing in complicated mixtures. While an extended analysis time is needed to simultaneously and accurately profile 92 linkages, the presented method provides a more substantially improved comprehensive analysis over the previous LC-MS/MS and GC-MS methods.¹⁷ Optimized partial methylation conditions were used to synthesize trisecting, bisecting, linear, and terminal species for hexoses, pentoses, deoxyhexoses, and

hexuronic acids using commercial monosaccharides as building blocks. The synthesized linkages were validated by deductive reasoning through analysis of residues derived from commercial disaccharide, oligosaccharide, and polysaccharide standards. Additional signals positively identified as synthesized glycosidic linkages were tentatively assigned for later evaluation pending the availability of commercial standards. The method's ability to accurately characterize a highly branched polysaccharide was validated by comparison to the known structure. Further validation for the ability to analyze polysaccharides within a complicated matrix was performed by comparison with our previous rapid linkage analysis method and GC-MS. Glycosidic linkages derived from fructose, N-acetylglucosamine, and N-acetylgalactosamine require a different synthetic approach, and methods for the creation of standards derived from these monosaccharides are currently being developed and will be the topic of future reports. The combined library and UHPLC/QqQ MS method developed in this work will allow for the accurate profiling and relative quantitation of linkages derived from disaccharides, oligosaccharides, and polysaccharides present in complicated matrices. Additionally, the concept of this work may provide as an orthogonal approach for the linkage profiling of N- and O-glycosylation, an analysis typically reliant on the availability of enzymes, and will be a topic of future reports.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b03101.

Effect of hydrolysis time on the deacetylation of α -D-glucose pentaacetate and the effect of acetylation time for 50 μ g of D-glucose (Figure S-1); and MRM chromatograms for bisecting species obtained by the partial methylation and PMP-labeling of arabinose, xylose, and ribose monosaccharides (Figure S-2) (PDF)

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Author Contributions

A.G.G. performed experimental design and drafted the manuscript. A.G.G. and E.N. performed all experiments and analysis of samples and produced all figures, tables, and schemes for the manuscript. J.J.C. aided in the preparation of the instrument and samples for analysis. M.J.A. and C.B.L. reviewed and edited the manuscript.

Author Contributions

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Notes

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