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A rapid-throughput adaptable method for determining the monosaccharide composition of polysaccharides

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ABSTRACT

Polysaccharides make up the largest non-water component of plant-based foods. Their ability to manipulate the gut microbiome and modulate the immune system has increased interest in the rapid elucidation of their structures. A necessary component for the structural characterization of polysaccharides is the determination of their monosaccharide composition. Current methods of monosaccharide analysis are not suitable for analyzing large sample-sets and are limited by their inability to analyze polysaccharides. We have developed a 96-well plate hydrolysis and derivatization procedure followed by a rapid and sensitive 10-min ultra-high performance liquid chromatography triple quadrupole mass spectrometry analysis capable of the absolute quantitation of 14 plant monosaccharides. Four polysaccharide standards, inulin, xyloglucan, arabinogalactan, and rhamnogalacturonan-I, which are commonly found in plants, were used to optimize and validate the method. The optimized conditions were applied to eight foods to show the method's reproducibility and ability to analyze complicated and insoluble polysaccharide mixtures. This approach will allow researchers to obtain accurate and absolute quantitation of monosaccharides in the large sample-sets that are required for agricultural, food, clinical, and nutrition-based studies.

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1. Introduction

Carbohydrates have been shown to be involved in many biological processes and are ubiquitous across all taxa. As food, carbohydrates can have strain-specific prebiotic properties [1–3], antioxidant activities [4,5], and immune modulating effects [6,7]. These activities are generally related to their fundamental structure. Despite the importance of carbohydrates, the current tools to characterize their structures lack speed, sensitivity, specificity, and reproducibility. For these reasons, many substances containing carbohydrates, specifically food, are not well characterized. Polysaccharides are highly diverse in structure, as they can be composed of monosaccharides that differ in stereochemistry, can be linked through several different hydroxyl groups in both α and β configurations, can be highly branched, and may be composed of polymers consisting of over 100,000 covalently linked monosaccharide residues [8,9].

The structural elucidation of any carbohydrate is largely dependent on determination of its monosaccharide constituents. Analysis of intact large polysaccharides has often yielded little structural information, while characterizing their monosaccharide compositions in the presence of complicated biological matrices has been cumbersome and subsequently ignored. The accurate quantification of the monosaccharide constituents of polysaccharides requires extensive depolymerization of the polysaccharide, chromatography capable of separating many isomers, and an analytical platform that can analyze non UV-active monosaccharides. Current methods for the analysis of monosaccharides have focused on broadening the diversity of the monosaccharides capable of

Abbreviations: GC, gas chromatography; MS, mass spectrometry; FID, flame ionization detector; HPAEC/PAD, high performance ion exchange chromatography pulsed amperometric detection; UHPLC/QqQ MS, ultra-high performance liquid chromatography triple quadrapole mass spectrometry; HPLC, high performance liquid chromatography; UV-Vis, ultraviolet- visible detection; PMP, 1-phenyl-3-methyl-5-pyrazolone; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Glc A, glucuronic acid; Gal A, galacturonic acid; TFA, trifluoroacetic acid; MRM, multiple reaction monitoring.

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being analyzed, while increasing the sensitivity and speed of analysis [10–16]. However, these methods are focused primarily on monosaccharides in their free form, not those that are depolymerized from polysaccharides.

To obtain the accurate monosaccharide composition of polysaccharides, the polymers must first be depolymerized in a manner that minimizes degradation of the nascent monosaccharides. There are several methods for liberating monosaccharides from polysaccharides including methanolysis, enzymatic hydrolysis, and acid hydrolysis. Willför et al. compared these methods in an inter-laboratory study and showed that acid hydrolysis released the highest concentration of neutral monosaccharides, while methanolysis prevented acidic monosaccharides from degrading [17]. Alternatively, enzymatic hydrolysis has been used in situations where easily degraded monosaccharides, such as sialic acids, needed to be liberated for accurate quantitation [18]. Although acid hydrolysis is the most effective and commonly used method, it requires a delicate balance of liberating monosaccharides without degrading them further. The effects of acid hydrolysis on several polysaccharides have been investigated by Wang et al., who concluded that each polysaccharide required different optimized hydrolysis conditions [19].

Chromatographic separation of monosaccharides has also improved considerably. Traditional methods for monosaccharide analysis includes gas chromatography (GC) paired with mass spectrometry (MS), which is the gold standard for this analysis, or flame ionization detection (FID). However, these methods rely heavily on volatility, which requires extensive derivatization techniques [10,12,17,20]. High-performance anion exchange chromatography paired with pulsed amperometric detection (HPAEC/PAD) has also been used for separating and detecting monosaccharides without derivatization. However, these methods suffer similarly from long run times, typically 45–90 min [14,17,21,22]. More recently, reversed phase HPLC techniques with ultraviolet-visible detection (UV–vis) have also been used to quantitate monosaccharides but require complete baseline separation between monosaccharides. These reverse-phase HPLC approaches require reducing end derivatization with a chromophore, such as 2-aminobenzene or 1-phenyl-3-methyl-5-pyrazolone (PMP), because monosaccharides typically do not exhibit UV activity [11,13,15]. Recently, methods employing PMP have been expanded further to utilize HPLC and ultra-high performance liquid chromatography (UHPLC) by coupling them to various mass spectrometers for detection and quantitation [23,24]. More recently, we showed that UHPLC/QqQ MS is useful for quantitating a large number of free monosaccharides in the sub-femtomole levels [16].

Despite the newer LC/MS-based methods for monosaccharide analysis, the analysis of polysaccharides remains time-consuming, relatively insensitive, and not highly reproducible. In this study, we have developed a comprehensive method for characterizing the monosaccharide composition of plant-based polysaccharides, specifically those found in food. The method involves a rapid-throughput 96-well plate hydrolysis, derivatization, and extraction workflow. Chromatographic separation and analysis are performed within a 10-min run using UHPLC/QqQ MS operated in dynamic multiple reaction monitoring mode (dMRM). The acid hydrolysis conditions were optimized with polysaccharide standards to efficiently liberate monosaccharides from polysaccharides, while minimizing degradation. This method represents the first 96-well plate-based monosaccharide analysis and is capable of preparing and analyzing over 100 samples in less than two days without sacrificing sensitivity and reproducibility. The effectiveness of our approach is demonstrated through the quantification of the polysaccharide-derived monosaccharides from eight common foods. These attributes make the prospect of using monosaccharide analysis as a tool in nutrition-based clinical trials and rapid screenings of agricultural products possible for the first time. Ultimately, this work will provide rapid, sensitive, and accurate quantitation of the monosaccharide components of polysaccharides, further enhancing our fundamental understanding of carbohydrate structures and their biological functions.

2. Experimental methods

2.1. Materials

D-galactose, D-mannose, D-glucose, D-allose, D-fructose, L-arabinose, D-ribose, D-xylose, L-fucose, L-rhamnose, *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), D-glucuronic acid (Glc A), D-galacturonic acid (Gal A), and 1-phenyl-3-methyl-5-pyrazolone (PMP), chloroform (HPLC grade), methanol (HPLC grade), and anthrone were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Honeywell (Muskegon, MI). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Geel, Belgium). Arabinogalactan (larch wood), inulin (chicory), xyloglucan (tamarind) and rhamnogalacturonan-I (potato) were purchased from Megazyme (Chicago, IL). Sulfuric acid was purchased from EM Science (Burlington, MA). Food samples were purchased from a local grocery store. Nano-pure water was used for all experiments.

2.2. Food and polysaccharide preparation

For the analysis of food, samples were lyophilized to complete dryness and ground to a fine powder in a KRUPS F203 Grinder (Millville, NJ). Solutions of 10 mg/ml were prepared and blended with 1.4 mm stainless steel beads in a Storm 24 Bullet Blender made by Next Advance (Averill Park, NY). The samples were heated for 1 h at 100 °C, and an aliquot was transferred to a 96-well plate for hydrolysis. For the analysis of purified polysaccharide standards, rhamnogalacturonan-I, xyloglucan, inulin, and arabinogalactan solutions were prepared at a concentration of 1.0 mg/ml and an aliquot was transferred to a 96-well plate for hydrolysis. Food samples were prepared in six replicates and purified polysaccharides standards were prepared in triplicate. Standard curve solutions containing 14 monosaccharides (fructose, allose, mannose, glucose, galactose, xylose, arabinose, ribose, fucose, rhamnose, glucuronic acid, galacturonic acid, GlcNAc, and GalNAc) were prepared at concentrations ranging from 1 ng/ml to 100 μg/ml.

2.3. Hydrolysis and derivatization

The food and polysaccharide standard solutions underwent acid hydrolysis with TFA. Upon completion of hydrolysis, samples were quenched with cold water. Conditions for PMP derivatization were modified from the method presented by Xu et al. [16] to be more amenable to a 96-well plate format. Briefly, aliquots of food, polysaccharide standards, and monosaccharide standard curves were added directly to polypropylene 96-well plates containing NH_4OH and PMP in methanol and incubated at 70 °C. Samples were then dried to completion by vacuum centrifugation and reconstituted in water. Two chloroform-water extractions were performed to remove excess PMP. The aqueous layer was collected and stored at −20 °C until the time of analysis.

2.4. Anthrone assay

The anthrone assay was modified to analyze microliter volumes from the method presented by Dreywood et al. [25] A 2 mg/ml solution of anthrone reagent in concentrated sulfuric acid was prepared. In 1.5 ml screw cap polypropylene tubes, 20 μl of the anthrone

solution was mixed with 10 μl of 1 mg/ml of each food sample. Amylopectin standards ranging from 0.1 to 1.0 mg/ml was prepared to construct a standard curve. The samples and the standards were vortexed before centrifugation, and incubated in a water bath at 100 °C for 10 min. The samples were vortexed and centrifuged for 30 s. A 1 μl aliquot was analyzed using an IMPLEN NanoPhotometer P300 in single wavelength mode set to 620 nm using a 1 mm pathlength.

2.5. Instrumental analysis

Samples were analyzed on an Agilent 6495 QqQ MS coupled to an Agilent 1290 Infinity II UHPLC. Separation was performed on an Agilent C18 column. Chromatographic solvents consisted of A: 25 mM ammonium acetate adjusted to pH 8.2 using NH_4OH in 5% acetonitrile in water (v/v) and B: 95% acetonitrile in water (v/v). The mass spectrometer was operated in positive ion mode, and dynamic multiple reaction monitoring (dMRM) mode was used for data acquisition. All other UHPLC and MS parameters were applied as described by Xu et al. [16] Data analysis was performed on the Agilent Mass Hunter B.08 quantitative analysis software and peaks were automatically integrated. To account for the addition of water during hydrolysis, the concentration of each monosaccharide was multiplied by a correction factor that is derived from the ratio of the monosaccharide residue molar mass to the monosaccharide molar mass. If free monosaccharides are analyzed without hydrolysis, this correction factor should not be used.

$$\textit{Bound Monosaccharide Concentration} = \textit{Free Monosaccharide Concentration} \times \frac{\textit{Monosaccharide Molar Mass}\ (g/mol) - 18\ (g/mol)}{\textit{Monosaccharide Molar Mass}\ (g/mol)}$$

3. Results and discussion

3.1. Overview of method

To accurately and precisely measure the monosaccharide constituents of food and plant products, solubilization and homogenization are necessary. Due to many polysaccharides having low solubility and others that have gelling properties, we first ground the dry samples to a fine powder before further homogenization with stainless steel beads. The samples were then heated in water to solubilize the remaining insoluble material further. An aliquot from the mixture was then transferred to a 96-well plate for the subsequent steps of the reaction. It should be noted that despite this rigorous pretreatment, sampling the heterogeneous solution may have still presented the largest source of error in the analysis.

In 96-well plates, samples were reacted with TFA at elevated temperatures to hydrolyze the glycosidic bonds. TFA was chosen because it can be easily removed by evaporation upon completion of hydrolysis. Evaporation was the preferred method of neutralization because the addition of a base would have produced salts that could further complicate the mass spectrometry analysis. Samples were derivatized with PMP to achieve better chromatographic separation with reverse phase liquid chromatography and to increase the ionization efficiency of the monosaccharides further. To remove the unreacted PMP and other hydrophobic material that may compete for ionization, samples were extracted with chloroform and water.

Samples were separated by reverse phase chromatography with a UHPLC method. The method achieved baseline separation of monosaccharide isomers in a rapid, 10-min run. The short run allows a 96-well plate to be completed in 17 h, thereby illustrating the rapid-throughput potential of this method. The mass spectrometer was run in dynamic multiple reaction monitoring (dMRM) mode to provide high selectivity and sensitivity. The precursor mass was selected in the first quadrupole, and two fragment ions were monitored in the third quadrupole. The quantifier ion, 175.1 *m/z*, corresponds to the mass of the PMP residue, while the qualifier, 217.2 *m/z*, is formed by cleavage of the monosaccharide C2-C3 bond and one of the C1-PMP bonds. Lastly, the peaks in the chromatogram were integrated, and the absolute concentrations were obtained using a standard curve. To ensure reproducible analysis over long runs, a quality control consisting of a 10 μg/ml standard pool was run every twelve samples. To show the reproducibility, eight quality controls were run over a 24-hour run that consisted of 96 unique samples. The relative standard deviation of the quality controls was 3.5% and suggests that the instrument was highly stable. The annotated dMRM chromatograms of the eight quality controls and the relative standard deviation of each monosaccharide are shown in Fig. 1. Each MRM transition contained several isomers, all of which were baseline resolved in just seven minutes.

3.2. Optimization of hydrolysis conditions

To deduce the optimal hydrolysis conditions, polysaccharide standards including arabinogalactan, inulin, xyloglucan, and rhamnogalacturonan-I were selected for optimization. These compounds are commonly found in food and provide a diverse combination of monosaccharide compositions, solubility, and rheological properties that broaden the utility of the method. To deduce the optimum temperature for acid hydrolysis, the polysaccharides were hydrolyzed at 75 °C, 100 °C, and 121 °C for two hours. When hydrolyzed at 100 °C, the highest concentration of monosaccharides were produced (Fig. 2). At 75 °C, lower concentrations of monosaccharides were observed, which indicated that the temperature was not sufficient to fully release all of the monosaccharides from the parent polysaccharides. Lower monosaccharide concentrations were also observed when hydrolyzed at 121 °C, which suggests that the conditions were too harsh and caused the released monosaccharides to degrade further, likely through caramelization and Maillard-type reactions. [26] For arabinogalactan, the highest yield of galactose (0.46 mg/mg) was obtained when hydrolyzed at 100 °C, and all three temperatures generated similar concentrations of arabinose (0.09 mg/mg). Inulin hydrolyzed at 100 °C yielded higher concentrations of fructose than the other two temperatures (0.07 mg/mg at 75 °C, 0.89 mg/mg at 100 °C and 0.50 mg/mg at 121 °C). A small amount of glucose was also detected in inulin with 100 °C, which generated the highest concentration (0.037 mg/mg at 75 °C, 0.042 mg/mg at 100 °C and 0.039 mg/mg at 121 °C). For xyloglucan, hydrolysis at 100 °C yielded the highest concentration of glucose (0.26 mg/mL). However, slightly higher concentrations of galactose and xylose were generated by hydrolysis at 121 °C (0.091 mg/mg at 100 °C compared to 0.099 mg/mg at 121 °C for galactose and 0.14 mg/mg compared to 0.15 mg/mg for xylose). For rhamnogalacturonan-I, hydrolysis at 121 °C generated slightly higher concentrations of galactose than at 100 °C (0.19 mg/mg compared to 0.11 mg/mg). Higher concentrations of rhamnose and galacturonic acid were produced by hydrolysis at 100 °C than 121 °C (0.042 mg/mg compared to 0.018 mg/mg for rhamnose, and 0.092 mg/mg compared to 0.050 mg/ml for galacturonic acid).

To deduce the optimum time for hydrolysis, the four polysaccharides were treated with TFA and incubated at 100 °C for times ranging from 0 to 240 min (Fig. 3). All four polysaccharides showed similar behavior; the monosaccharides increased in concentration as time increased before decreasing at later times. Inulin was the least stable polysaccharide with fructose concentrations peaking at 1.0 mg/mg at just 30 min, before dropping

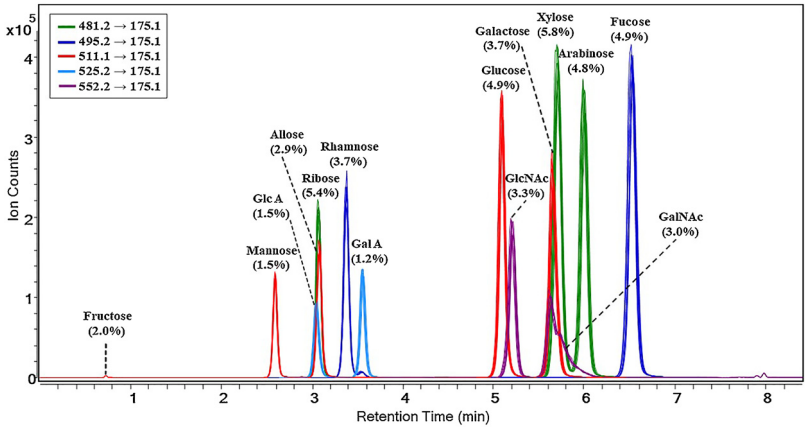

Fig. 1. The dynamic multiple reaction monitoring chromatograms of 14 monosaccharides in eight quality controls run every twelve samples during a 24-h run. The relative standard deviation of each monosaccharide is shown. Each transition is shown in a different color.

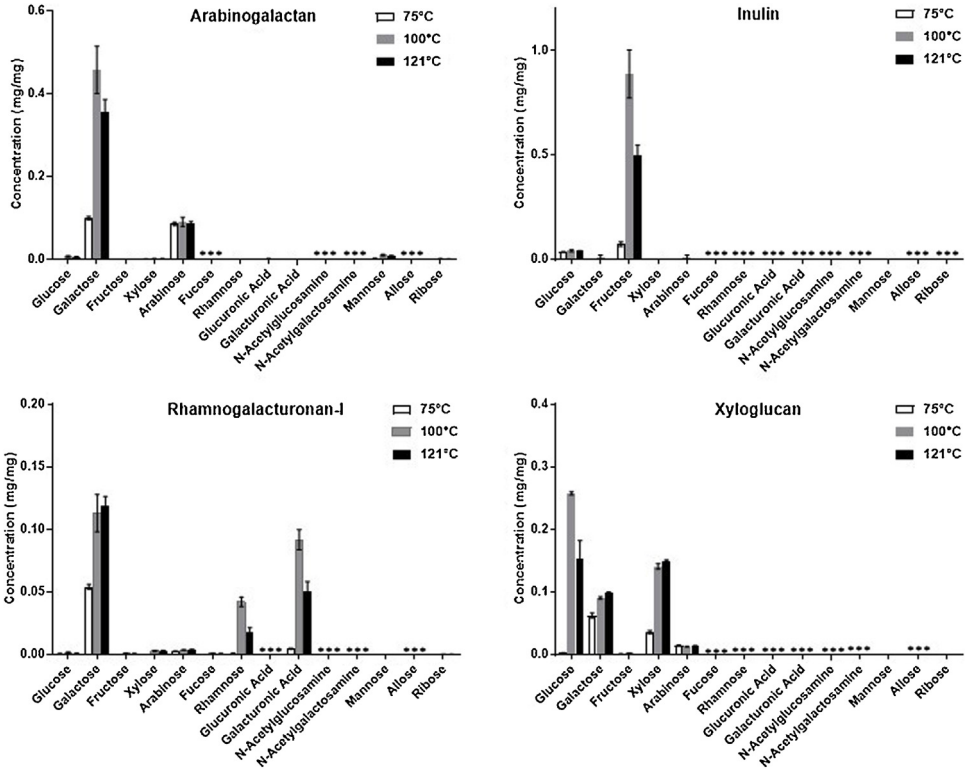

Fig. 2. Absolute monosaccharide composition of arabinogalactan, inulin, rhamnogalacturonan-I, and xyloglucan hydrolyzed at 75 °C, 100 °C, and 121 °C. Data are presented as the average ± standard deviation ($n = 3$). (*) Denotes values that are below the limit of quantitation (LOQ).

to 0.025 mg/mg at 150 min. The monosaccharide concentrations of arabinogalactan and xyloglucan peaked at 90 min of hydrolysis and only degraded slightly thereafter. Rhamnogalacturonan-I yielded the highest concentrations of monosaccharides at 120 min of hydrolysis. From a mechanistic point of view, the acid hydrolysis appears to first trim the more accessible branched groups before hydrolyzing the backbone. This phenomenon is most apparent in xyloglucan, and rhamnogalacturonan-I. In xyloglucan, the galactose and xylose groups increased to a maximum concentration at 90 min of hydrolysis, while the glucose initially lagged before it increased in abundance over the entire hydrolysis period. For rhamnogalacturonan-I, the galactose branches were first hydrolyzed before the alternating rhamnose and galacturonic acid backbone was depolymerized to reach a maximum at 150 min. In each case, the standard deviations remained low during the initial release of monosaccharides but increased as the released monosaccharides degraded. Each monosaccharide and linkage type have unique acid reactivity and stability, making a single hydrolysis condition inadequate for characterizing the complete monosaccharide composition. When highly selective hydrolysis is needed, different time points may need to be analyzed. For all but inulin, two hours appeared adequate for optimum hydrolysis and was thus applied to the analysis of food polysaccharides.

3.3. Validation of the method on known polysaccharides

The method was tested by comparing our monosaccharide analysis of rhamnogalacturonan-I, xyloglucan, arabinogalactan, and inulin to that provided by the manufacturer (Megazyme). It is worth noting that Megazyme used a GC-FID method to determine the monosaccharide composition, which is incapable of simultaneously measuring fructose, neutral monosaccharides, and

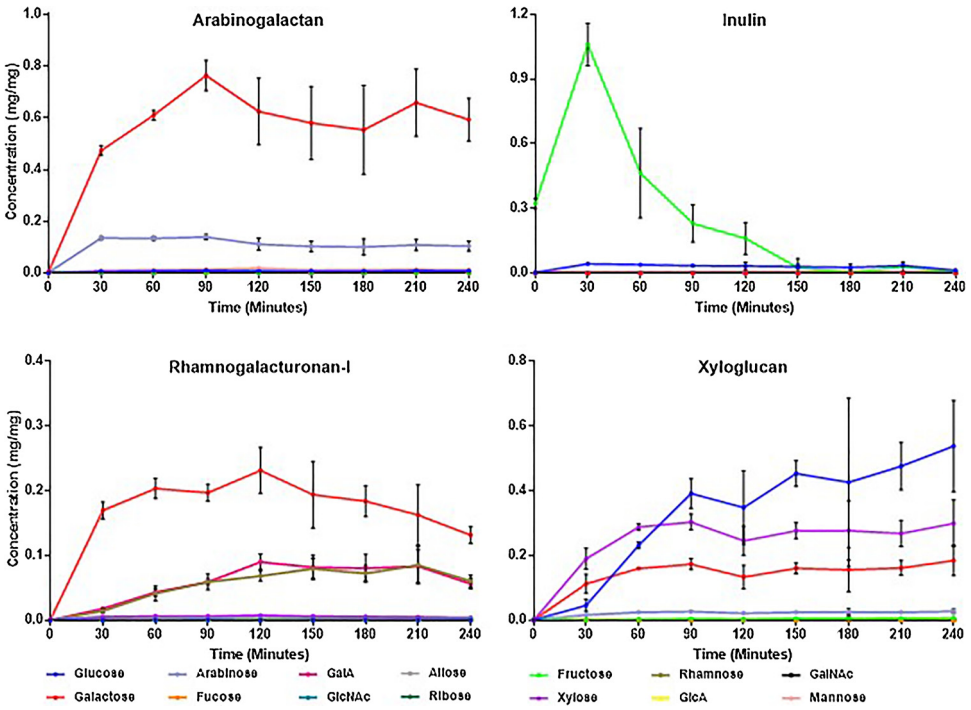

Fig. 3. Monosaccharide liberation and degradation trends of arabinogalactan, inulin, rhamnogalacturonan-I, and xyloglucan hydrolyzed for 0–240 min. Error bars represent standard deviation ($n = 3$).

hexuronic acids [17,19]. In general, the monosaccharide compositions matched very well with the manufacturer supplied values. Both analyses of xyloglucan yielded almost identical compositions. Our method showed 50.9% glucose (versus 49% stated by the manufacturer), 17.9% galactose (17%) and 27.8% xylose (31%). Both results are consistent with the known structure of xyloglucan [27]. Arabinogalactan similarly yielded highly consistent values with 79.6% galactose (versus 81%) and 15.8% (14%) arabinose. For rhamnogalacturonan-I, the UHPLC/QqQ MS method showed a lower relative amount of galacturonic acid, 35.6% (versus 60%). This difference could be attributed to the difficulty of using the GC-FID method to measure hexuronic acids, which may not have been included in their analysis. Lastly, inulin was determined to have 93.7% fructose and 4.5% glucose. The manufacturer did not provide a monosaccharide composition for this polysaccharide, likely due to the difficulty in analyzing fructose by GC. Nonetheless, the values obtained here are in accordance with the known structure of inulin [28].

3.4. Monosaccharide stability under optimized conditions

The stability of monosaccharides under acidic conditions was explored to determine the extent of degradation under the acid hydrolysis conditions. All of the monosaccharide standards (glucose, galactose, mannose, allose, xylose, arabinose, ribose, fucose, rhamnose, fructose, glucuronic acid, galacturonic acid, GlcNAc, and GalNAc) were subjected to hydrolysis conditions in triplicate for two hours at 100 °C and compared to triplicate nascent monosaccharide standards as their control.

The extent of monosaccharide degradation is shown in Fig. 4. The results suggest that the neutral monosaccharides exhibit higher resistance to acid than the acidic or N-acetylated monosaccharides. Fructose showed almost no observable level of degradation. Of the neutral aldoses, hexoses showed the least amount of degradation (17.7–31.8%), while pentoses showed slightly more degradation (22.8–30.7%), and deoxyhexoses showed greater degradation with 24.0% for fucose and 28.1% for rhamnose. The acidic sugars, glucuronic and galacturonic acid degraded at vastly different rates, 49.0%, and 28.6%, respectively. For the N-acetylated sugars, N-acetylgalactosamine underwent the highest level of degradation (79.9%), with N-acetylglucosamine degrading 45.7%. Interestingly, glucose and galactose had similar acid reactivity, while galacturonic acid was much more stable than glucuronic acid, and N-acetylglucosamine was more stable than N-acetylgalactosamine. Despite the variation in reaction conditions, similar trends were observed previously by Wang et al. [19].

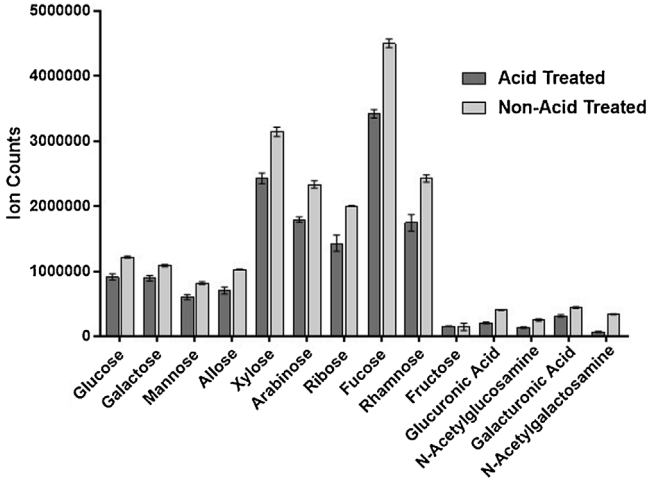

Fig. 4. Comparison of 14 monosaccharides that underwent incubation with TFA for 2 h at 100 °C and those that did not undergo acid treatment. Data are presented in average ± standard deviation ($n = 3$).

Interestingly, the stabilities of the monosaccharide standards under hydrolysis conditions did not always equate to increased abundances after acid hydrolysis. The most extreme example is fructose and inulin. Under acidic conditions, fructose is relatively stable (Fig. 4). However, under acidic conditions, inulin produced fructose that decreased over time (Fig. 3), suggest-

Table 1
Absolute quantitation of 14 monosaccharides in eight foods by dry mass %. Data are presented as the average ± standard deviation (n = 6).

Monosaccharide	Red Rice Conc. (%)	Jasmine Rice Conc. (%)	Soy Flour Conc. (%)	Rye Flour Conc. (%)	Sweet Potato Conc. (%)	Cucumber Conc. (%)	Blood Orange Conc. (%)	Blood Orange Peel Conc. (%)
Glucose	77.7 ± 4.6	86.2 ± 16.8	11.4 ± 0.7	39.4 ± 8.2	67.0 ± 5.2	19.3 ± 2.6	29.1 ± 2.5	25.9 ± 3.1
Galactose	0.8 ± 0.1	0.7 ± 0.1	8.2 ± 0.6	1.0 ± 0.1	1.5 ± 0.2	2.1 ± 0.2	1.5 ± 0.3	2.3 ± 0.3
Fructose	10.1 ± 1.0	9.9 ± 0.64	18.0 ± 1.0	16.9 ± 1.6	27.6 ± 2.3	49.3 ± 7.5	52.6 ± 9.0	35.2 ± 4.7
Xylose	1.0 ± 0.2	0.3 ± 0.04	1.8 ± 0.3	12.0 ± 4.3	0.4 ± 0.2	1.5 ± 0.2	0.5 ± 0.1	1.6 ± 0.3
Arabinose	1.4 ± 0.3	0.4 ± 0.1	7.0 ± 1.1	8.8 ± 1.6	1.7 ± 0.2	2.4 ± 0.4	3.6 ± 0.5	9.0 ± 2.9
Fucose	0.03 ± 0.004	0.01 ± 0.0005	0.7 ± 0.1	0.05 ± 0.01	0.03 ± 0.005	0.3 ± 0.04	0.2 ± 0.01	0.4 ± 0.08
Rhamnose	0.02 ± 0.004	ND —	0.6 ± 0.06	0.03 ± 0.004	0.2 ± 0.02	0.2 ± 0.03	1.4 ± 0.1	3.7 ± 0.7
Glucuronic Acid	ND —	ND —	0.14 ± 0.011	ND —	ND —	0.01 ± 0.005	0.01 ± 0.005	0.02 ± 0.002
Galacturonic Acid	ND —	ND —	0.08 ± 0.005	ND —	0.03 ± 0.007	0.1 ± 0.01	0.04 ± 0.02	0.1 ± 0.01
GlcNAc	0.02 ± 0.004	0.01 ± 0.005	0.08 ± 0.006	0.02 ± 0.004	ND —	0.04 ± 0.009	0.02 ± 0.005	0.03 ± 0.009
GalNAc	ND —	ND —	ND —	ND —	ND —	0.02 ± 0.006	0.01 ± 0.004	ND —
Mannose	0.08 ± 0.005	0.09 ± 0.01	1.0 ± 0.2	0.5 ± 0.05	0.09 ± 0.01	0.6 ± 0.07	0.2 ± 0.03	0.4 ± 0.06
Allose	ND —	ND —	ND —	ND —	ND —	0.02 ± 0.005	ND —	ND —
Ribose	0.1 ± 0.005	0.1 ± 0.008	0.4 ± 0.04	0.09 ± 0.007	0.06 ± 0.009	0.2 ± 0.04	0.05 ± 0.005	0.06 ± 0.01
Total	91.3 ± 4.5	97.7 ± 17.4	49.3 ± 3.6	78.8 ± 10.9	98.6 ± 7.6	76.1 ± 10.9	89.3 ± 10.2	78.8 ± 11.0

ing that the degradation of inulin under acidic conditions may not have necessarily produced only fructose but possibly other non-monosaccharide side products. The other monosaccharides appeared to behave more consistently. The galactose standard was found to degrade by 17.7% in a two-hour period, while in xyloglucan, rhamnogalacturonan-I, and arabinogalactan the average loss due to degradation was 20.3%. Galactose in arabinogalactan showed the highest rate of release at 30 min of hydrolysis and a monosaccharide loss of 37.9% over the subsequent two-hours. In xyloglucan, the galactose maximum was reached at two-hours, and 18.1% degradation was observed after an additional two hours of hydrolysis. Moreover, in rhamnogalacturonan-I, galactose concentrations were stable from 30 to 120 min and only degraded by 4.8%. Similar trends of released monosaccharides being stabilized by complex mixtures were observed in glucose, arabinose, xylose, and galacturonic acid. As most monosaccharides were more stable in mixtures than independently, we chose not to hydrolyze our standard curves because this would have overestimated the monosaccharide concentrations in our samples.

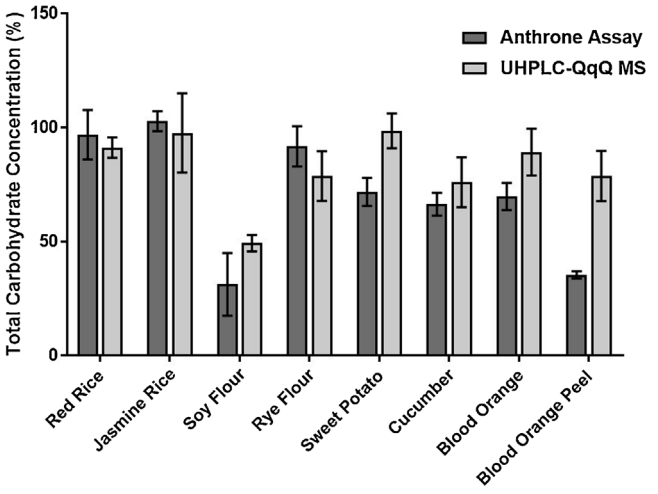

Fig. 5. Comparison of UHPLC-QqQ dMRM analysis ($n = 6$) and the anthrone assay ($n = 3$) for the total carbohydrate concentration of eight foods. Data are presented as the average ± standard deviation.

3.5. Analysis of food polysaccharides

The method was used to examine a small group of common foods. Polysaccharides often have varying levels of solubility, which may complicate analysis and affect reproducibility. To determine whether the method was capable of accurately quantifying the monosaccharides in complex matrices, eight structurally different foods were chosen and analyzed in six replicates. The foods selected included grains, starches, legumes, fruits, and vegetables. Food samples were treated with a series of bead blasting and heating steps to increase solubility and disaggregate the polysaccharides. This appeared to increase the solubility and homogeneity, however, the samples were still not completely solubilized. The results are summarized in Table 1. Red and jasmine rice contained primarily glucose, 77.7% and 86.2% by dry mass, respectively, and fructose, 10.1%, and 9.9%. Another grain, rye, showed a much more diverse composition corresponding to 39.4% glucose, 16.9% fructose, 12.0% xylose, and 8.8% arabinose. Sweet potato was found to be composed almost entirely of glucose (67.0%) and fructose (27.6%). Defatted soy flour was observed to have the most diversity with at least five abundant monosaccharides including fructose (18.0%), glucose (11.4%), galactose (8.23%), arabinose (7.01%), and xylose (1.76%).

Blood orange flesh and peel were analyzed separately to show that this method is capable of determining differences in the carbohydrate biogeography of the plant. The carbohydrate composition of the flesh and skin consisted largely of fructose (52.6% and 35.2%, respectively) and glucose (29.1% and 25.9%). The flesh had lower amounts of rhamnose than the skin 1.4% and 3.6%), arabinose (3.64%, and 9.02%), and xylose (0.5% and 1.6%). Cucumber contained fructose (49.3%) and glucose (19.3%). Galactose, xylose, and arabinose were minor components, 1.5%, 1.5%, and 2.4%, respectively.

To validate the hydrolysis and subsequent analysis of monosaccharides in complicated food matrices, we compared the total carbohydrate concentration determined with the UHPLC-MS analysis to that obtained using the anthrone assay (Fig. 5). The anthrone assay is a standard method used for absolute carbohydrate quantitation. First, concentrated sulfuric acid converts the monosaccharides into 5-(hydroxymethyl)furan-2-carbaldehydes, which are subsequently derivatized with anthrone for observation by UV–vis spectroscopy [25,29,30].

We probed the specificity of the anthrone assay towards different monosaccharides and found that they can give vastly different responses as shown in Supplemental Fig. 1. Glucose gave the best response while both hexuronic acids and N-acetylated monosaccharides provided little to no response. Similar results have been described previously [31,32]. Despite the known limitations, the anthrone assay is still frequently used [33,34]. The UHPLC/QqQ method gave slightly lower values for glucose-rich foods: red rice (91.3% vs. 96.3% for anthrone), jasmine rice (97.7% vs. 102%), and rye flour (78.8% vs. 91.8%). For foods with more diverse monosaccharide compositions, our UHPLC/QqQ monosaccharide analysis gave higher values: soy flour (49.3% vs. 31.3% for anthrone), sweet

potato (98.6% vs. 71.9%), cucumber (76.1% vs. 66.5%), blood orange (89.3% vs. 69.9%), and blood orange peel (78.8% vs. 35.4%). These results suggest that the UHPLC/QqQ method can provide accurate absolute carbohydrate concentration of foods when the individual monosaccharide components are summed together.

4. Conclusions

The UHPLC/QqQ MS method for the monosaccharide analysis of polysaccharides is a valuable new method for carbohydrate analysis. The method is well suited for rapid-throughput implementation with sample preparation being done in a 96-well format. A 10-min analysis allows for over 140 samples to be analyzed in less than 24 h. Validation was performed by comparing the UHPLC/QqQ MS analysis to the known structures of several purified polysaccharides with diverse structures that encompass traditionally difficult monosaccharides, such as fructose and galacturonic acid. Despite carbohydrates being the largest component of most plant-based foods, our understanding of their structures is rudimentary. Acid hydrolysis paired with monosaccharide analysis provides a general method that can be used for rapidly characterizing the polysaccharides in food products. The optimized hydrolysis conditions were applied to eight different foods, and their monosaccharide compositions were quantified. The method will allow monosaccharide analysis to become a standard analytical tool capable of rapidly analyzing many samples. In part, this method addresses growing requests by the microbiome community for the development of analytical methods that can better characterize and classify dietary fibers [35–38].

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijms.2018.12.009.

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