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Authors

Couture, Garret

Luthria, Devanand

Chen, Ye

et al.

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Multi-glycomic characterization of fiber from AOAC methods defines the carbohydrate structures

Garret Couture[1,2], **Devanand L. Luthria**[3], **Ye Chen**[1,2], **Nikita P. Bacalzo Jr.**[1,2], **Fakir S. Tareq**[3], **James Harnly**[3], **Katherine M. Phillips**[4], **Pamela R. Pehrsson**[3], **Kyle McKillop**[3], **Naomi K. Fukagawa**[3], **Carlito B. Lebrilla**[1,2]

[1]Department of Chemistry, University of California Davis, Davis, CA 95616, USA

[2]Foods for Health Institute, University of California Davis, Davis, CA 95616, USA

[3]USDA Agricultural Research Service, Beltsville Human Nutrition Research Center, Beltsville, MD 20705, USA

[4]Department of Biochemistry, Virginia Tech, Blacksburg, Virginia, United States of America

Abstract

Dietary fiber has long been known to be an essential component of a healthy diet and recent investigations into the gut microbiome-health paradigm have identified fiber as a prime determinant in this interaction. Further, fiber is now known to impact the gut microbiome in a structure-specific manner, conferring differential bioactivities to these specific structures. However, current analytical methods for food carbohydrate analysis do not capture this important structural information. To address this need, we utilized rapid-throughput LC-MS methods to develop a novel analytical pipeline to determine the structural composition of soluble and insoluble fiber fractions from two AOAC methods (991.43 and 2017.16) at the total monosaccharide, glycosidic linkage, and free saccharide level. Two foods were chosen for this proof-of-concept study: oats and potato starch. For oats, both AOAC methods gave similar results. Insoluble fiber was found to be comprised of linkages corresponding to β-glucan, arabinoxylan, xyloglucan, and mannan while soluble fiber was found to be mostly β-glucan with small amounts of arabinogalactan. For raw potato starch, each AOAC method gave markedly different results in the soluble fiber fractions. These observed differences are attributable to the resistant starch content of potato starch and the different starch digestion conditions used in each method. Together these tools are a means to obtain the complex structures present within dietary fiber

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Author Contributions: G.C. wrote the manuscript. G.C. and D.L.L. performed all experiments and produced all figures and tables. Y.C., N.P.B., and F.S.T. aided in performing experiments. J.H., K.M.P., P.R.P., K.M., N.K.F., and C.B.L. aided in experimental planning and edited the manuscript.

Conflicts of Interest: The authors declare no competing interests.

Supplementary Materials: The Supplementary Materials include Table S1 which contains the total monosaccharide compositions of the raw oat, its dietary fiber fractions, and the arabinoxylan quality control sample run along with the oat samples. Supplementary Table S2 contains the glycosidic linkage profiles of raw oats, its dietary fiber fractions, and the arabinoxylan quality control samples run along with the oat samples. Supplementary Table S3 contains the free saccharide compositions of raw oats and its dietary fiber fractions. Supplementary Table S4 contains the total monosaccharide compositions of the potato starch, its dietary fiber fractions, and the arabinoxylan quality control sample run along with the samples. Supplementary Table S5 contains the glycosidic linkage profiles of potato starch, its dietary fiber fractions, and the arabinoxylan quality control samples run along with the samples. Supplementary Table S6 contains the free saccharide compositions of potato starch and its dietary fiber fractions. Supplementary Figure 1 illustrates the differences in total glucose and glucose linkages between IDF from oat analyzed by each AOAC method 991.43 and 2017.16.

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while retaining “classical” determinations such as soluble and insoluble fiber. These efforts will provide an analytical framework to connect gravimetric fiber determinations with their constituent structures to better inform gut microbiome and clinical nutrition studies.

Keywords

Dietary Fiber; Carbohydrates; Liquid Chromatography; Mass Spectrometry; Gut Microbiome; Nutrition; Food Chemistry

INTRODUCTION

The term “dietary fiber” was first introduced in the 1950s to define the portion of our diets that we cannot digest; namely cellulose, hemicellulose, and lignin.[1] This definition was iteratively refined through the 1970s as fiber’s purported health benefits were postulated and explored.[2–5] In 2009, the World Health Organization and Codex Alimentarius arrived at an official definition to harmonize nomenclature efforts and health claims. As the definitions of dietary fiber evolved, so too did the analytical methods for measuring it. The determination of dietary fiber in food first began by applying methods for crude fiber analysis in animal feeds.[6] These methods were then refined by the addition of digestive enzymes to measure digestible components such as starch separately from dietary fiber.[7] Further expansion resulted in the delineation of soluble and insoluble fiber components as well as lignin.[8] Since the 1970s, these analytical methodologies continued to be refined and harmonized into the methods defined by the Association of Official Analytical Chemists (AOAC) most commonly employed today. In 2015, the United States Food and Drug Administration (FDA) announced its definition of fiber as “non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by the FDA to have physiological effects that are beneficial to human health.” Within this definition, there lies an enormous amount of structural and functional diversity. Despite constant refinement of the methodologies, quantifying and characterizing these structures within dietary fiber remains a significant challenge.

Dietary fiber has garnered particular attention in recent years due to increased interest in the gut microbiome. Numerous reports have determined that fiber polysaccharides are a major driver of gut microbial ecology and heavily influence the plethora of microbial metabolites produced and thus host health.[9–12] This interaction between fiber and the gut microbiota is dependent upon fiber structure. Specifically, gut microbes possess genes encoding for only certain glycosyl hydrolases and polysaccharide lyases that are specific for the degradation of particular monosaccharide and glycosidic linkage residues.[13, 14] For example, the hemicellulose arabinoxylan (comprised of a β1➞4 xylopyranose backbone with branches of α1➞2,3 arabinofuranose) would require a particular set of β-xylosidases and α-arabinosidases for degradation by gut microbes while xyloglucan (comprised of a β1➞4 glucose backbone with branches of α1➞6 xylose β1➞2 galactose, and others), would require β-glucosidases, α-xylosidases, β-galactosidases, and even α-fucosidases.[15, 16] Thus, these hemicellulosic polysaccharides would potentially have completely different

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bioactivities. Further, the same polysaccharide from different sources contain unique monosaccharide and linkage profiles and these differences in fine structure also impact the gut microbiome differently.[17, 18] Many other factors may also affect the structure of the polysaccharides in food such as time of harvest, post-harvest processing, and the method of cooking used.[8, 19] Thus, analytical methods capable of characterizing and quantifying the carbohydrate structures in food are needed to understand the complex relationship between fiber and the gut microbiome. Rapid-throughput liquid chromatography-mass spectrometry (LC-MS) methods for monosaccharide and glycosidic linkage analysis have already proven essential in developing and understanding the mechanisms behind microbiota-directed therapeutic foods.[20, 21] However, there are currently no analytical methods that utilize a multi-glycomic approach to integrate the free saccharide, total monosaccharide, and glycosidic linkage compositions of dietary fiber in food.

Despite efforts towards a generally applicable definition of dietary fiber, the term remains ambiguous and carries a different meaning to various stakeholders. To consumers and dieticians, fiber is a necessary dietary component for optimal health. To many food companies, fiber may be an opportunity for marketing and product improvement. To plant scientists, fiber refers to the structures comprising the plant cell wall. To food scientists and chemists, fiber is a group of carbohydrates possessing glycosidic linkages preventing its digestion by human enzymes but allowing its fermentation by the gut microbiome. An ideal analytical framework for dietary fiber analysis would bridge these gaps and provide both “classical” enzymatic–gravimetric determinations as well as specific structural information utilizing monosaccharide and linkage analyses.

In this proof-of-concept study, two commonly employed AOAC methods (991.43 and 2017.16) were used to determine the total dietary fiber content of raw oats and potato starch utilizing a dietary fiber analyzer. The resulting soluble and insoluble fractions were determined, isolated, and subjected to comprehensive structural analysis employing three recently developed LC-MS-based methods to determine their total monosaccharide, glycosidic linkage, and free saccharide compositions. The products of each starch digestion were quantified and characterized, providing insight towards the applicability of each method. This analytical pipeline is a significant step forward in the information obtained from classical dietary fiber determinations and is unique in that is captures macroscopic, nutritional definitions such as “soluble fiber” and “insoluble fiber” while also quantifying and defining the structural composition of the oligo- and polysaccharide components of these fractions. This feature can effectively bridge the gaps between food chemistry, clinical science, and the gut microbiome, thus representing a path forward for dietary fiber analysis as well as its definition in the future.

MATERIALS AND METHODS

Trifluoroacetic acid (TFA), chloroform (HPLC grade), ammonium acetate, ammonium hydroxide solution (NH_4OH) (28–30%), sodium hydroxide pellets (semiconductor grade, 99.99% trace metals basis), dichloromethane, anhydrous dimethyl sulfoxide (DMSO), iodomethane, 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP), methanol (MeOH, HPLC grade), fructose, ribose, rhamnose, mannose, allose, glucuronic acid (GlcA), galacturonic

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acid (GalA), glucose, galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), xylose, arabinose, fucose, maltose, sucrose, and raffinose were purchased from Sigma-Aldrich (St. Louis, MO). Rye arabinoxylan, maltotetraose, maltopentaose, maltohexaose, kestose, stachyose, and verbascose were purchased from Megazyme (Bray, Ireland). Acetonitrile (HPLC grade) was purchased from Honeywell (Muskegon, MI). Sodium hydroxide (reagent grade), maleic acid, acetic acid (glacial), hydrochloric acid, D-sorbitol, calcium chloride ($CaCl_2.2H_2O$), deionized water, acetone (reagent grade), and ethanol (99%) were purchased from Fisher Scientific (Pittsburg, PA, USA). Dietary fiber analyzer bags (SDF bags (ANKOM #DF-S), IDF bags (ANKOM #DF-I)) and enzymes (thermostable α-amylase (*Bacillus licheniformis*, ANKOM concentrate enzyme #TDF81), porcine pancreatic α-amylase (PAA, ANKOM concentrate enzyme #TDF86), protease (*Bacillus licheniformis,* ANKOM concentrate enzyme #TDF82), amyloglucosidase (AMG, *Aspergillus niger*, ANKOM concentrate enzyme #TDF85)) were obtained from ANKOM Technology (Macedon, NY, USA). MES-Tris buffer solution (0.05 M, pH 8.2) was prepared using reagent MES (2-(N-morpholino)ethanesulfonic acid) and Tris (Tris(hydroxymethyl)aminomethane) obtained from Fisher Scientific (Pittsburgh, PA, USA). The sodium maleate buffer solution (50 mM, pH 6.0) was prepared using maleic acid, sodium hydroxide, and calcium chloride. The rapid integrated total dietary fiber assay kit (K-RINTDF) was obtained from the Neogen part of Megazyme (Lansing, MI, USA). Tris base was purchased from Sigma-Aldrich (St. Louis, MO, USA). Oat sample was purchased from the company ANKOM Technology (Macedon, NY, USA) and raw potato starch (PenPure^{R} 10) was obtained from Ingredion$_{TM}$ (Westchester, Illinois, USA).

Fiber content determination.

Determination of insoluble (IDF) and soluble dietary fiber (SDF) was performed by AOAC 991.43 and 2017.16 methods using an automated ANKOM^{TDF} dietary fiber analyzer.[1,2] In brief, samples (1 g) were weighed in IDF filter bags (5 replicates) and set on the serial Ankom fiber analyzer. In the sixth bag, the oat reference standard was analyzed to monitor the performance of the instrument. The fiber analyzer was programmed to deliver buffer and enzymes according to the AOAC methods.

For methods AOAC 991.43, digestion was sequentially performed with enzymes (α-amylase, protease, and amyloglucosidase (AMG)) under controlled temperature as previously reported.[1] In brief, the fiber analyzer was programmed to deliver 40 mL of MES-Tris buffer solution and 1 mL of enzymes (α-amylase, protease, and AMG) were sequentially added in each bag. The time and temperature were set to 30 min at 95 °C for α-amylase digestion, and 30 min at 60 °C for protease digestion. The hydrochloric acid was pumped to adjust the pH to 4.0–4.7 to terminate the digestion reaction. After completion of the digestions, the digested fiber materials filtered through the IDF bag to SDF bags. At this stage, the instrument was set to deliver 225 mL 95% ethanol at 60 °C to each digested sample for precipitation of soluble dietary fiber with ethanol. After 60 min, the solution was filtered through the SDF filter bags, and the filtrate was collected in the glass container. The precipitate was rinsed twice with 15 mL of 78% ethanol and 95% ethanol. The IDF and SDF bags were removed from the fiber analyzer and rinsed with acetone and air-dried for 30

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min followed by drying in an oven at 100–102 °C for at least 90 min. The dried bags were weighed to determine the crude IDF and SDF contents in the samples.

For the AOAC 2017.16 method, the following modifications were done before fiber analysis.[3] A 35 mL of sodium maleate buffer solution (50 mM, pH 6.0 and 2 mM $CaCl_2$) was used instead of 40 mL MES-Tris buffer (0.05 M, pH 8.2) in AOAC 991.43. In addition, 1 mL of 100 mg/mL of sorbitol, 2 mL of PAA/AMG enzyme (PAA (4 KU/5 mL) plus AMG (1.7 KU/5 mL)) were used in AOAC 2017.16 as compared to 1 mL of α-amylase (150 Ceralpha/mL; 10.8 U/mg in 50% glycerol + 0.09% sodium azide) in AOAC 991.43. The time and temperature were set to 4 h at 37 °C for the PAA/AMG digestion. Furthermore, the total incubation time was 5 h for AOAC 2017.16, whereas the total incubation time was 90 min for AOAC 991.43. After 4 h, the instrument delivered Tris base solution 3 mL (0.75 M) to adjust the pH 8.2 to terminate the reaction. The termination reaction incubation time was set to 30 min at 60 °C. The instrument was set to deliver 1 mL protease enzyme and the mixture was incubated for 30 min. The mixture pH was adjusted to 4.2 to terminate the reaction. After digestion, the soluble dietary fiber passed through the IDF filter bags to SDF filter bags. The SDF filter bags were pre-loaded with diatomaceous earth. The fiber analyzer was programmed to deliver 225 mL of 95% ethanol at 60 °C to each digested sample and the mixture was incubated for 60 min for precipitation of SDF. The precipitated was washed twice with 15 mL 78% ethanol and 95% ethanol and the samples were processed in the same way as reported above for AOAC 991.43. The filtrate considered at soluble dietary fiber soluble (SDFS) in ethanol was initially concentrated in a rotary evaporator followed by lyophilization. The dried IDF, SDF, and the concentrated filtrate residue were analyzed using mass spectrometric analysis. SDFS was determined via UHPLC-QqQ MS analysis by summing the concentrations of all oligosaccharides (saccharides with degree of polymerization [DP] > 2) measured by the method.

Determination of protein and ash content.

Undigested protein content was determined using a combustion method on a rapid MAX N exceed Dumas analyzer from Elementar. Only one sample was used to check the amount of undigested protein and duplicate analysis was done for ash content analysis. Initially, the bags were sealed from the top and the bottom. For protein analysis outside plastic covering was removed. For the determination of the ash content, the sealed bags were placed in a preweighed crucible and incinerated in the Barnstead thermolyne muffle furnace (Type 48000, Thermo Scientific, Walham, MA, USA) at 600 °C. Blank bags without samples were also separately incinerated in the same way to do the blank correction. For undigested protein determination, the sealed bags were further sealed at three positions. These were then cut into three small bags that were used for the determination of nitrogen content using Elemantar Combustion Analyzer (Elemntar Americas Inc., Ronkonkoma, NY, USA).[4] The total protein of the sample was determined and summed to determine total nitrogen content in a bag. Only a single analysis for nitrogen and duplicated analysis for ash were carried out for each sample and the average of the two runs was used for ash corrections.

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Preparation of Dietary Fiber Fractions for Glycomic Analysis.

The oats, potato starch, and insoluble dietary fiber samples were homogenized by bullet-blending with an Omni Bead Ruptor Elite (Kennesaw, GA) before a 10 mg aliquot was weighed out into 1.5 mL screw-cap Eppendorf tubes. An arabinoxylan polysaccharide standard was also prepared in the same way and used as a control for monosaccharide and linkage analysis. The whole oat and potato starch samples were precipitated with 80% EtOH and the supernatant removed and saved for free saccharide analysis before further homogenization. A stock solution of 10 mg/mL was prepared by adding 1 mL of nanopure water. The stock solution then underwent a bullet blending procedure followed by heat treatment (1 h at 100°C) and another round of bullet blending to ensure homogeneity. Soluble dietary fiber and soluble dietary fiber precipitate samples were contained in a dry mixture with diatomaceous earth. These mixtures were transferred to 15 mL Falcon tubes and 10 mL of nanopure water was added. The samples were then vortexed thoroughly and the resulting suspensions were incubated at 100 °C for 1 hr to solubilize the carbohydrates. The tubes were then centrifuged, and a 5 mL aliquot was diluted to 10 mg/mL by original dry weight for analysis. Waste and soluble dietary fiber supernatant samples were reconstituted in nanopure water to make 50 mg/mL stock solutions which were then diluted to 10 mg/mL for analysis.

Quantitative Monosaccharide Compositional Analysis.

Methods were adapted from previous publications with some adjustments.[20–22] Aliquots of each stock solution and an arabinoxylan polysaccharide standard (used as a quality control) were subjected to acid hydrolysis with 4 M TFA for 1 h at 121˚C in 96-well plates. Hydrolysis was quenched by the addition of cold nanopure water. A 10 uL aliquot from each sample was then derivatized alongside an external calibration curve (0.001 to 100 μg/mL) containing 14 monosaccharides by adding 100 uL of 0.2 M PMP in methanol, 100 uL of ammonia solution (28–30 % w/v), and heating to 70 °C for 30 min. The derivatized glycosides were dried to completeness by vacuum centrifugation and extracted twice with chloroform to remove excess PMP. A 1 uL aliquot of the aqueous layer was injected into an Agilent 1290 Infinity II UHPLC system equipped with an Agilent Poroshell HPH C18 column (50 × 2.1 mm i.d., 1.8 μm particale size) and corresponding guard column. Separation of the PMP-labeled monosaccharides was achieved using a constant flow rate of 0.9 mL/min and a 2 min isocratic elution at 11 % B followed by a 1.6 min flush at 99% B, and 0.8 min equilibration for a total run time of 4.6 min. Solvent A consisted of 25mM ammonium acetate in 5% acetonitrile with pH adjusted to 8.2 using ammonia solution. Solvent B consisted of 95% acetonitrile in water. Mass spectral analysis was carried out on an Agilent 6495B triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA) operated in positive ion mode while using dynamic multiple reaction monitoring (dMRM). The total monosaccharide content in each sample was determined by comparison to the external calibration curve.

Glycosidic Linkage Analysis.

A permethylation procedure was adapted from Galermo et al.[23, 24] Aliquots of 5 uL were transferred from each sample stock solution to a 96-well plate and permethylated using

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iodomethane (40 uL) in a solution of DMSO (150 uL) containing saturated NaOH (5 uL). The samples were allowed to react on a shaker at room temperature for 50 min under argon before being quenched by the addition of cold water and then DCM. NaOH and DMSO were removed by repeated extraction with cold nanopure water. The upper aqueous layers were discarded while the bottom organic layer containing permethylated products was dried to completion by vacuum centrifugation. The permethylated samples were then subjected to acid hydrolysis at 100˚C for 2 hr with 4 M TFA and subsequently dried by vacuum centrifugation. The released permethylated monosaccharide residues were derivatized with PMP following the previously described procedure. Once dried, the samples were then reconstituted in 100 uL of 70% aqueous methanol. Separation and analysis of the permethylated glycosides were carried out on an Agilent 1290 Infinity II UHPLC system equipped with an Agilent Zorbax RRHD Eclipse Plus C18 column (150 × 2.1 mm i.d., 1.8 μm particle size) and corresponding guard. Mass spectral analysis was also carried out on an Agilent 6495B triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA) operated in multiple reaction monitoring (MRM) mode. For analysis, 1 μL of sample was injected onto and separated using a 15 min binary gradient with a constant flow rate of 0.45 mL/min. Mobile phase A and B were the same as those used for monosaccharide analysis except the pH of mobile phase A was adjusted to 7.7. The following binary gradient was used: 0.00−5.00 min, 21.00% B; 5.00−9.00 min, 21.00−22.00% B; 9.00−11.00 min, 22.00% B; 11.00−13.60 min, 22.00−24.50% B; 13.60−13.61 min, 24.50− 99.00% B; 13.61−13.80 min, 99.00% B; 13.80−13.81 min, 99.00−21.00% B; 13.81−15.00 min, 21.00% B. Glycosidic linkages were identified by comparing their MRM transitions and retention times to an established library.

Free saccharide analysis.

The 10 mg/mL stock solutions of each sample were diluted first with water and then into 75% ACN after centrifugation at 10,000 rpm for 5 min. A standard curve containing two monosaccharides, three disaccharides, and seven oligosaccharides ranging in concentration from 1 to 200 μg/mL was also prepared. A 5 μL aliquot was then injected onto the same Agilent UHPLC-QqQ MS instrument this time fitted with a Waters BEH Amide column (150 × 2.1 mm i.d., 2.5 μm particle size) and corresponding guard. Free saccharides were separated using a 25 min gradient elution: 0–4.0 min, 95% B; 4.0–5.5 min, 95 – 80% B; 5.5–16.0 min, 80–60% B; 16.0–19.0 min, 60% B; 20.0–25.0 min, 95% B. Mobile phase A consisted of 10 mM ammonium acetate in 10 % aqueous ACN while mobile phase B consisted of 10 mM ammonium acetate in 90 % ACN. The pH of both mobile phases was adjusted to 10.2 with aqueous ammonia. The 6495B QqQ MS was operated in negative ion mode and used single ion monitoring (SIM) for detection. Free saccharides in each sample were determined by comparison to the external calibration curve.

RESULTS

Two food samples (oats and potato starch) were analyzed by AOAC methods 991.43 and 2017.16 on an Ankom Dietary Fiber Analyzer (Figure 1). The resulting fractions were dried, weighed, and structurally elucidated using a comprehensive multi-glycomics workflow consisting of quantitative total monosaccharide and free saccharide analyses as well as a

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glycosidic linkage analysis (Figure 2). The monosaccharide analysis monitored the prevalent monosaccharides in food and provided quantitative information on the carbohydrates present in each fiber fraction. The glycosidic linkage analysis monitored nearly 100 linkages and found over 50, comprising the preponderant linkages found in food. This analysis provided structural information on the saccharides found in the foods and their fiber fractions. Additionally, a quantitative free saccharide analysis was performed to capture free sugars (glucose, fructose, sucrose, maltose, and lactose) and low molecular weight oligosaccharides (raffinose, kestose, stachyose, verbascose, and maltooligosaccharides DP 4 to ~10) in each fraction. These results added a rich, integral layer of information capable of identifying, quantifying, and structurally elucidating the mono-, di-, oligo-, and polysaccharide components of each fraction in unprecedented detail.

Enzymatic-Gravimetric Determination of Dietary Fiber Fractions

The calculated average percentages of dietary fractions measured with both AOAC methods 991.43 and 2017.16 for oats and potato starch are provided in Table 1. Potato starch did not contain enough insoluble dietary fiber (IDF) to measure. AOAC method 991.43 does not include a soluble dietary fiber supernatant (SDFS) fraction.

IDF is defined as fiber that is insoluble in water which encompasses many polysaccharide components with diverse structures as well as lignin. SDFP contains fiber that is soluble in water and insoluble in ethanol which may also be comprised of a large number of polysaccharide and oligosaccharide components. SDFS is the component of fiber that is soluble in ethanol and is limited to small oligosaccharides.

Multi-glycomic Analysis of Oats

The monosaccharide and glycosidic linkage compositions of the oat sample and each of its dietary fiber fractions are provided in Figure 3. Monosaccharide analysis showed that the oat sample contained mostly glucose (580 μg/mg by dry weight) with small amounts of xylose (16 μg/mg), arabinose (17 μg/mg), galactose (6 μg/mg), and mannose (1 μg/mg). The total carbohydrate content was 620 μg/mg by dry weight (Supplementary Table S1). Glycosidic linkage analysis revealed that the majority of the glucose was 4-linked (66 %) with a smaller contribution from 3-linked glucose (3.5%). The large 4-linked component is consistent with a high amount of starch while the 3-linked glucose is more consistent with β-glucan as 3-linked is not found in starch. Linkage analysis also yielded xylose that were 4-linked (0.5%) and branched 3,4-linked (0.3%) with arabinose that were terminal (t-ara*f*, 2.8%) consistent with the presence of arabinoxylan (Supplementary Table S2). The insoluble dietary fiber (IDF) fractions from both AOAC methods yielded similar carbohydrate abundances and compositions with glucose, xylose, and arabinose being the main constituents along with small amounts of mannose. Furthermore, linkage analysis of the IDF fractions produced nearly equal amounts of 3- and 4-glucose (suggesting β-glucan) and 4- and 3,4-xylose and t-arabinofuranose (suggesting arabinoxylan). The presence of 4-mannose further suggested the presence of β-mannan. Note that while we could not determine the polysaccharides unambiguously, we inferred the identities from the linkages as is commonly done in the field.[25] Henceforth to facilitate the discussion, we provide polysaccharide identities that were inferred from the linkages.

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Due to the high sensitivity of the analysis, xyloglucan linkages such as t-galactose, t-xylopyranose, and 4,6-glucose were also detected despite their low abundances. Although the IDF from both methods were similar in composition, 2017.16 gave significantly higher total glucose than 991.43. Furthermore, 4-, 4,6- and t-glucose were more abundant in the 2017.16 IDF (although only t- and 4,6-glucose reached statistical significance) suggesting the increased glucose relative to 991.43 was likely from starch (Supplementary Figure 1a, b), and likely resistant starch.

Analysis of the soluble dietary fiber (SDF, 991.43) and soluble dietary fiber precipitate (SDFP, 2017.16) revealed compositions composed of 3- and 4-glucose in ratios of about 1:3. Similar ratios are consistent with β-glucan.[26] Similarly, small amounts of 6-, 4,6- and t-galactose along with t-arabinose pointed to branched arabinogalactans. Small amounts of 4- and 3,4-xylose suggested the presence of arabinoxylan as a minor component. Note that the amylase and amyloglucosidase added for the digestion of starch in the original sample were not removed, and the monosaccharide and linkage compositions of their mannose-containing N-glycans were also visible. An abundance of 2-, 3-, and t-mannose further supports this notion. Mannose and the N-glycan associated linkages were also abundant in the blank samples from both methods with 2017.16 having more of these components than 991.43.

The monosaccharide analysis of the waste of 991.43 and soluble dietary fiber supernatant (SDFS) of 2017.16 fractions were found to contain nearly all glucose with the majority being terminal (t-) and minor components 4-linked. These findings pointed towards the presence of free glucose monomers along with small concentrations of maltooligosaccharides- derived from the enzymatic starch digestions in each method. Enzyme was also present in these fractions as evidenced by the t- and 3-linked mannose residues.

Free saccharide analysis of the waste and SDFS confirmed the majority of these fractions was free glucose (Figure 4, Supplementary Table S3) revealed differences in the profiles of starch breakdown products from the enzymatic digestions. Namely, the waste fraction from 991.43 contained more free saccharide from maltose and maltooligosacccharides. Lower abundances of these compounds were found in the SDFS fraction from 2017.16. Free saccharide analysis of the SDF from 991.43 and SDFP from 2017.16 gave opposite results with more free saccharides found in the SDFP than in the SDF. Sucrose and raffinose family oligosaccharides(stachyose and raffinose) were found in original oat samples. They were also found in the waste and SDFS fractions. Both the waste from 991.43 and SDFS from 2017.16 contained glucose, maltose, and maltooligosaccharides from the enzymatic digestions. However, the maltooligosaccharides from each method displayed different retention, suggesting distinct oligosaccharide products.

Multi-glycomic Analysis of Potato Starch

The compositions of the potato starch and its AOAC fractions are illustrated in Figure 5. Potato starch was found to be nearly 100 % glucose by dry weight with 4-linked, t-, and 4,6-linked glucose being the predominant linkages present (Supplementary Tables S4 and S5) and consistent with starch. There was insufficient material collected from the IDF fractions of either method to perform glycomic analysis.

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Monosaccharide analysis showed SDF contained only minute amounts of carbohydrates, while SDFP was found to possess the majority of the carbohydrates from the original sample, unlike the oat samples. The large discrepancy is likely due to the differences in the enzymatic starch digestion conditions in each method. AOAC 2017.16 utilizes a milder digestion than 991.43, resulting in large oligomers that precipitate out into SDFP. Linkage analysis confirmed that both SDF and SDFP contained maltodextrins arising from starch digestion. However, the procedure used in 2017.16 resulted in only partial digestion, leaving high molecular weight maltodextrins (DP>10, highest monitored in the method) in the SDFP fraction. Free saccharide analysis (Figure 6, Supplementary Table 6) further confirmed this finding. Potato starch contained small amounts of endogenous glucose, maltose, and maltooligosaccharides ranging from DP 3–10, while these free saccharides were absent in the SDF from 991.43, indicating efficient digestion in the latter. The SDFP from 2017.16, however, contained relatively large amounts of maltooligosaccharides.

The waste and SDFS fractions of potato starch from each method also exhibited major differences. The waste from 991.43 contained approximately three times the amount of total glucose than the SDFS fraction of 2017.16 (Figure 6c). Based on the ratio of 4- to t-glucose linkages, the average DP of the saccharides in the SDFS was greater than those in the waste fraction. Free saccharide analysis confirmed that SDFS indeed contained more maltooligosaccharides than the waste fraction. Additionally, the same maltooligosaccharides present in SDFP were found in SDFS. These compounds differed greatly from those found in the waste of 991.43. Specifically, the oligosaccharides in the fractions from 2017.16 followed the linear maltooliogsaccharide ladder, while the oligosaccharides in the waste from 991.43 yielded different retention times suggesting different linkages. Indeed, closer inspection of the linkage data from the waste fraction revealed the presence of 6-glucose in addition to 4-glucose (Figure 5, Supplementary Table 5), suggesting that the starch digestions from each method produced distinct oligosaccharide products.

DISCUSSION

Dietary fiber is recognized as an essential component of a healthy diet.[27, 28] Despite its importance, relatively little advancements have been made towards the analysis of fiber since its earliest common definition nearly 50 years ago.[1] Currently, most methods for the determination of fiber are gravimetric measurements while total carbohydrates are not typically measured at all and are instead determined by difference after subtraction of moisture, protein, fat, and ash. Uncovering the structure-function relationships between dietary fiber, gut microbes, and human health is currently an active and important area of research, however current methods for fiber analysis are clearly not granular enough for this purpose.[20, 21, 29–32] The role of dietary fibers cannot be understood without knowing structures particularly in a number of functions including modulating gut microbiome.[33–36] The paucity of available methods for dietary fiber arises from the inherent difficulty of carbohydrate analysis as it requires a suite of historically low throughput and/or low sensitivity techniques and instrumentation.[37, 38] However, advancements in methods utilizing rapid throughput, high sensitivity, liquid chromatography-mass spectrometry (LC-MS) have made it possible to provide quantitative structural information on the

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carbohydrates in hundreds of food samples rapidly using 96-well plate formats.[20, 21, 31, 32, 39] These nascent methods make it feasible to perform in-depth carbohydrate analysis on large sample collections. Still, the output from these analyses often lack the explicit context that many in the field of nutrition and clinical science may recognize, replacing familiar terms like “total/soluble/insoluble fiber” with descriptions involving monosaccharides and glycosidic linkages. The latter descriptions have proven to be necessary details, but they can be difficult to reconcile with current dietary recommendations and classical determinations. Thus, it is important that these new approaches to carbohydrate analysis be coupled to classical methods involving isolation and gravimetric analysis of dietary fiber fractions such as IDF, SDF(P), and SDFS.

In the present work, two common foods (oats and potato starch) were analyzed on the Ankom Dietary Fiber Analyzer using two commonly employed AOAC methods: 991.43 and 2017.16. The resulting fiber fractions were recovered and subjected to a suite of rapid throughput LC-MS based methods for the comprehensive analysis of their carbohydrate contents at the monosaccharide, linkage, and free saccharide levels. This proof-of-concept study revealed an avenue to bridge the gap between classical definitions of fiber (IDF, SDF, etc.) and the higher resolution pictures required for gut microbial studies that are provided by LC-MS analyses. The resulting data can ultimately be used to make more meaningful and specific connections between dietary fiber, the gut microbiome, and host health.

From the analysis of the raw foods, oats were found to contain starch, β-glucan, arabinoxylan, mannan, and xyloglucan. Sucrose and the α-galactooligosaccharides stachyose and raffinose were also obtained from the same sample. As expected, potato starch was comprised solely of starch with small amounts of maltooligosaccharides. Separation of the carbohydrate fractions in the two AOAC methods 991.43 and 2017.16 followed by LC-MS analysis revealed the location of the respective components. In both AOAC methods, the IDF fraction from oats was found to be a diverse mix of polysaccharides with nearly equal abundances of arabinoxylan and β-glucan as well as smaller amounts of β-mannan and xyloglucan. Soluble oat β-glucan has garnered much attention for its role in ameliorating metabolic diseases and some cancers. However, the results suggest that β-glucan is only partially soluble with the largest component being in the soluble fractions of both methods.[40, 41] After β-glucan, arabinoxylan was also primarily in the IDF. Arabinoxylan is another important dietary fiber found in grains and has been shown to modulate the gut microbiome.[42, 43] Its fine structural detail is known to affect its function in modulation of metabolic functions.[17] Less abundant components of the oats including β-mannan and xyloglucan were also detected through their specific and respective linkages (4-mannose for β-mannose and t-galactose, t-xylopyranose, and 4,6-glucose for xyloglucan) in the IDF fraction.[44] These minor components are also known to be extensively utilized by gut microbes.[16, 45] Both AOAC methods yielded similar IDF abundances and compositions, however the total glucose in IDF from 2017.16 (188.5 μg/mg) was higher than 991.43 (154.2 μg/mg) with statistical significance ($p = 0.005$, Student’s t-test). Increased relative abundances of the starch-associated linkages 4-, 4,6-, and t-glucose in 2017.16 IDF suggested that starch was greater in the IDF of 2017.16 compared to 991.43. The former utilizes a much gentler starch digestion, and the additional starch was likely resistant starch that escaped the “softer” enzymatic digestion of 2017.16. One limitation of the

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methodology employed here is the inability to quantify cellulosic glucose which is likely a major component of IDF. However, this would require hydrolysis with sulfuric acid (H_2SO_4). TFA was used here to make the sample preparation more directly amenable to mass spectral analysis, but future developments could employ clean-up steps such as C18 solid phase extraction (SPE) of PMP-derivatized glycosides to remove the salts created from H_2SO_4 hydrolysis.

The SDF(P) fractions were comprised almost entirely of β-glucan. The differences in solubility between the measured β-glucans across fractions could arise from different molecular weights, linkage distributions, and their chemical interactions within the grain.[44, 46] The ratio of 3- and 4-linked glucose to terminal glucose was significantly lower in the IDF (15.6) than the SDF(P) fractions (25), suggesting variations in degree of polymerization as a contributing factor.

Another challenging facet of defining dietary fiber arises from the concept of resistant starch (RS), which is defined analytically as the component of starch not digested after exposure to amylase and amyloglucosidase digestion. Four types of RS are defined (RS1, RS2, RS3, and RS4). RS1 is starch that is physically inaccessible to enzyme as in partially milled grains and seeds. RS2 is resistant to digestion due to its native conformation in raw foods that typically becomes digestible through cooking. RS3 is indigestible due to the process of retrogradation during which amylose chains form double helices that resist gelatinization upon cooking. It commonly occurs in foods that were cooked and subsequently cooled. RS4 is starch that is chemically modified by cross-linking or derivatization, thereby impeding the activity of amylase on the substrate.[47] Potato starch was chosen as a model food to apply the current methodology towards understanding how RS behaves in AOAC methods 991.43 and 2017.16. As a raw and processed starch product, potato starch is expected to contain only RS2.[47] The AOAC methods used here differ greatly in the conditions used to digest starches present in foods. Starch digestion in 991.43 is carried out at elevated temperature with heat-stable amylase and is meant to hydrolyze all starch within a sample. The digestion in method 2017.16, however, is carried out under conditions mimicking physiological (37 °C, pH 7.2) and is meant to hydrolyze only digestible starch while defining undigested starch as “resistant.”[44] The biological meaning of this analytically resistant starch is a topic of some debate, but nonetheless provides a means of quantifying these components in foods.[48] By coupling these AOAC methods to the suite of presented LC-MS methods, it was determined that 991.43 was indeed effective at total starch digestion, leaving only a minute amount of bound glucose in the SDF fraction of potato starch. Most of the hydrolysate was found in the waste fraction, indicating that nearly all of the starch had been hydrolyzed to glucose, maltose, and small oligosaccharides. The small amount of bound glucose that was detected in the SDF could further be described as belonging to long chain maltodextrins as the ratio of 4-linked to terminal glucose was only slightly lower than that of the potato starch itself. This was contrary to 2017.16, where the majority of the bound glucose from potato starch was found in the SDFP rather than the SDFS. There results indicated that digestion hydrolyzed the starch into large oligosaccharides that were in turn soluble in water but insoluble in ethanol. Again, the ratio between 4-linked and terminal glucose in the linkage analysis suggested that the compounds escaping complete digestion were maltodextrins. Furthermore, the free saccharide analysis revealed

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that a large fraction (~28%) of these maltodextrins exhibited DPs ranging from 3–10. The waste fraction from method 991.43 and SDFS fraction from 2017.16 also differed in their carbohydrate abundances and compositions. These fractions contained free glucose, maltose, and oligosaccharides resulting from the starch digestion. The waste fraction contained significantly more total carbohydrate than the SDFS fraction mostly due to increased free glucose.

CONCLUSIONS

Current methodologies for measuring dietary fiber and inclusion in food labels create a form of nutritional “dark matter” by providing only gravimetric determinations of inherently complex biomolecules. The oligo- and polysaccharide structures contained within the broad definitions of soluble and insoluble fiber vary widely between different foods, imbuing unique and structure-dependent bioactivities upon interaction with the gut microbiome. Quantifying and characterizing these structures in food will be integral in delineating the role of fiber-microbe interactions in human and animal health. The integrated methods described here provide a means to quantify the complex carbohydrate structures in food while retaining familiar and clinically relevant determinations such as “insoluble/soluble fiber.” Isolation of the dietary fiber fractions from oats and potato starch using two commonly employed AOAC methods (991.43 and 2017.16) on a commercial Fiber Analyzer allowed for their subsequent structural characterization at the monosaccharide, glycosidic linkage, and free saccharide levels using rapid-throughput LC-MS methods. The analysis of oats revealed that the non-cellulosic insoluble fiber from oats was composed of arabinoxylan and β-glucan (evidenced by 3- and 4-glucose) with small amounts of xyloglucan and mannan while the soluble fiber fraction was chiefly composed of β-glucan and trace amounts of arabinogalactan as derived from both AOAC methods. For potato starch, the fractions obtained from each AOAC method were found to be markedly different mostly due to the nature of their starch digestions. The harsher digestion from 991.43 hydrolyzed potato starch produced mainly free glucose and maltose that were collected in the “waste” fraction. The milder digestion from 2017.16, meant to capture resistant starch, was found to hydrolyze potato starch mostly to higher molecular weight maltodextrins that were captured in the SDFP fraction, as well as glucose, maltose, and maltooligosaccharides collected in the SDFS fraction. Together, these findings provide new and comprehensive insight regarding the structures present as dietary fiber in these food products. In the future, LC-MS analyses that quantify and structurally define the glycans within dietary fiber will supersede existing gravimetric methods to provide the details necessary to understand the positive health effects of fiber. This proof-of-concept study will serve as a prelude towards more complex and diverse foods leading to more thorough understanding of dietary fiber and its effect on the gut microbiome. We propose that food labeling requirements include knowledge of specific carbohydrates and that the information be included in AOAC and CODEX tables. Both the LC-MS and McCleary method should be combined in future food analysis.

Supplementary Material

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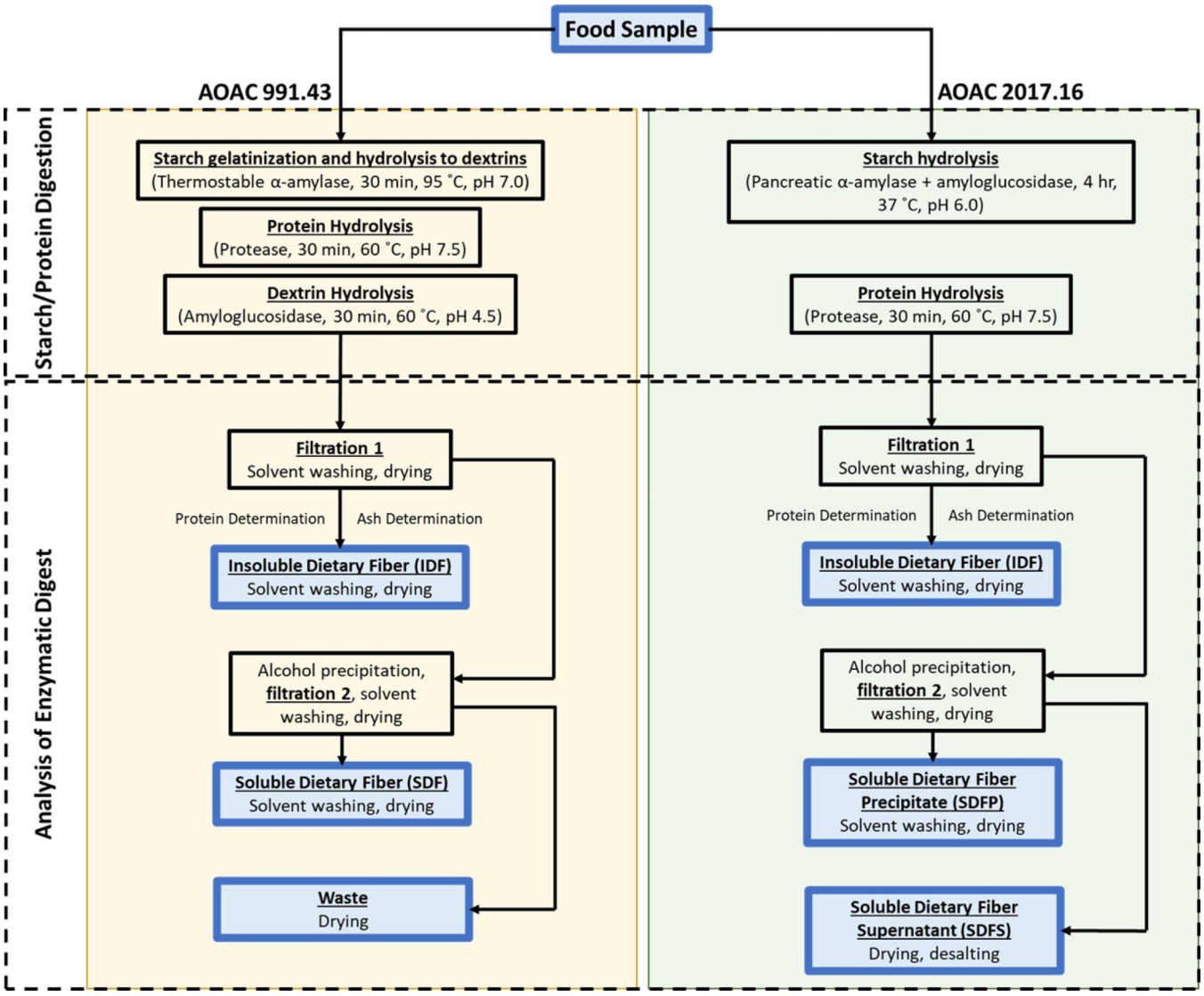

Figure 1. Overview of AOAC methods 991.43 and 2017.16.

Fractions highlighted in blue were collected, weighed for gravimetric determinations, and subjected to a comprehensive glycomic analysis that provided total monosaccharide, linkage, and free saccharide compositions.

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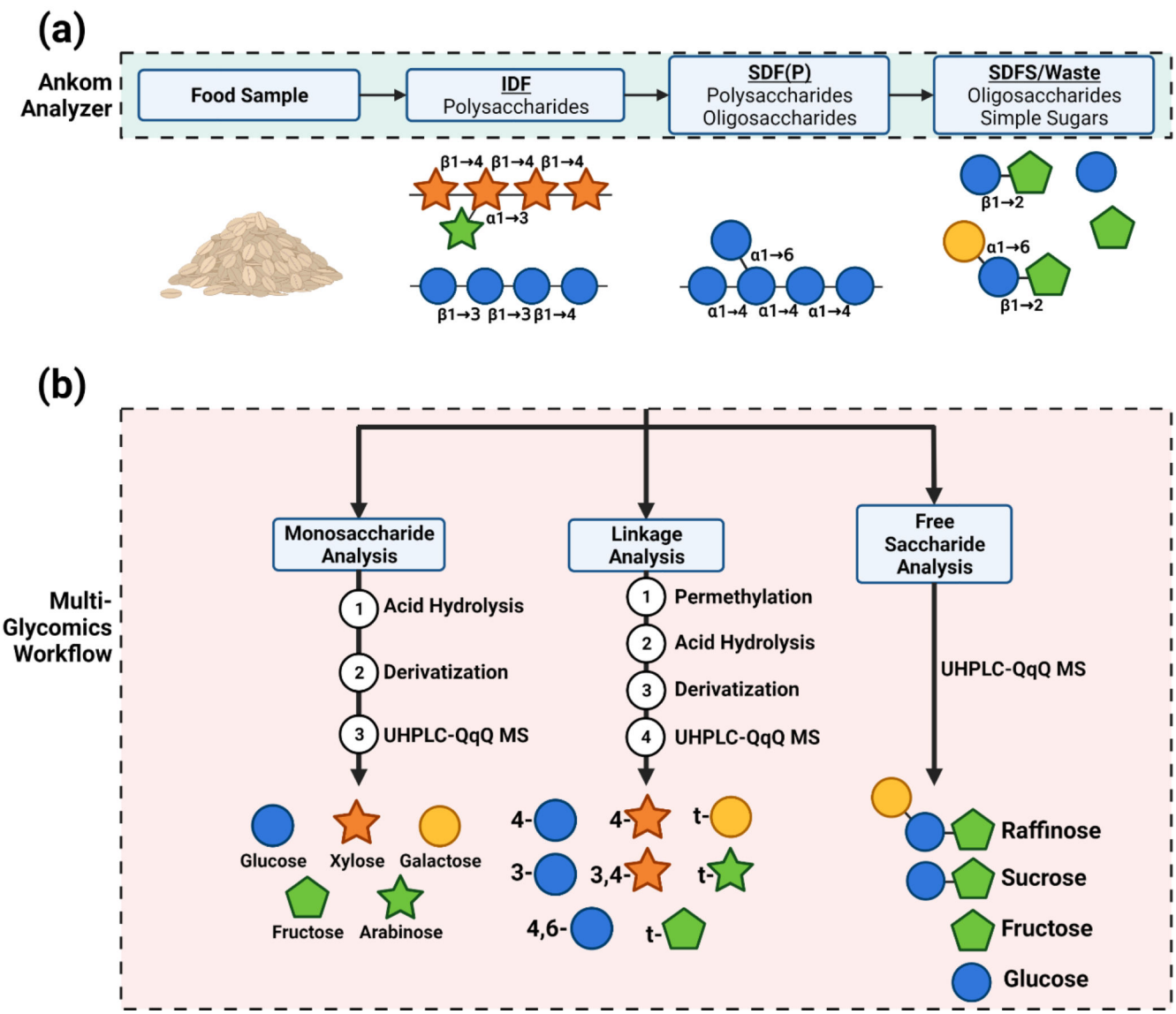

Figure 2.

Overview of the multi-glycomic workflow used to analyze AOAC fiber fractions. (a) The enzymatic-gravimetric AOAC methods fractionate the native carbohydrate components from food into IDF, SDF(P), and SDFS. Select structures present in oat are shown as examples. (b) Each fraction was then subjected to quantitative monosaccharide compositional, glycosidic linkage, and free saccharide analyses.

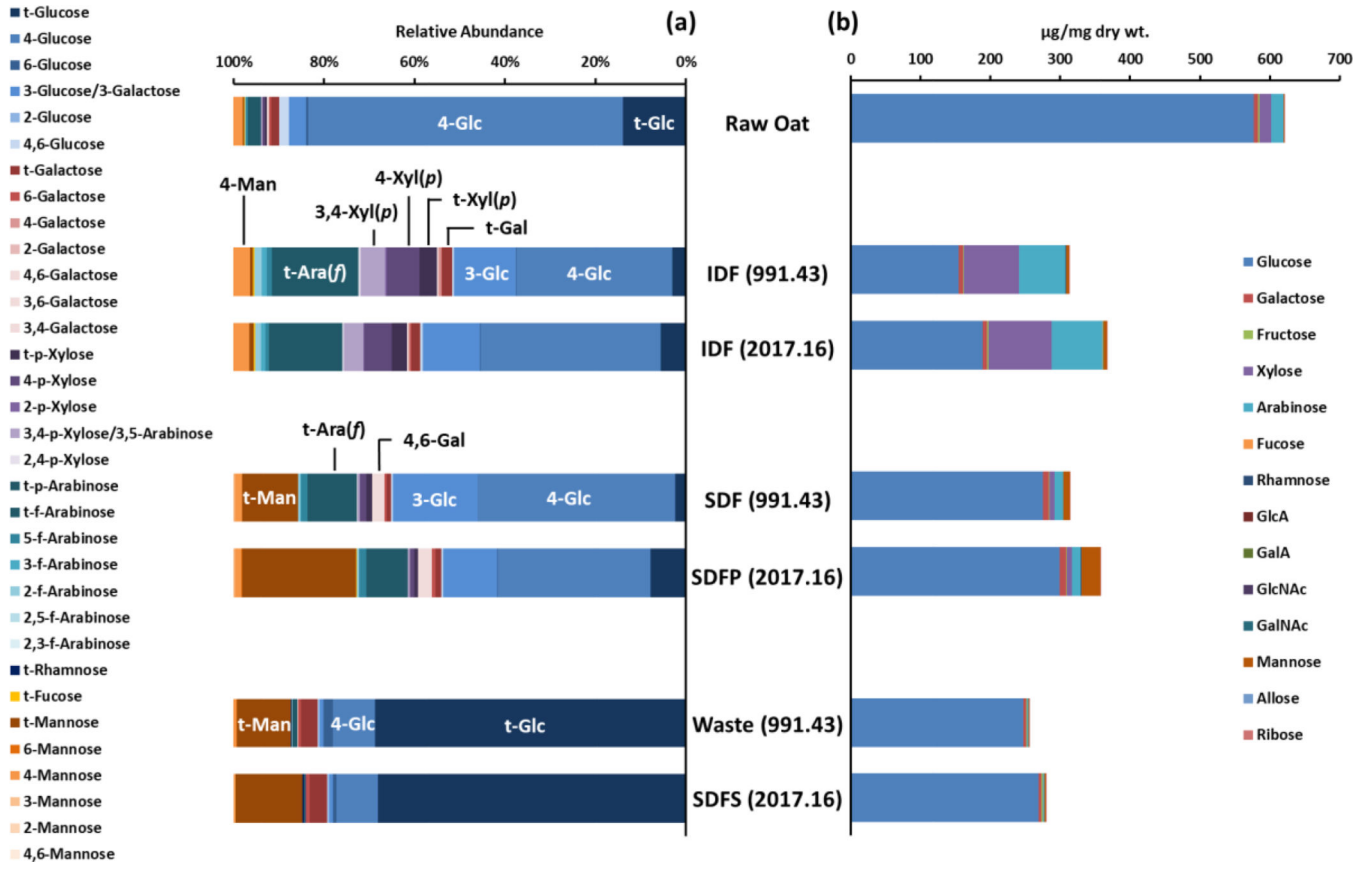

Figure 3.

(a) Glycosidic linkage and (b) monosaccharide compositions of oat and its dietary fiber fractions from each AOAC method. Each bar represents an average of three technical replicates. The “t-“ denotes a terminal linkage.

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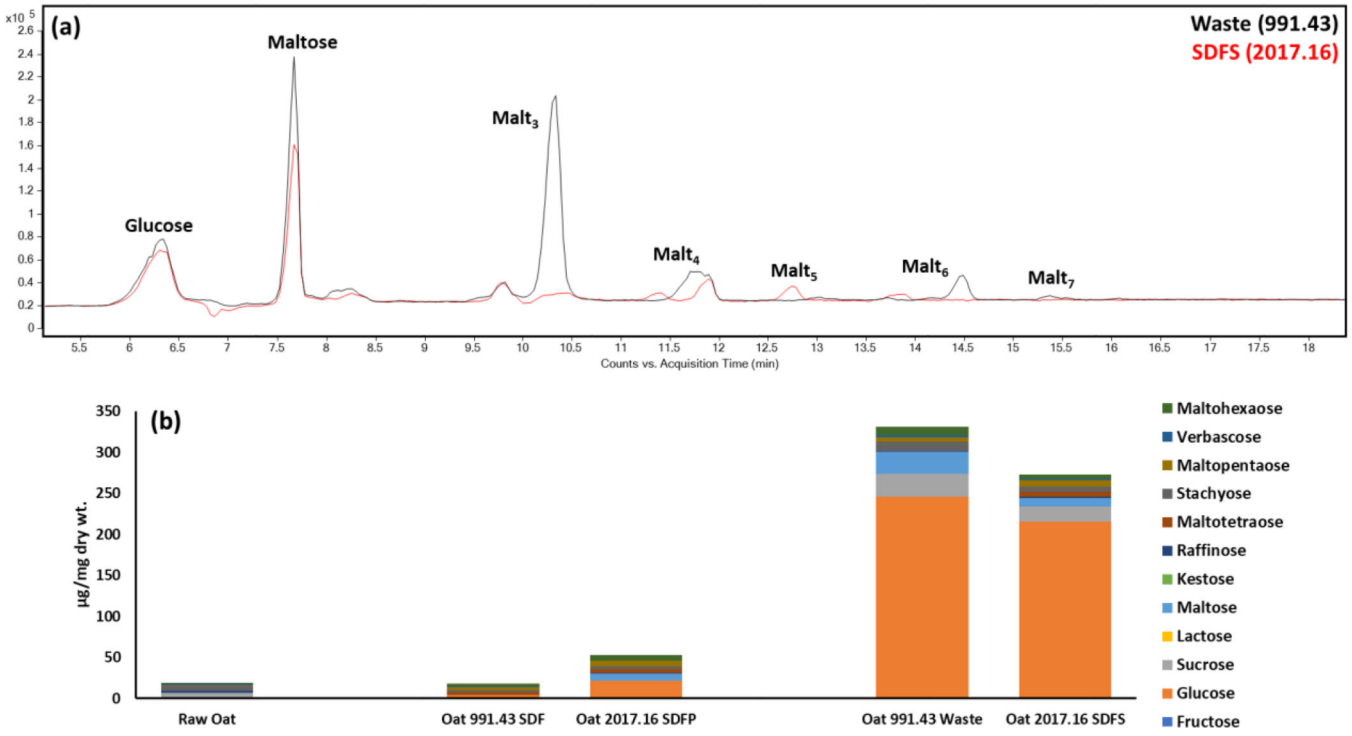

Figure 4a-b.

(a) Total ion chromatogram depicting the free saccharides present in oat waste (991.43) and SDFS (2017.16). (b) Quantified results of free saccharides in the raw oat sample, SDF, SDFP, waste, and SDFS fractions.

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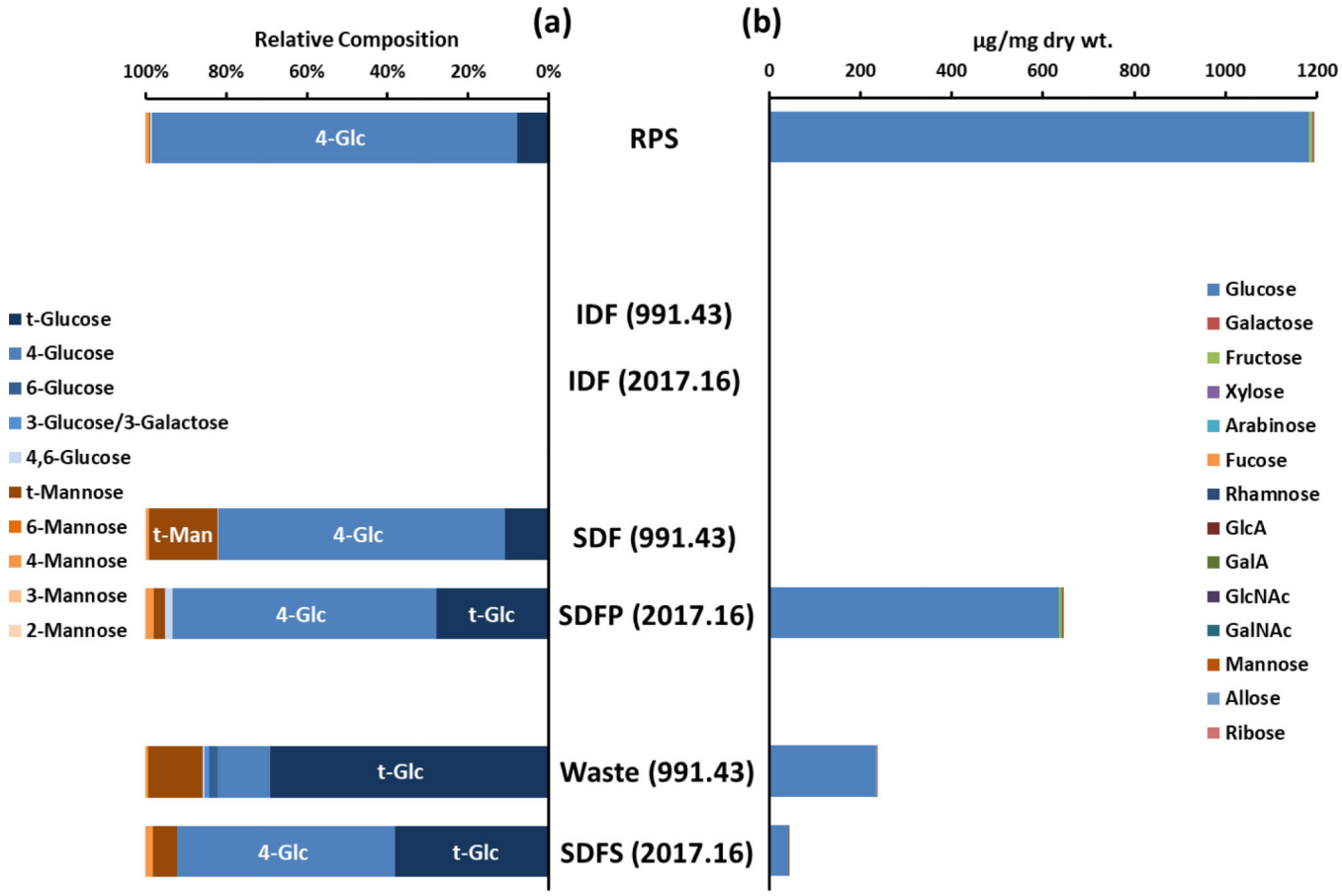

Figure 5a-b. (a) Glycosidic linkage and (b) monosaccharide compositions of potato starch and its dietary fiber fractions from each AOAC method.

There was insufficient material in the IDF fraction from either method to perform glycomic analysis. Each bar represents an average of three technical replicates. The "t-" denotes a terminal linkage.

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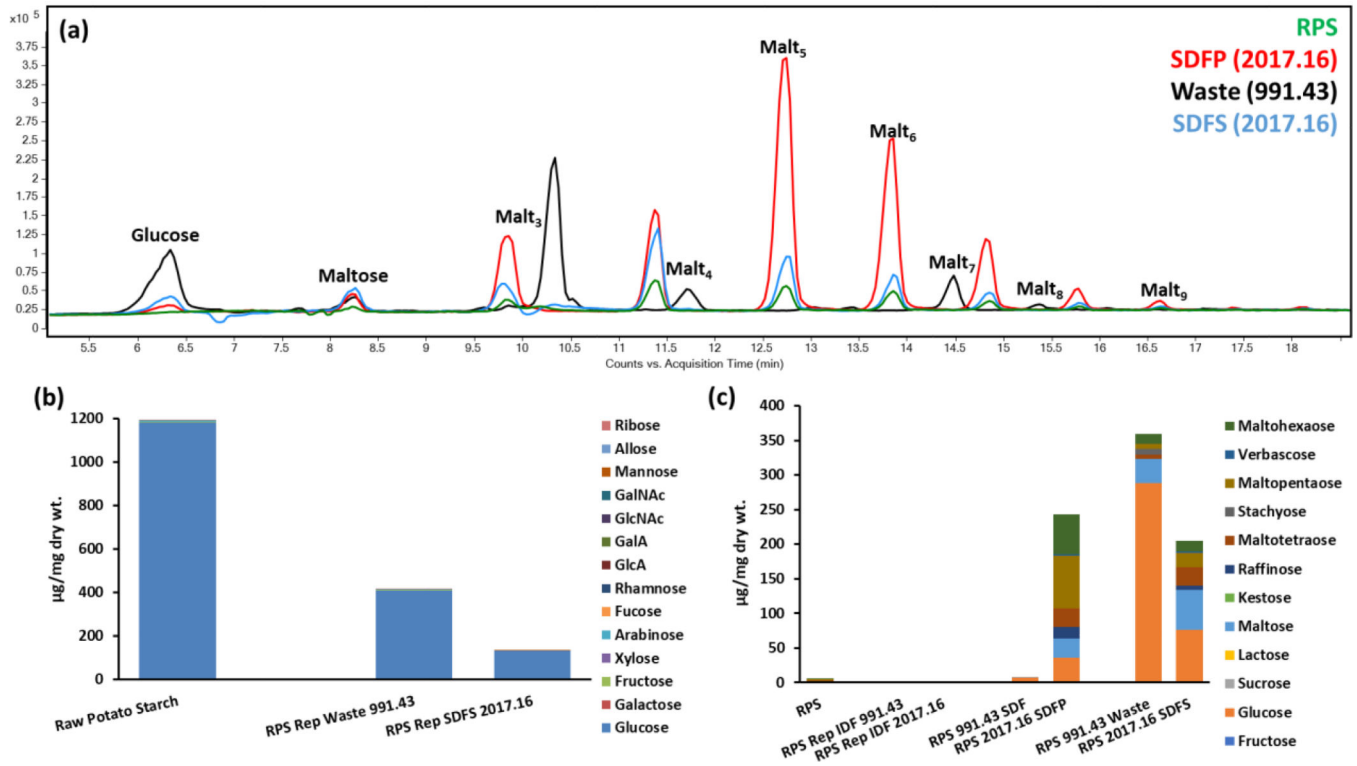

Figure 6a-c.

(a) Total ion chromatogram depicting the free saccharides present in potato starch, SDFP (2017.16), waste (991.43) and SDFS (2017.16). (b) Total monosaccharide composition of potato starch, waste (991.43), and SDFS (2017.16). (c) Quantified free saccharide results for potato starch, SDFP, waste, and SDFS fractions.

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Table 1.

IDF (insoluble dietary fiber), SDF (soluble dietary fiber), SDFP (soluble dietary fiber precipitated with ethanol), SDFS (soluble dietary fiber soluble in ethanol) and, TDF (total dietary fiber) percent in oat and potato starch samples. Both samples were analyzed in triplicate.

Samples	(%) IDF (mean ± SD)	(%) SDFP (mean ± SD)	(%) SDFS (mean ± SD)	(%) TDF (mean ± SD)
AOAC 991.43				
Oat	11.91 ± 0.42	8.64 ± 0.51	NA	20.55 ± 0.92
Potato Starch	NQ	0.35 ± 0.02	NA	0.35 ± 0.02
AOAC 2017.16				
Oat	10.25 ± 0.07	8.32 ± 0.53	2.84 ± 0.23	21.41 ± 0.23
Potato Starch	NQ	69.72 ± 0.19	7.10 ± 1.50	76.82± 1.5

NA = Not applicable, NQ = Not quantifiable, and SD = standard deviation