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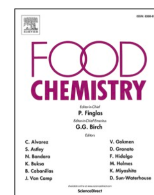

Chromatographic preparation of food-grade prebiotic oligosaccharides with defined degree of polymerization

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ABSTRACT

Prebiotic oligosaccharides are of widespread interest in the food industry due to their potential health benefits. This has triggered a need for research into their sensory properties. Such research is currently limited due to the lack of available food-grade oligosaccharide preparations with specific degree of polymerization (DP). The aim of this study was to develop economical approaches for the preparation and characterization of prebiotic oligosaccharides differing with respect to composition and DP. Such preparations were prepared by chromatographic fractionation of commercially available prebiotic mixtures using microcrystalline cellulose stationary phases and aqueous ethanol mobile phases. This approach is shown to work for the preparation of food-grade fructooligosaccharides of DP 3 and 4, galactooligosaccharides of DP 3 and 4, and xylooligosaccharides of DP 2–4. Methods for the characterization of the different classes of oligosaccharides are also presented including those addressing purity, identity, total carbohydrate content, moles per unit mass, and DP.

1. Introduction

Prebiotics are currently defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). This definition encompasses commercially available prebiotic oligosaccharide products, which are mixtures containing different-sized oligosaccharides. In recent years, the interest in prebiotic ingredients continues to expand due to their beneficial impact on human health and the related marketing value. Potential health benefits include improving digestion and gastrointestinal health and cardiovascular function, reducing adherence of pathogenic bacteria to intestinal epithelial cells, and reducing the risk of colorectal cancer (Davani-Davari et al., 2019). Dietary prebiotics are also incorporated into foods for their organoleptic effects (Guimarães et al., 2020; Wang, 2009), such as reduced-calorie fat replacers or bulking agents.

For a food ingredient to be classified as a prebiotic, it must: 1) be able to withstand food processing treatments such as high temperatures and low pH, 2) be able to withstand digestive processes before reaching the colon, 3) be selectively fermented by beneficial bacteria in the colon, 4) promote growth and proliferation of beneficial bacteria, and 5) provide benefit to the host’s well-being and health (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004; Wang, 2009). Although not all prebiotics are carbohydrates (e.g., flavonols), the majority of prebiotics are oligosaccharides, a subset of carbohydrates (Davani-Davari et al., 2019). Oligosaccharides, in turn, are often defined as having 3–10 units (Cummings & Stephen, 2007). Herein, short-chain prebiotic oligosaccharides refer to short-chain carbohydrates containing 3–4 glycosyl residues.

Prebiotics are obtained in the diet through a variety of natural sources, including fruits (e.g., banana, nectarine, watermelon), vegetables (e.g., onion, soybeans, asparagus, wheat, garlic), honey, and maternal milk (for types and sources of prebiotics, see Al-Sheraji et al., 2013; Gänzle, 2011; Jovanovic-Malinovska, Kuzmanova, & Winkelhausen, 2014). However, the quantity of prebiotic oligosaccharides present in most natural sources is low relative to the amounts thought necessary to elicit their beneficial effects (Davani-Davari et al., 2019), although there are some exceptions (e.g., chicory root, Jerusalem artichoke). As such, there is a growing market for prebiotic oligosaccharide-fortified food products (Fonteles & Rodrigues, 2018; Manning & Gibson, 2004). The most prevalent prebiotic oligosaccharide ingredients in food products are fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (Al-Sheraji et al), with xylooligosaccharides (XOS) gaining in popularity over the past few years (Vázquez, Alonso, Domínguez, &

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Parajó, 2000). These prebiotic oligosaccharides are also sold and consumed in a wide range of supplements (Carlson, Erickson, Lloyd, & Slavin, 2018).

Prebiotic oligosaccharides differ from one another with respect to chemical structure (see Fig. 1). Structural differences include their unique glycosyl residues (glucose, fructose, galactose, and xylose), glycosidic linkages [β(1 → 2), β(1 → 3), β(1 → 4), or β(1 → 6)], and degree of heterogeneity. FOS refers to oligosaccharides of D-fructose residues linked by β(2 → 1) bonds with a terminal sucrose (fructose-α(2 → 1)-glucose) (Hidaka, Hirayama, Tokunaga, & Eida, 1990; Loo et al., 1999; Yun, 1996). GOS, on the other hand, are oligosaccharides made up of D-galactose linked through β(1 → 2), β(1 → 3), β(1 → 4), or β(1 → 6) bonds with a terminal lactose (galactose-β(1 → 4)-glucose) (Gänzle, 2011; Splechtna et al., 2006). Due to the nature of the synthetic process for the production of GOS, which involves β-galactosidase-catalyzed transgalactosylation, the resulting GOS is a heterogeneous mixture comprised of GOS differing with respect to glycosidic linkages and chain lengths; essentially all of the constituent GOS contain lactose (galactose-glucose) at the reducing end (Nauta et al., 2009). XOS are made up of D-xylose linked through β(1 → 4) bonds (Loo et al., 1999). Within each class of prebiotic oligosaccharide, the number of glycosyl residues making up the chains can differ, resulting in homologs with different degrees of polymerization (DP).

The functional properties of prebiotic oligosaccharides are becoming of greater importance due to their increased prevalence in foods. For example, it is relevant to understand sensory properties of different prebiotic oligosaccharides, and how these sensory properties differ with chain length, particularly given recent findings that humans can taste oligosaccharides derived from starch (Lapis et al., 2014, 2016; Pullicin, Penner, Lim, & Glendinning, 2017). Moreover, prebiotic oligosaccharides with different structural properties could confer different health benefits (Belorkar & Gupta, 2016; Davani-Davari et al., 2019). Studying the sensory and functional properties of specific prebiotic oligosaccharides has been challenging because prebiotic oligosaccharides are commonly sold as a mixture of oligosaccharides differing with respect to DP and also including mono- and disaccharides (e.g., glucose, sucrose, xylose). Therefore, the fractionation of prebiotic oligosaccharides based on size is necessary to investigate the relationships between the DP of a prebiotic oligosaccharide and its sensorial and functional properties.

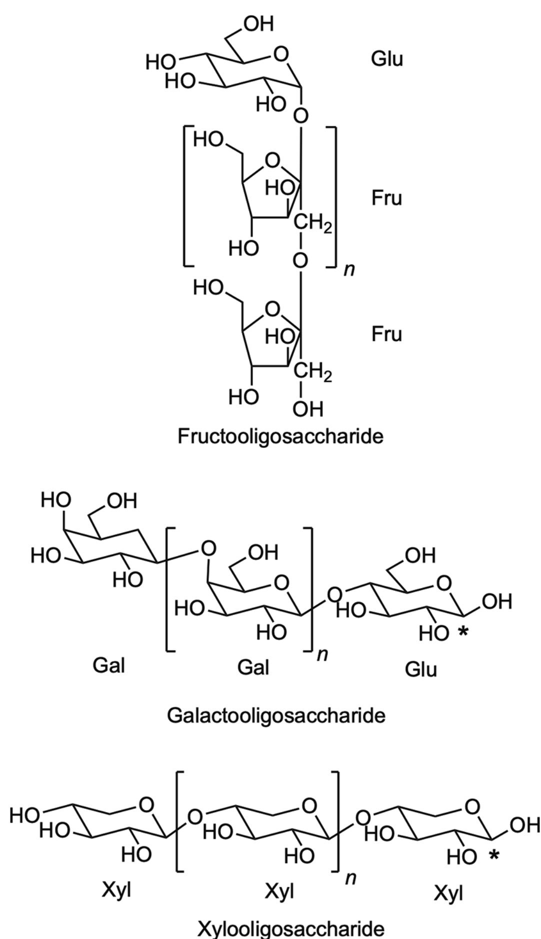

Fig. 1. Structures of common prebiotic oligosaccharides. The following terms refer to the respective monosaccharides; Fru: fructose, Glu: glucose, Gal: galactose, Xyl: xylose.* denotes the reducing end of the oligosaccharide.

Balto et al. (2016) recently fractionated food-grade maltopolysaccharides (MPS) and maltooligo-saccharides (MOS) from starch and corn syrup solids based on their differential solubilities in aqueous ethanol solutions. That approach was subsequently improved to enable the preparation of food-grade MOS preparations with reduced DP heterogeneity. This was accomplished by incorporating a chromatographic fractionation step based on the interaction of MOS with cellulose-based stationary phases (Pullicin, Ferreira, Beaudry, Lim, & Penner, 2018). The method developed by Pullicin et al. (2018) was adapted in this study to allow the preparation of lower molecular weight FOS, GOS, and XOS oligosaccharides of defined DP. Cellulose was used as the chromatographic stationary phase and aqueous ethanol as the mobile phase. Both the stationary and mobile phases can be obtained as food grade materials and thus the method is appropriate for the preparation of prebiotic oligosaccharides suitable for human testing.

The study reported in this paper had two objectives. The first was to develop fractionation methods for the preparation of research-ready, food-safe, DP-defined FOS, GOS, and XOS for use in human foods and/or human testing. The second objective was to develop a series of relatively straight-forward analytical methods for the characterization of oligosaccharide preparations. This second objective addresses the difficulty that arises when working with oligosaccharides differing with respect to chemical makeup.

2. Materials and methods

2.1. Materials

Prebiotic oligosaccharide starting materials used in this study were NUTRAFLORA® P-95 (FOS; Ingredion Inc., Bridgewater, NJ), BIOLIGO™ GL-5700 IMF (GOS; Ingredion Inc. Bridgewater, NJ), and PreticX 95 (XOS; AIDP Inc., City of Industry, CA). Saccharide analytical standards were glucose monohydrate and maltose monohydrate from Spectrum Chemical (Gardena, CA); D-fructose, sucrose, 1-kestose (FOS DP3), nystose (FOS DP4), D-galactose, D-lactose, and D-xylose from Sigma-Aldrich (St. Louis, MO); and xylobiose (XOS DP2), xylotriose (XOS DP3), and xylotetraose (XOS DP4) and 6′-galactosyllactose (GOS DP3) from Megazyme (Bray, Ireland). Solvents were ACS/USP-grade 100% ethanol from Pharmco Aaper (Shelbyville, KT), butanol (*n*-butanol ≥ 99%, FCC, FG) from Sigma-Aldrich (St. Louis, MO), HPLC/ACS-grade acetonitrile (CAS 75–05-8) from Fischer Scientific (Fair lawn NJ), deionized (DI) water (18.2 Ω, produced using a Millipore Direct-Q® 5 UV-R water purification system), and deuterium oxide 99.96% (Cambridge Isotope Laboratories, Tewksbury, MA). Chemical reagents included 1-naphthol (ReagentPlus® ≥99%), L-serine (ReagentPlus® ≥99% HPLC) (CAS 56–45-1), ACS-grade calcium carbonate (CAS 471–34-1), and thiourea (CAS 62–56-6) from Sigma-Aldrich (St. Louis, MO); sodium carbonate (CAS 497–19-8), ACS-grade sodium bicarbonate (CAS 144–55-8), and disodium 2,2′-bicinchoninate Pierce BCA solids from Thermoscientific (Rockford, IL); ACS-grade copper (II) sulphate pentahydrate (CAS 7758–99-8) from Avantor (Center Valley PA); ACS-grade anthrone (CAS 90–44-8) from Alfa Aesar (Ward Hill, MA); and ACS-grade sulfuric acid (CAS 7664–93-9) from EMD Millipore (Billerica, MA). Other materials used include

microcrystalline cellulose (Avicel PH-101) from UPI Chem (Somerset, NJ), and TLC silica gel 60 plates from EMD Millipore (Billerica, MA).

2.2. Methods

Column chromatography was done using a column with 73 mm I.D × 305 mm length with 1 L reservoir and fritted disc (Synthware, Pleasant Prairie, WI).

2.2.1. Fractionation of FOS

Column ready sample was prepared by adding 350 mg of FOS powder to 5 ml 85 % aqueous ethanol solution and stirring at 400 rpm and 30 °C until a clear solution was achieved. The stationary phase was prepared using 300 g of microcrystalline cellulose (Avicel PH-101; UPI Chem, Somerset, NJ) mixed with 70% aqueous ethanol and carefully poured down the previously wetted walls of the column to prevent splashing. The column was then rinsed with 70% ethanol using compressed air at ∼ 2 psi until the elute turned from yellow to clear and colorless. Final column height was about 20 cm. The column was equilibrated with 100 ml 85% ethanol and allowed to drain until the solvent reached the top of the packing, before the sample was carefully loaded onto the column using a pipette. A one-step gradient was used for the mobile phase. The initial eluent was 1.9 L of 85% aqueous ethanol; the second eluent was 1.5 L of 80% ethanol. The first 1000 ml of eluate typically consisted of glucose, fructose, and sucrose, which were discarded; subsequent eluate was collected in 15 ml fractions.

2.2.2. Fractionation of GOS

Column ready sample was prepared by dissolving 675 mg of GOS syrup (74% solids) in 5 ml 80 % aqueous ethanol solution and stirring at 400 rpm at a temperature of 30 °C until a clear solution was achieved. The stationary phase was prepared using 250 g of microcrystalline cellulose. Method for column preparation was similar to FOS column preparation (see 2.2.1). Final column height was about 17 cm. The column was equilibrated with 100 ml 85% ethanol, before the sample was carefully loaded onto the column. The initial eluent was 0.9 L of 85% aqueous ethanol; the second eluent was 1.5 L of 80% ethanol. The first 1200 ml of eluate typically consisted of glucose, galactose, and lactose, which were discarded; subsequent eluate was collected in 15 ml fractions.

2.2.3. Fractionation of XOS

Column ready sample was prepared by dissolving 1 g of XOS powder in 5 ml 70 % aqueous ethanol solution and stirred at 400 rpm at a temperature of 30 °C until it became a clear liquid. The stationary phase was prepared using 250 g of microcrystalline cellulose. Method for column preparation was similar to FOS column preparation (see 2.2.1). The column was equilibrated with 100 ml 75% ethanol, before the sample was carefully loaded onto the column. The initial eluent was 0.9 L of 75% ethanol; followed by 1.5 L of 65% ethanol; and lastly 0.5 L of 55% ethanol. The first 300 ml of eluate consisted of xylose and was discarded; subsequent eluate was collected in 15 ml fractions.

2.2.4. Solvent removal and drying

Ethanol was removed from samples using a rotary evaporator (Büchi Rotovapor R-205, Büchi Labortechnik AG) equipped with a water bath set at 55 °C (Buchi B-490) and a vacuum pump (Chemglass Scientific Apparatus/10 Torr). Samples were initially concentrated to a thick syrup, then washed by resuspending the preparation in additional water and then re-concentrating. This washing process was done twice in order to achieve the desired ethanol removal (Balto et al., 2016; Pullicin et al., 2018). The resulting concentrated samples were stored at –23 °C until being lyophilized (Labconco Freezone Freeze Dryer, Hampton, NH).

2.3. Chemical analysis

2.3.1. Thin layer chromatography

Thin layer chromatography (TLC) was used for initial verification of the chromatographic resolution of oligosaccharide fractions. A capillary spotter was used to deliver eluate onto TLC plates; concentrated spots were obtained by spotting each sample 3 times on a single location. Plates were thoroughly dried before being placed in the solvent chamber. Mobile phases were mixtures of ethanol, butanol, water (ratios were dependent upon the nature of the oligosaccharides). The mobile phase for FOS was 69:14:17 (ethanol, butanol, water; Robyt & Mukerjea, 1994); XOS was 5:3:2 (ethanol, butanol, water; López-Hernández, Rodríguez-Alegría, López-Munguía, & Wacher, 2018); and GOS was 3:5:2 (ethanol, butanol, water; Rabiu, Jay, Gibson, & Rastall, 2001). The staining solution used for FOS and XOS was 5% H_2SO_4 in ethanol with 0.5% α-Naphthol based on the staining solution used for maltodextrins (Robyt & Mukerjea, 1994). However, the staining solution was found to leave very faint and indistinguishable coloring for GOS. Hence, the staining solution for GOS was 35% H_2SO_4 in ethanol with 0.5% α-Naphtol (Manucci, 2009). In all cases, staining solution was applied by immersion and color development occurred as a result of heating prior dipped plates using a heat gun (General Lab Supply Co., Wayne, NJ) (Manucci, 2009; Rabiu et al., 2001; Tanriseven & Doğan, 2002). TLC plates were analyzed using JustQuantify online software (Sweday, Sodra Sandy, Sweden).

2.3.2. High performance liquid chromatography – evaporative light scattering detector (HPLC-ELSD)

The purity and identity of the oligosaccharide fractions were evaluated via High Performance Liquid Chromatography (HPLC) equipped with evaporative light scattering detection (ELSD). Lyophilized samples were initially dissolved in DI water and then acetonitrile was added to make an oligosaccharide in 60% acetonitrile/40% water solution. Analysis was performed using Prominence UFLC-HPLC system (Shimadzu, Columbia, MD) equipped with a system controller (CMB-20A), degasser (DGU-20A), solvent delivery module (LC-20AD), autosampler (SIL-10A), column oven (CT20-A), and evaporative light scattering detector (ELSD-LT II; kept at 60 °C with nitrogen gas pressure of 350 kPa) on a HILICpak VN-50 4D analytical column and a HILICpak VN-50G 4A guard column (Shodex, New York, NY) for analysis of all samples. The column oven was set to 30̊C for the analysis of FOS; and 60̊C for analysis of GOS and XOS. Standard curves were prepared using commercially available xylose, xylobiose, xylotriose, and xylotetraose for XOS DP 1–4; fructose, sucrose, 1-kestose, and nystose for FOS DP 1–4, and galactose, lactose, and 6′-galactosyllactose for GOS DP 1–3. Peak integrations were done using the manufacturer's LC-solution software (Shimadzu, Kyoto, Japan).

2.3.3. Reducing ends assay

Reducing end assays were performed to determine the moles of reducing ends present per given amount of XOS preparations; that data in turn was used to calculate average DP. Reducing ends per unit weight XOS preparation were quantified using the BCA/copper-based assay as described by Kongruang, Han, Breton, and Penner (2004). BCA working reagent was prepared with equal amounts of solution A and solution B. Solution A contained 54.28 g/L (512 mM) Na_2CO_3, 24.2 g/L (288 mM) of $NaHCO_3$, and 1.942 g/L (5 mM) of disodium 2,2′-bicinchoninate in DI water. Solution B contained 1.248 g/L (5 mM) $CuSO_4{\cdot}5H_2O$ and 1.262 g/L (12 mM) of L-serine in DI water. Solutions A and B were kept refrigerated in amber bottles until ready for use. Assays were initiated by adding 0.5 ml of BCA working reagent to test tubes containing 1 ml of aqueous carbohydrate preparation. Tubes were immediately topped with a glass marbles, vortexed, and placed into 100 °C water bath for 15 min. Tubes were then immersed in an ambient temperature water bath to be brought to room temperature. Solutions were then transferred into cuvettes and the absorbance measured at 560 nm using a Shimadzu 160

UV–Vis spectrophotometer. Calibration curves were produced using known concentrations of xylose standard. Assays were done in triplicate. New BCA working reagent was prepared fresh each day.

2.3.4. Glucose assay

Moles of FOS and GOS per given amount of preparations were determined by quantifying the number of glucose molecules present following acid-catalyzed hydrolysis of the oligosaccharide preparations. The assay is based on FOS and GOS having a single glucose moiety per molecule. Oligosaccharide preparations were hydrolyzed as 1 mg/ml solutions in 1% H_2SO_4 (FOS; Nguyen, Sophonputtanaphoca, Kim, & Penner, 2009) and 2% H_2SO_4 (GOS; Sophonputtanaphoca, Pridam, Chinnak, Nathong, & Juntipwong, 2018) contained in marble-capped test tubes. Hydrolysis tubes were incubated in a boiling water bath for 90 min (FOS) and 60 min (GOS), followed by immersion in an ice bath for 5 min. Samples were then left to equilibrate to room temperature before being neutralized through the addition of calcium carbonate ($CaCO_3$). Neutralized hydrolysates were used for subsequent glucose determination using the glucose oxidase/peroxidase method as described by the supplier (Sigma Aldrich); the neutralized hydrolysate was also used for chromatographic analyses primarily aimed at verifying complete oligosaccharide hydrolysis. Analytical grade glucose, lactose and sucrose were used as standards. Acid hydrolyses were done in triplicate and glucose assays were done on each hydrolyzed sample.

2.3.5. Total carbohydrate assay

The carbohydrate content of oligosaccharide preparations was determined using spectrophotometric anthrone/sulfuric acid-based assays (Haldar, Sen, & Gayen, 2017). Specifics of the assays used for the different oligosaccharide preparations were altered based on the unique reactivities of FOS, GOS, and XOS (see Results and Discussion). In all cases, a 0.1% (w/v) anthrone solution was prepared in 98% ice cold sulfuric acid and allowed to equilibrate for 15–20 min before use. Anthrone reagent for XOS also contained 1% (w/v) thiourea for color stabilization. Four ml of anthrone reagent was pipetted into test tubes containing 1.0 ml aqueous carbohydrate solution. Test tubes were immediately capped with marbles and placed in a boiling water bath for 3 min. Sample-containing tubes were then placed in an ambient temperature water bath for 10 min prior to taking absorbance measurements at 672 nm (FOS and GOS) and 465 nm (XOS) using a Shimadzu 160 UV–Vis spectrophotometer. Calibration curves were produced using aqueous samples of glucose, xylose, fructose, and galactose prepared at 0–1 mg/ml. All samples were assayed in triplicate. Anthrone reagent was prepared fresh on the days of the analyses.

2.3.6. Nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) was used to verify that the spectra of the experimental oligosaccharide preparations matched those of the corresponding analytical standards. NMR was also used to verify removal of residual ethanol by identifying the CH3 group at 1.17 ppm (Fulmer et al., 2010). A Bruker AVIII 400 MHz 2-channel spectrometer with 5 mm dual carbon (DCH) cryoprobe with a z-axis gradient was used to analyze samples at room temperature dissolved in D_2O. Topspin 2.1 computer software was used to acquire spectra.

3. Results and discussions

All prebiotic oligosaccharides were chromatographically fractionated using food-grade, cellulose-based stationary phases and aqueous-ethanol mobile phases. Fractionations within the distinct classes of oligosaccharides (FOS vs. GOS vs. XOS) differed with respect to mobile phase ethanol contents, sample loads, run times, and flow rates. This approach allowed the economic preparation of well-defined prebiotic oligosaccharides suitable for use in human studies. The characterization of the resulting oligosaccharide preparations was based on chemical, chromatographic (HPLC) and spectrophotometric (NMR) methods for the determination of carbohydrate content, number-average DP, chemical identity and solvent removal.

3.1. Fractionation methods for the preparation of prebiotic oligosaccharides

The fractionation of food grade prebiotic oligosaccharides is particularly challenging because of the similarity in the structures of the compounds being fractionated (XOS and FOS differ only with respect to DP, and GOS with respect to DP and glycosidic linkage) and the limited availability of cost-effective food-grade materials for use as chromatographic stationary and mobile phases. Food-/pharmaceutical-grade microcrystalline cellulose was used for the stationary phase and aqueous ethanol for the mobile phase. This study made use of the fact that the economical preparation of purified prebiotic oligosaccharides does not require baseline resolution since the commercially available starting materials are relatively inexpensive. In the present case, specific oligosaccharide recoveries ranged from 30 to 75% (see Table 1). Lower recovery values reflect greater peak overlap (i.e., lower resolution); the lower resolution was accounted for in this work by collecting relatively small volumes of column eluent as separate fractions and then pooling only those fractions having clean DP profiles [DP profiles of individual fractions were determined by thin layer chromatography (TLC); see Method section for the TLC parameters]. This approach limits recoverable oligosaccharides to those from the center of elution peaks; the better the resolution the greater the center cut of the elution peak available for oligosaccharide recovery (see Fig. 2 for chromatograms and associated fractions recovered). Highest recoveries thus corresponded to those oligosaccharides for which there was the greatest resolution, that being the XOS in the present chromatographic system.

The representative chromatograms depicted in Fig. 2 illustrate the resolution obtained for each of the oligosaccharide preparations. Sample loads for typical chromatograms were 350, 675 and 1000 mg in 5 ml of the noted aqueous-ethanol solutions for FOS, GOS and XOS, respectively. Sample loads were dictated by the required resolution; XOS was chromatographed at the highest sample load (4 mg per g microcrystalline cellulose; Table 1) because XOS were resolved to the greatest extent in this system. The % ethanol content of the different mobile phases was dictated by the solubility of the oligosaccharides in aqueous-ethanol solutions (relative solubilities in 80% ethanol were FOS > GOS > XOS); increasing the % ethanol content of aqueous solutions corresponded to a decrease in oligosaccharide solubility in all cases. Elution volumes for different oligosaccharides of equivalent DP were similar, but slightly greater for XOS (e.g., compare elution volumes for DP4 components of each oligosaccharide preparation in Fig. 2). The somewhat greater elution volume for XOS is consistent with stronger associations with the cellulose stationary phase, particularly when noting that the mobile phase used for XOS chromatography was the lowest in % ethanol (i.e., XOS had the weakest mobile phase; that being the mobile phase least likely to promote oligosaccharide-cellulose interactions (Pullicin et al., 2018). Chromatographic run times for the various oligosaccharides were in the range of ten hours, with a general trend of elution times increasing with increasing sample loads and decreasing mobile phase ethanol contents. The amounts of the purified oligosaccharides obtained per chromatographic run are given in Table 1 along with relevant associated parameters.

3.2. Analytical methods for the characterization of prebiotic oligosaccharides

The starting materials for the preparation of the individual prebiotic oligosaccharides were commercially available food-grade heterogeneous prebiotic products (heterogeneous with respect to DP, for XOS and FOS, and with respect to DP and glycosidic linkage for GOS). Hence, the analyses performed on the oligosaccharide fractions obtained via chromatography focused on DP, which was ascertained by measuring

Table 1
Chromatographic parameters for fractionation of prebiotic oligosaccharides. [a]

Targeted Oligosaccharide	Composition of mobile phase gradient [b] (% ethanol)	Sample load [c]	Average recovered oligosaccharide per chromatographic run [d] (mg)	Percent oligosaccharide recovered from loaded sample [e]
FOS DP3	85 → 80	1.2	40	30
FOS DP4	85 → 80	1.2	80	49
GOS DP3	85 → 80	2.7	100	47
GOS DP4	85 → 80	2.7	120	75
XOS DP2	75 → 65 → 55	4.0	270	44
XOS DP3	75 → 65 → 55	4.0	190	76
XOS DP4	75 → 65 → 55	4.0	110	73

[a] Stationary phase was microcrystalline cellulose. Starting materials were commercially available heterogeneous preparations of FOS (NUTRAFLORA® P-95), GOS (BIOLIGO™ GL-5700 IMF), and XOS (PreticX 95). FOS = fructooligosaccharides; GOS = galactooligosaccharides; XOS = xylooligosaccharides.
[b] aqueous ethanol solutions.
[c] calculated as mg oligosaccharide preparation loaded onto column divided by grams of stationary phase in column.
[d] amount of collected target oligosaccharide for one chromatographic column run in milligrams rounded to the closest ten milligrams.
[e] calculated by dividing the recovered grams of targeted oligosaccharide by the estimated amount of targeted oligosaccharide applied to the column and then multiplying by 100.

carbohydrate content, moles per unit weight carbohydrate, HPLC profiles and NMR spectra.

The high carbohydrate content of the purified oligosaccharide preparations (close to 100% carbohydrate in all cases; Table 2) was anticipated given the nature of the starting material. Nevertheless, verification of each preparations' carbohydrate content was necessary to justify subsequent calculations based on these values (i.e., determination of the average DP of oligosaccharides within a given preparation; see below). The quantification of carbohydrate content was done using anthrone/sulfuric acid-based assays which are themselves based on the reaction of sugar-derived furan derivatives with the anthrone reagent to produce quantifiable colored compounds (Brummer & Cui, 2005). Sugars differ with respect to their reactivities under the assay's reaction conditions. Hence, the assay must be adapted for the different classes of oligosaccharides to which it is applied; this includes the wavelength used for quantification and the applicable calibration standards (Table 2). The absorption maxima of the products resulting from the color forming reactions of the pentoses have distinctly shorter wavelengths than those for the hexoses. The wavelength used for XOS quantification in this study was 465 nm, which differs from that used for the quantification of FOS and GOS (the wavelength corresponding to maximum sensitivity for FOS and GOS was 672 nm). The wavelengths used herein for the quantification of these prebiotic oligosaccharides are analogous to those reported as optimum for the analysis of the corresponding monosaccharides (Haldar et al., 2017). Choosing the appropriate calibration standard is another important aspect of anthrone/sulfuric acid-based assay design. The data depicted in Figure S1 illustrate that the different sugars, even those within the same classes (e.g., aldohexoses), have somewhat different color yields under equivalent reaction conditions (this being in general agreement with data of Haldar et al., 2017). Hence, it is prudent to use calibration standards that best reflect the composition of the presumed oligosaccharides in the analyte mixture. In the present case, we know the general structure of the oligosaccharides (Fig. 1), so we can deduce logical representative monosaccharide mixtures (Table 3). The importance of this is illustrated in **Fig. S1** by comparing the standard curves for glucose and galactose with that of lactose (a disaccharide composed of glucose and galactose); the lactose curve being approximately equidistance between the glucose and galactose curves.

The moles of oligosaccharides per unit weight purified preparation was determined using two approaches (Table 3). FOS and GOS both have single terminal glucose residues (Fig. 1). Thus, the moles of glucose resulting from complete hydrolysis of a unit weight of the parent oligosaccharide is equal to the moles of said oligosaccharide in that amount of preparation. Acid-catalyzed hydrolysis was used to convert the parent oligosaccharides into their constituent monosaccharides (Nguyen et al., 2009; Sophonputtanaphoca et al., 2018). The glucose content of the resulting solution was then determined using the spectrophotometric glucose oxidase/peroxidase (GOP) assay (Raba & Mottola, 1995). A second approach was used to quantify the moles of XOS per unit weight preparation; this being the quantification of reducing ends. The GOP assay was not considered for the analysis of XOS preparations because they do not contain a defined number of glucose moieties per molecule. Instead, the BCA/copper-based reducing sugar assay was applied using xylose as the calibration standard (Waffenschmidt & Jaenicke, 1987). This approach could have been used for the quantification of the GOS preparations as well since they are reducing oligosaccharides; although in this study those preparations were assayed as specified above using the GOP assay. FOS preparations are not reducing oligosaccharides, so the reducing sugar assay was not applicable for those preparations. The results from the quantification of the moles per unit weight oligosaccharide preparation (Table 3) combined with the % carbohydrate content of the different preparations (Table 2) were used to calculate the average DP of each fractionated oligosaccharide preparation (Table 3). The combined results from the reducing sugar and total sugar assays suggest that the GOS-DP4 sample was slightly contaminated with GOS-DP3. The measured average DP for the GOS-DP4 preparation was somewhat lower than the theoretical value of 4 (measured value is 3.7; Table 3). The presence of small amounts of GOS-DP3 in the GOS-DP4 preparation is also in agreement with the collected fractions depicted by the shaded regions in Fig. 2; the implication being that the amount of GOS-DP3 in the GOS-DP4 preparations can be lowered if narrower bands of eluate are pooled for collection (the trade-off being between purity and yield, as discussed above).

The DP values (Table 3) obviously provide information as to the size of the oligosaccharides, but they can also be indicative of the purity of the samples. They are only 'indicative' in the sense that a pure preparation of DP4 would give the same average DP as a 1:1 M mixture of DP3 and DP5. Thus, it is prudent to verify purity using an alternative method. Herein we used chromatography. Representative chromatograms of the different preparations are depicted in Fig. 3. The dominant single peak for each of the preparations reflects the samples' purity. We attribute the relatively short, broad peak for GOS-DP4 as reflecting the mixed glycosidic linkages in this preparation. The identity of the purified oligosaccharide preparations was further verified in this work by confirming that the retention times of chromatographed samples agree with those of commercially available analytical standards.

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was also used to verify the nature of the oligosaccharide preparations obtained via chromatography. The approach was to compare the spectra of the prepared samples with either those of commercially available analytical standards in the cases of FOS and XOS, or published chemical shift data for analogous compounds in the case of GOS; recall that glycosidic linkages of GOS starting materials are heterogeneous in nature, while

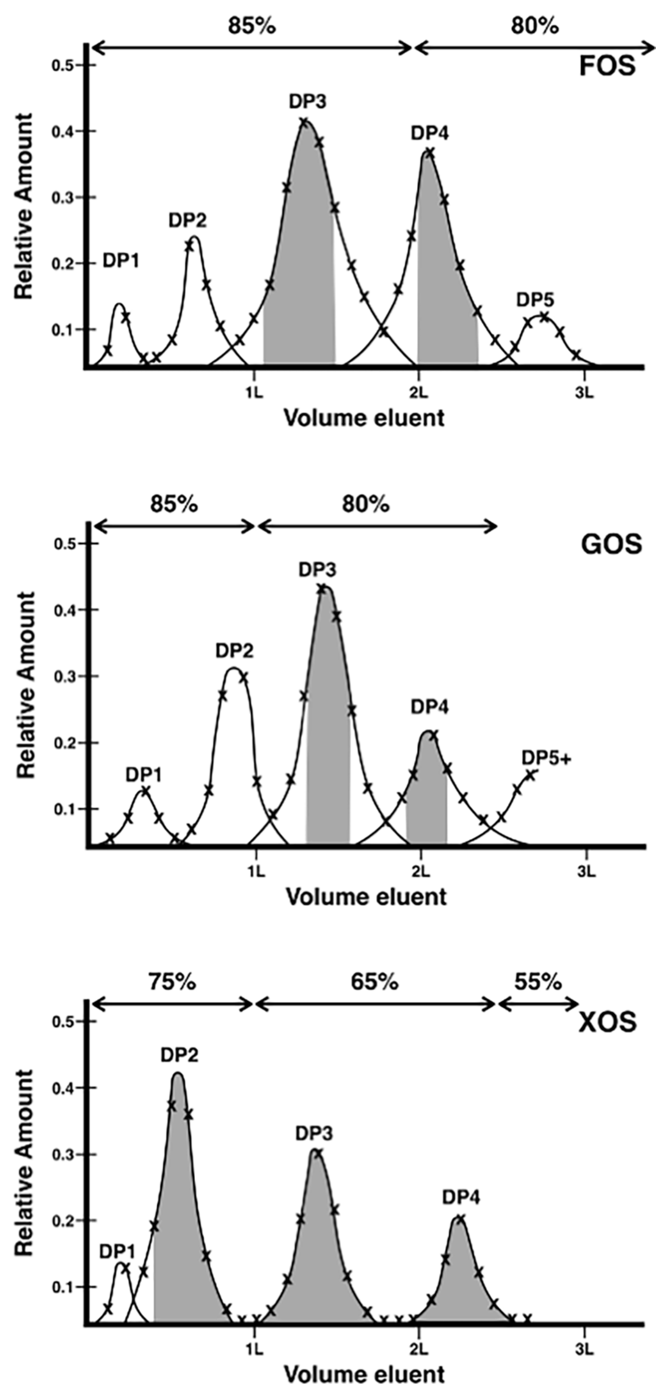

Fig. 2. Representative chromatogram illustrating the fractionation of fructooligosaccharides (FOS), galactooligosaccharides (GOS), and xylooligosaccharides (XOS). 'Relative amounts' of specific oligosaccharides in the eluent were determined using thin layer chromatography (TLC)/densitometry; every sixth 15 ml fraction was analyzed in this manner (resulting data points are indicated as 'x' in chromatograms). Numerical values for 'Relative Amount' are relative to a 4 mg/ml standard corresponding to the oligosaccharide that was included in each TLC run. Percent values listed across the top x-axis represent mobile phase gradient composition (% ethanol). Purified oligosaccharide preparations were obtained by pooling the fractions within the shaded region of each chromatographic peak.

commercially available GOS standards are homogeneous molecules. The spectra of the prepared FOS and XOS samples matched those of the standards for the presumed compounds (see Fig. S2, **A** and **C**). The spectra of the prepared GOS-DP3 samples matched those recently published by van Leeuwen et al. (2014a; 2014b), where a heterogenous starting material, relative to glycosidic linkages, was also used (see Fig. S2, **B**).

Table 2
Total carbohydrate content of oligosaccharide preparations based on anthrone/sulfuric acid-based spectrophotometric quantification.

Targeted Oligosaccharide [a]	Percent Carbohydrate [bc]	Assay Parameters	
		Spectrophotometer Wavelength (nm)	Calibration Standard composition [d]
FOS DP3	98.3 ± 1.49	672	1 Glucose : 2 Fructose
FOS DP4	99.1 ± 0.19	672	1 Glucose : 3 Fructose
GOS DP3	100.6 ± 0.74	672	1 Glucose : 2 Galactose
GOS DP4	99.9 ± 1.89	672	1 Glucose : 3 Galactose
XOS DP2	99.7 ± 0.51	465	Xylose
XOS DP3	100.8 ± 1.55	465	Xylose
XOS DP4	100.2 ± 0.29	465	Xylose

[b]All color-development reaction mixtures contained 1 ml aqueous carbohydrate solution in 4 ml reagent solution (reagent solution: 0.1% (w/v) anthrone in 98% H_2SO_4 with or without added thiourea) and were reacted.
[c]Percent carbohydrate values are means per ± SD in triplicate, calculated based on dry weight basis.
[a] FOS = fructooligosaccharide; GOS = galactooligosaccharides; XOS = xylooligosaccharide.
[d] Calibration standards were made up with the following ratios of monosaccharides.

Table 3
Moles of oligosaccharide per unit weight and average degree of polymerization (DP) of purified prebiotic oligosaccharide preparations.

Targeted Oligosaccharide [a]	Moles of oligosaccharide per 100 g oligosaccharide preparation [b]	Average molecular weight [c] (g/moles)	Average DP [d]
FOS DP3	0.201 ± 0.003	497.5	3.0
FOS DP4	0.155 ± 0.009	645.2	3.9
GOS DP3	0.198 ± 0.002	505.1	3.0
GOS DP4	0.163 ± 0.003	613.5	3.7
XOS DP2	0.347 ± 0.005	288.2	2.0
XOS DP3	0.247 ± 0.012	404.9	2.9
XOS DP4	0.182 ± 0.020	549.5	4.0

GOS/FOS: 180 g/mol for a single hexose unit + n (162 g/mol remaining hexose units) = 'Average molecular weight'; 'Average DP' = n + 1.
XOS: 150 g/mol for single xylose unit + n (132 g/mol remaining xylose units) = 'Average molecular weight'; 'Average DP' = n + 1.
[a] FOS = fructooligosaccharide; GOS = galactooligosaccharides; XOS = xylooligosaccharide.
[b] Values are means per ± SD in triplicate, calculated based on dry weight basis based on quantification of terminal residues.
[c] Average molecular weight calculated as 100 g of sample divided by the Moles per 100 g oligosaccharide preparation.
[d] The following equations were used to calculate the average DP of the different oligosaccharide preparations.

4. Conclusion

This study is the first to address the important issue of obtaining relatively low-cost, size-defined prebiotic oligosaccharides suitable for human testing. Here we show that such oligosaccharides can be obtained via chromatographic fractionation of commercially available food-grade prebiotic oligosaccharide mixtures using microcrystalline cellulose stationary phases and aqueous-ethanol mobile phases. The specifics of productive chromatographic conditions differ depending on

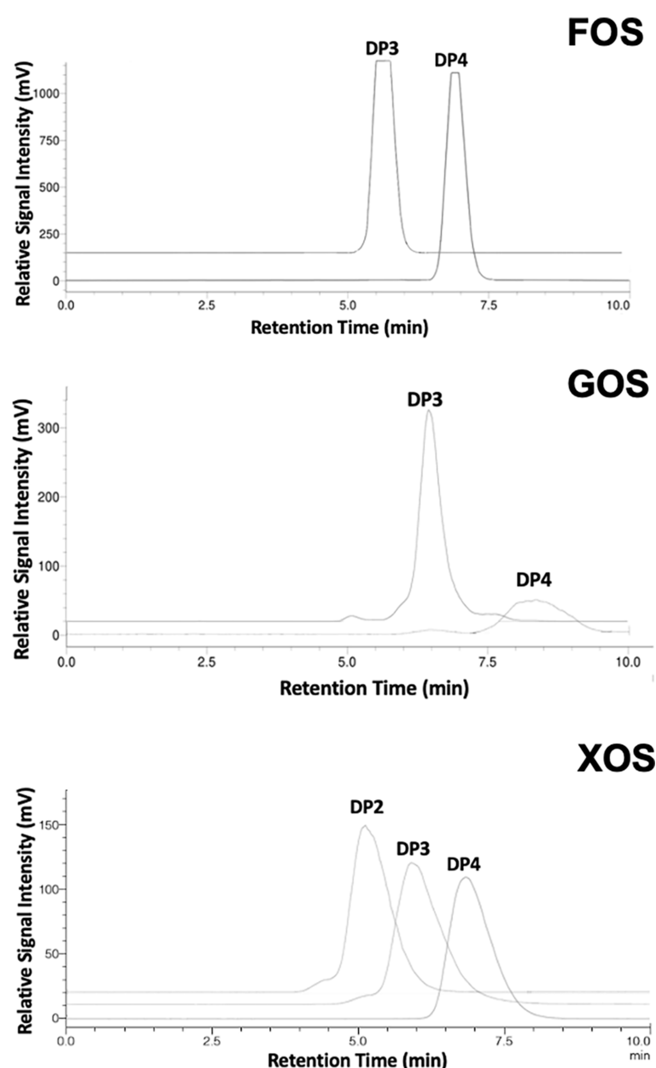

Fig. 3. Chromatograms from HPLC-ELSD depicting the fractionated oligosaccharides. FOS = fructooligosaccharide, GOS = galactooligosaccharide, XOS = xylooligosaccharide, DP = degree of polymerization.

the class of oligosaccharides being fractionated. This approach is shown to be successful in obtaining FOS, GOS and XOS of DP3 and DP4. XOS of DP2 (xylobiose) is also readily prepared using the described method; FOS and GOS of DP2 were not a focus of this study since they are readily available as relatively high purity food-grade products (FOS-DP2 is sucrose; GOS-DP2 is lactose). Importantly, the fractionation was focused on stimuli in the DP range of 2–4. Available data suggest that in the case of maltooligosaccharides DP2 and DP3 are sweet, while DP4 and longer are not (Pullicin et al., 2017). With the methods outlined here, we were able to successfully produce DP-defined fractions of FOS, GOS, and XOS in sufficient quantities for sensory testing (Lapis, Penner, & Lim, 2016; Pullicin et al., 2017).

The second objective of this study was to develop analytical methods for the characterization of size-defined prebiotic oligosaccharides differing with respect to constituent composition; the focus again being on FOS, GOS and XOS. A series of methods were outlined for measuring the total carbohydrate content, moles per unit weight and DP of each of the aforementioned classes of oligosaccharides. Furthermore, it is shown how HPLC and NMR can be used in a complimentary manner to further establish each preparations' purity and identity. The combined methods presented herein provide an excellent starting point for the economical preparation of size-defined, physiologically relevant, prebiotic oligosaccharides for use in human sensory testing. Such sensory study would be critical to understand how chemical properties of oligosaccharides (e.g., glycosyl residues, DP) alter their taste properties.

CRediT authorship contribution statement

Megan C.Y. Ooi: Investigation, Formal analysis, Visualization, Writing – original draft. **Xiaojie Zhang:** Investigation, Formal analysis. **Christopher M. Beaudry:** Methodology, Writing – review & editing. **Juyun Lim:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Michael H. Penner:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131542.

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