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ARTICLE

In-Depth Characterization of Prebiotic Galacto-oligosaccharides by a Combination of Analytical Techniques

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A commercial prebiotic galacto-oligosaccharide mixture (Vivinal GOS) was extensively characterized using a combination of analytical techniques. The different techniques were integrated to give complementary information on specific characteristics of the oligosaccharide mixture, ranging from global information on degree of polymerization (DP) to the identity and concentration of individual oligosaccharides. The coupling of high-performance anion-exchange chromatography (HPAEC) to mass spectrometry (MS) was determined to be especially suitable to assign the DP of individual oligosaccharides on the basis of their *m*/*z* values as well as their quantification using external standards. The combination of NMR spectroscopy and methylation analysis after isolation using size exclusion chromatography (SEC) and hydrophilic interaction liquid chromatography (HILIC) was used for identification. All DP2 compounds could be identified and quantified in this way as well as the main DP3 compounds.

KEYWORDS: Galacto-oligosaccharides; prebiotics; identification; quantification; high-performance anion-exchange chromatography; mass spectrometry; NMR; HILIC; GC-MS; analysis

INTRODUCTION

The analysis of complex oligosaccharide mixtures is still a challenge in the field of analytical chemistry. Various analytical techniques have been applied to complex mixtures of oligosaccharides in the literature, the choice of analytical technique depending on the required level of detail, the type of carbohydrate product, and perhaps availability (*1*, *2*). Due to the nature of carbohydrates and the characteristics of the synthesis of oligosaccharides, mixtures of oligosaccharides may contain hundreds of individual compounds including many isomers. These compounds might differ in the type, number, and order of monomers in the chain and in the linkages between them.

Galacto-oligosaccharides (GOS) are nondigestible oligosaccharides, and prebiotic properties of these oligosaccharides have been established in several studies, both in vitro (*3*) and in vivo (*4*). The consensus is that these substrates have a selective stimulatory effect on bifidobacteria (*5*). GOS is produced by the enzymatic transgalactosylation of lactose using β-galactosidase (lactase, EC 3.2.1.23), giving several oligomers of different chain lengths (*6*), ranging from DP2 to DP10 with a terminal glucose. The main raw material for the commercial production of GOS is whey-derived lactose (*7*). Whey-derived lactose is formed in large amounts as a product of the dairy industry. The structures and product ratio of GOS are dependent on the source of the enzyme, substrate concentration, pH, and temperature (*6*, *8*). In aqueous systems, transgalactosylation has to compete with hydrolysis, and as a result GOS mixtures always contain significant amounts of unreacted lactose and monosaccharides (*6*, *9*).

Although GOS are known to comprise a number of isomeric di-, tri-, tetra-, and pentasaccharides, the structures of these compounds have not been investigated in detail. The structural analysis of GOS has been a subject in several papers. Many of these papers focus on the synthesis and production of GOS using various enzymes and conditions, but the analysis of GOS in these studies is only a means of getting a rough description of the product or to determine a few specific compounds. Methods most often used for the analysis of GOS are high-performance anion-exchange chromatography (HPAEC) for separation (*10*−*17*) and NMR and methylation GC-MS for structural identification (*5*, *10*, *11*, *16*−*20*). Besides these more common analytical techniques for galacto-oligosaccharides, a range of other more infrequently applied techniques have been reported more recently, such as GC-MS after oximation and silylation (*21*) or LC-based separations other than HPAEC (*12*, *19*, *20*, *22*). However, none of these studies were aiming at identifying and quantifying all compounds in GOS. With that perspective the studies by Kimura et al. (*23*) and Fransen et al. (*10*) are especially interesting; these authors put quite an effort into the structure elucidation of individual oligosaccharides in GOS. The methods used by these authors to analyze their GOS products include a chromatographic separation of GOS followed by characterization or identification of compounds of interest with ^{13}C NMR or methylation analysis (GC-MS). Especially the work of Kimura et al. (*23*) must be mentioned here, in which carbohydrates are derivatized prior to analysis. However, this derivatization step is used only by these authors, and therefore it is very

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difficult to use their data for comparison to the literature or vice versa.

In this study several analytical techniques were combined to obtain in-depth information on the composition of a commercial prebiotic GOS mixture, including the identification and quantification of individual GOS.

MATERIALS AND METHODS

Materials. Commercially available and relevant reference compounds, including lactose, galactose, and glucose, of carbohydrates were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Reference compounds of β-D-galactose-(1→4)-β-D-galactose and β-D-galactose-(1→3)-β-D-galactose were obtained from Megazyme International Ltd. (Wicklow, Ireland). The prebiotic galacto-oligosaccharide product Vivinal GOS was supplied by FrieslandCampina Domo (Zwolle, The Netherlands) and consists of approximately 75% dry matter (DM).

Size Exclusion Chromatography (SEC). SEC analyses and preparative fractionations were performed on an AKTA explorer 100 system (GE Healthcare, Diegem, Belgium) equipped with a refractive index (RI) detector and a fraction collector. An XK 50/100 column (GE Healthcare) equipped with a thermostatic jacket was packed with 1800 mL of Biogel P2 fine (Bio-Rad, Veenendaal, The Netherlands). The bed height was 92.5 cm. Aliquots of 2.6 g of GOS (~2 g of dry matter) were diluted to 20 mL with Milli-Q water and applied to the column using a 50 mL superloop (GE Healthcare) The column was eluted with Milli-Q water at a flow rate of 2 mL/min at 60 °C (0.8 mL/min on sample application). DP fractions with a volume of 10 mL were collected and subsequently freeze-dried.

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC-PAD analyses were performed on a DX-500 BIO-LC system (Dionex, Sunnyvale, CA), equipped with a PAD [E_1 = 0.1 V (t_1 = 500 ms), E_2 = 0.6 V (t_2 = 80 ms), E_3 = −0.6 V (t_3 = 50 ms)] and a Kontron 560 autosampler (Beun de Ronde, Abcoude, The Netherlands). Oligosaccharide fractions (50 μL aliquots) were separated on CarboPac PA1 column with dimensions 250 mm × 4 mm i.d. (Dionex) with a CarboPac PA1 guard column with dimensions 50 × 4 mm i.d. (Dionex) at a flow rate of 1 mL/min at 22 °C. The eluents used for the analysis were (A) 500 mM NaOAc + 100 mM NaOH, (B) 100 mM NaOH, and (C) Milli-Q water. Two different gradients were used. In *gradient 1*, eluents A and B were mixed to form the following gradient: 100% B from 0 to 5 min followed by 0−26% A in 73 min. After each run, the column was washed with 100% A for 6 min and re-equilibrated for 10 min at 100% B. For improved separation of some DP2 peaks an isocratic flow of eluents B and C 15:85% v/v was applied for 67 min followed by a wash step with 100% A for 6 min and re-equilibration at 15% B:85% C for 17 min (*gradient 2*).

HPAEC-MS. The HPAEC-MS system was setup using an LTQ linear ion-trap system consisting of a Surveyor AS autosampler, a Surveyor MS pump, and an LTQ LT-10000 mass detector with an Opton ESI probe (Thermo Fisher Scientific, San Jose, CA).

The HPAEC separation was performed on a Carbopac PA1 column with dimensions 250 mm × 2 mm i.d. (Dionex) operated at 30 °C using the same eluents and gradients as described earlier for HPAEC-PAD. The flow rate was 215 μL/min, and the injection volume was 3 μL. An ASRS-2 mm suppressor (Dionex) was used as an in-line desalter to convert the eluate into an ESI-compatible solution. The membrane was continuously regenerated with acid generated by electrolysis of water. Milli-Q water was fed from an air-pressurized bottle into the regenerant chamber at a flow rate of ~6 mL/min. A regenerant current of 150 mA was applied.

The flow was reduced to approximately 50 μL/min prior to MS detection using a T-split and a restriction column. Mass detection was carried out using electrospray ionization in both positive and negative ionization modes (ESI spray voltage, 3.5 kV; heated capillary temperature, 275 °C; sheath gas, 30; auxiliary gas, 5; full scan range, m/z 150−2000; number of microscans, 3; maximum injection time, 200 ms).

HPAEC-PAD after Hydrolyis. Oligosaccharides were hydrolyzed at 90 °C for 1−2 h to monosaccharides in sulfuric acid (0.8 M). After hydrolysis, the samples were diluted 40 times prior to analysis with HPAEC-PAD. HPAEC-PAD equipment was the same as described above. Oligosaccharide fractions (50 μL aliquots) were separated on a CarboPac MA1 column with dimensions 250 mm × 4 mm i.d. (Dionex) with a CarboPac MA1 guard column with dimensions 50 mm × 4 mm i.d. (Dionex) at a flow rate of 0.4 mL/min at 22 °C. An isocratic system with 65% of Milli-Q water and 35% of 1000 mM NaOH was used during 60 min.

Table 1. DP Composition (Weight Percent) of GOS Obtained from SEC and Gal/Glc Ratio Obtained from HPAEC-PAD after Hydrolysis

	wt %	Gal/Glc (mol)
GOS		0.9
DP1	22.5	0.1
galactose	1.3	
glucose	21.2	
DP2	37.4	1.1
lactose	10.0	
other	27.4	
DP3	22.1	2.0
DP4	10.8	2.9
DP5	4.9	3.6
DP6	1.9	4.6
DP7	0.6	5.3
DP8	0.2	6.5

Hydrophilic Interaction Liquid Chromatography (HILIC). Preparative HILIC fractions for DP2 and DP3 were obtained using an AKTA explorer 100 system (GE Healthcare) equipped with an RI detector and a Frac950 fraction collector. Aliquots of 100 mg of each dried DP fraction were dissolved in 5 mL of Milli-Q water and diluted with acetonitrile to the corresponding eluent concentration; after dilution, the temperature was readjusted to 21 °C. Twenty-five milliliters of diluted DP fraction was separated on a Prevail Carbohydrate ES column with dimensions 300 mm × 20 mm i.d., 9 μm (Grace-Alltech, Deerfield, IL) using a 50 mL superloop (GE Healthcare) The column was eluted isocratically at a flow rate of 10 mL/min at 21 °C. The eluents used for the DP2 and DP3 fractions were acetonitrile/H_2O = 80:20 (v/v) and acetonitrile/H_2O = 76.5:23.5 (v/v), respectively. Fractions with a volume of 10 mL were collected. The fractions for each peak of up to eight runs were combined. The acetonitrile was evaporated using a rotatory evaporator prior to freeze-drying.

Methylation Analysis. Linkage analysis was performed by analysis of the partially methylated alditol acetates (PMAA). The oligosaccharides were first reduced using an aqueous solution of $NaBH_4$ for 2 h at room temperature. After neutralization with acetic acid and removal of boric acid by repeated coevaporation with methanol, the oligosaccharides were permethylated using CH_3I and solid NaOH in DMSO, as described by Ciucanu and Kerek (*24*). After hydrolysis with 2 M TFA (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBH_4$ as described above, followed by acetylation with 1:1 acetic anhydride/pyridine (2 h, 120 °C). After evaporation to dryness, water was added, and the final product was obtained by liquid−liquid extraction with ethyl acetate, which was dried with anhydrous Na_2SO_4 before analysis. The PMAAs were analyzed by GC-MS (Focus PolarisQ, Thermo Fisher Scientific) on a DB-225 capillary column with dimensions 30 m × 0.25 mm i.d., 0.25 μm (J&W Scientific, Folsom, CA) held at 50 °C during injection and then programmed at 40 °C/min to 215 °C and held for 40 min. Helium at a constant flow of 2 mL/min was used as carrier gas. Assignment of linkages was carried out using reference compounds.

NMR. ^{1}H NMR and ^{13}C NMR experiments were performed on a Bruker DMX-600 spectrometer equipped with a TCI-zGrad Cryoprobe (Bruker, BioSpin, Bremen, Germany). Prior to NMR analysis, the lyophilized samples (minimum ~10 mg) were dissolved in D_2O (99.97 atom % D, Euriso-Top) and lyophilized, and each residue was dissolved in 0.55 mL of D_2O. The probe temperature was 25 °C. Chemical shifts are expressed in parts per million downfield from the signal for 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured relative to that of the internal standard acetone (δ 2.225 at 25 °C for ^{1}H and δ 31.55 at 25 °C for ^{13}C). HDO-suppressed 1D ^{1}H NMR spectra were recorded with a sweep width of 8992 Hz at 600.13 MHz in data sets of 64K points and 32 transients. Proton-decoupled ^{13}C NMR spectra were acquired at 150.92 MHz. Typically, 512 transients of 64K data points with a sweep width of 34.722 Hz and a relaxation delay of 5 s were recorded. Signals in ^{1}H NMR and ^{13}C NMR spectra were assigned by comparison with the literature using a homemade database.

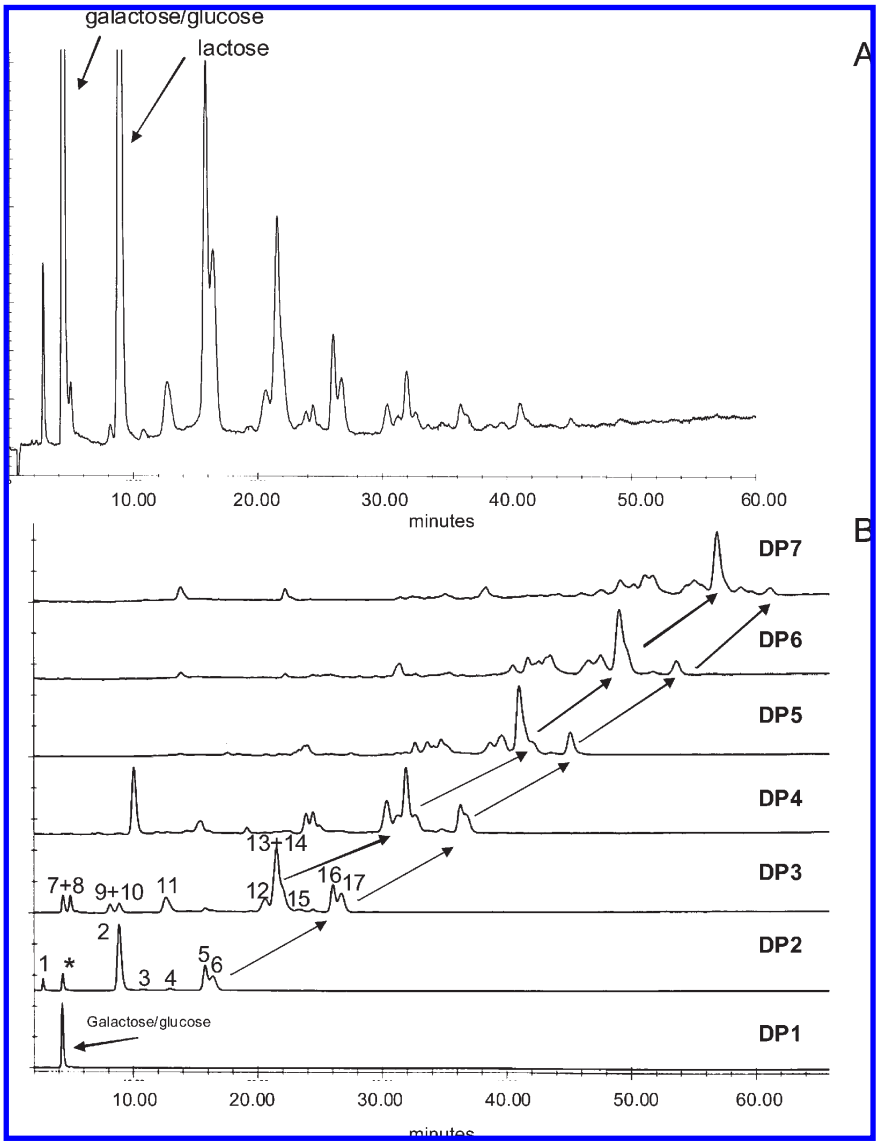

Figure 1. HPAEC-PAD chromatograms of (**A**) GOS and (**B**) DP fractions obtained by SEC.

RESULTS AND DISCUSSION

SEC. GOS were first separated by SEC to determine the distribution in the degree of polymerization (DP) and to obtain DP fractions to be used for further analysis. With RI detection a nicely separated distribution of peaks could be observed, starting from monosaccharides (DP1) at high elution volumes to higher oligosaccharides at lower elution volumes (data not shown). From the SEC-RI profile the distribution of DPs in GOS was calculated (**Table 1**). It can be seen from **Table 1** that the amount of monosaccharides in GOS was around 22%, disaccharides including lactose 37%, DP3 22%, and DP4 11%. Higher DPs are present below 5%, and up to DP7 could be reliably quantified. Traces of DP8−DP10 are present in GOS, but quantification was difficult due to the low response. The amounts presented in **Table 1** for DP8 should be considered as maximum amounts. Fractions up to DP8 were collected for further analysis.

HPAEC-PAD. Analysis of GOS by HPAEC-PAD shows a complex pattern of peaks, which decrease in intensity with increasing retention time as can be seen in **Figure 1**. Only a few peaks could be identified on the basis of reference compounds, that is, glucose, galactose, and lactose. **Figure 1** also shows the HPAEC-PAD chromatograms of DP1−DP7 SEC fractions from which it can be clearly seen that the complexity of the sample increases dramatically with increasing DP. For DP2 approximately 7 peaks can be observed, and for DP3 already more than 12, whereas for DP6 and DP7 a very broad distribution of many coeluting peaks is visible. This emphasizes the large number of oligosaccharides present in GOS. Interestingly, it can also be seen from **Figure 1** that there are similarities in the peak patterns between the different DP fractions, which indicate the presence of homologue oligosaccharide series.

One drawback of HPAEC-PAD is the lack of more specific chemical information that can be obtained from the PAD detector. For example, it is not possible to verify the DP of the peaks observed in **Figure 1**, and further separation by SEC followed by HPAEC-PAD analysis is necessary to obtain this information. A solution to this problem is the coupling of HPAEC and mass spectrometry (HPAEC-MS).

HPAEC-MS. HPAEC-MS has only been described a few times in recent literature and is a promising technique to obtain information on molecular mass and monosaccharide composition, although the level of information that can be obtained highly depends on the specific application (*28*−*30*).

A HPAEC-MS system was set up on the basis of existing LC-MS equipment. To successfully apply this method, it is essential that the salts from the mobile phases are removed prior to MS

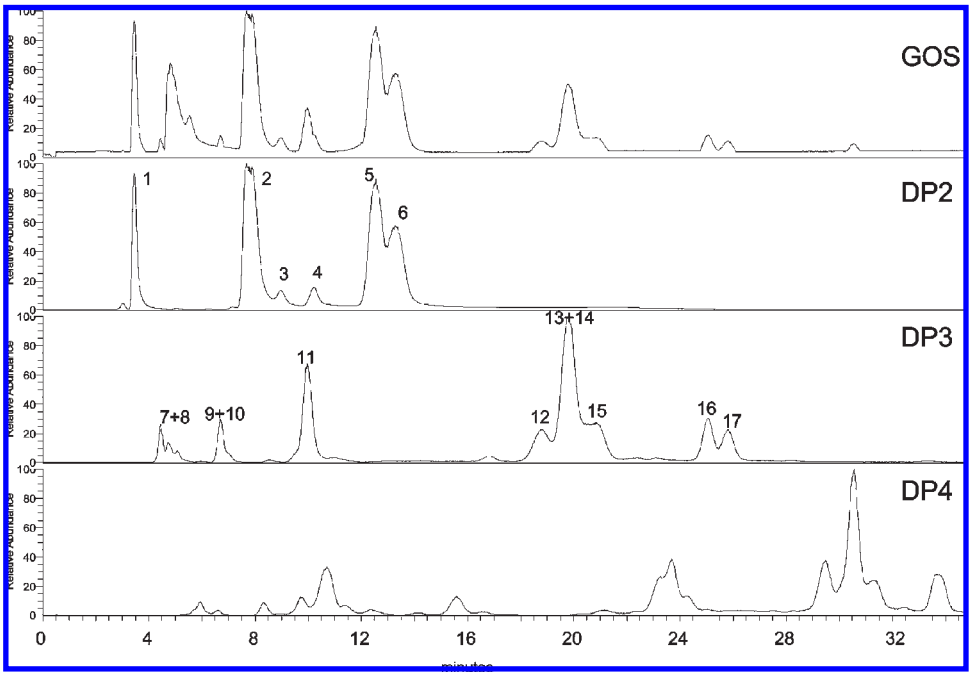

Figure 2. HPAEC-ESI-MS base peak chromatogram of GOS and extracted ion chromatograms ($[M + Na]^+$) corresponding to DP2 (*m*/*z* 365.4), DP3 (*m*/*z* 527.4), and DP4 (*m*/*z* 689.6).

detection. This is done using a suppressor that exchanges Na^+ ions with H^+ions. **Figure 2** shows a base peak HPAEC-MS chromatogram of GOS using ESI in the positive ionization mode. A similar chromatogram was obtained for ESI in the negative ionization mode but with lower sensitivity. In the positive ionization mode the main ions observed are $[M + H]^+$ and $[M + Na]^+$. It can be seen in **Figure 2** that the pattern of peaks observed with HPAEC-MS is very similar to that observed with HPAEC-PAD (**Figure 1**). The small differences in retention time are mainly due to different column dimensions and system volumes. The mass spectrometer gives molecular mass information allowing focusing on oligosaccharides with a certain DP by extracting their *m*/*z* related ions. **Figure 2** shows such extracted ion HPAEC-MS chromatograms for *m*/*z* values corresponding to DP2, DP3, and DP4 in GOS. These extracted ion chromatograms again show very similar patterns of peaks as compared to the HPAEC-PAD chromatograms for the SEC fractions corresponding to DP2, DP3, and DP4 SEC fractions (**Figure 1**). Close inspection of the HPAEC-PAD and HPAEC-MS chromatograms of DP2 shows that one of the peaks observed in the SEC DP2 fraction with HPAEC-PAD is glucose/galactose (*, **Figure 1**). Also, trace amounts of DP1 and DP3 compounds can be present after SEC fractionation, complicating the interpretation of HPAEC-PAD chromatograms. HPAEC-MS again can easily detect the presence or absence of DP1 and DP3 in DP2 oligosaccharides, and therefore annotation of individual peaks to a specific DP is very straightforward.

In a similar way it can be shown that DP3 and DP4 peaks eluting around the same time as DP2 peaks (**Figures 1** and **2**) are really DP3 and DP4 oligosaccharides and are not due to contamination of these fractions with lower DP fractions or artifacts as a result of sample preparation.

The advantage of HPAEC-MS is that the DP assignment of all oligosaccharides in a mixture can be done in a single experiment compared to HPAEC-PAD, for which SEC fractionation is required. Another advantage of HPAEC-MS is its suitability to quantify oligosaccharides, which will be described later.

The complexity of GOS is further demonstrated by **Figure 3**, which shows an extracted chromatogram corresponding to DP2 using HPAEC gradient 2, and it can be observed that peak 2 in **Figures 1** and **2** can be in fact assigned to three different compounds, one of them being lactose. Hence, it can be concluded that the DP2 fraction of GOS contains eight different peaks.

HPAEC-PAD after Hydrolysis. Acidic hydrolysis followed by HPAEC-PAD analysis was carried out for GOS and the DP fractions obtained by SEC to determine their Gal/Glc ratio. Because the single building block for the synthesis of GOS is lactose, that is, Gal-(1→4)-Glc, it is anticipated that only Gal and Glc units were present with a Gal/Glc ratio close to 1. **Table 1** shows that this indeed seems to be the case while also confirming that the galacto-oligosaccharides are elongated by the incorporation of Gal, leading to an increase in Gal/Glc ratio with increasing DP.

HILIC. Separation of GOS by SEC resulted in fractions of oligosaccharides with different DPs. However, analysis by HPAEC showed that within a DP fraction many compounds can be present. To facilitate the identification of oligosaccharides, further separation of DP fractions is necessary. Separation of DP fractions by HILIC, using a polar stationary phase with a mobile phase gradient from acetonitrile to water, resulted in triplets of peaks, indicating that HILIC separates within a DP fraction on properties other than molecular weight (data not shown). HILIC separation of DP fractions could easily be scaled up due to the absence of any salts in the mobile phases used.

The preparative HILIC fractions were subsequently analyzed by HPAEC-PAD to correlate the peaks in these fractions with the original patterns of peaks observed for the DP fractions after SEC with HPAEC-PAD. **Figure 4** shows stacks of HPAEC-PAD chromatograms for the SEC fraction and HILIC fractions of DP2. It can be clearly seen that separation of DP2 with HILIC leads to a couple of fractions that contain only one or two major DP2 peaks. For example, fraction 5 contains mainly peak 2a, whereas fraction 3 contains mainly peak 5. Furthermore, the three peaks that coelute, that is, lactose, 2a, and 2b, are also separated from each other by HILIC. The presence of only one or two major peaks in the HILIC fraction facilitates further analysis of these fractions by methylation analysis and NMR to identify the individual DP2 peaks.

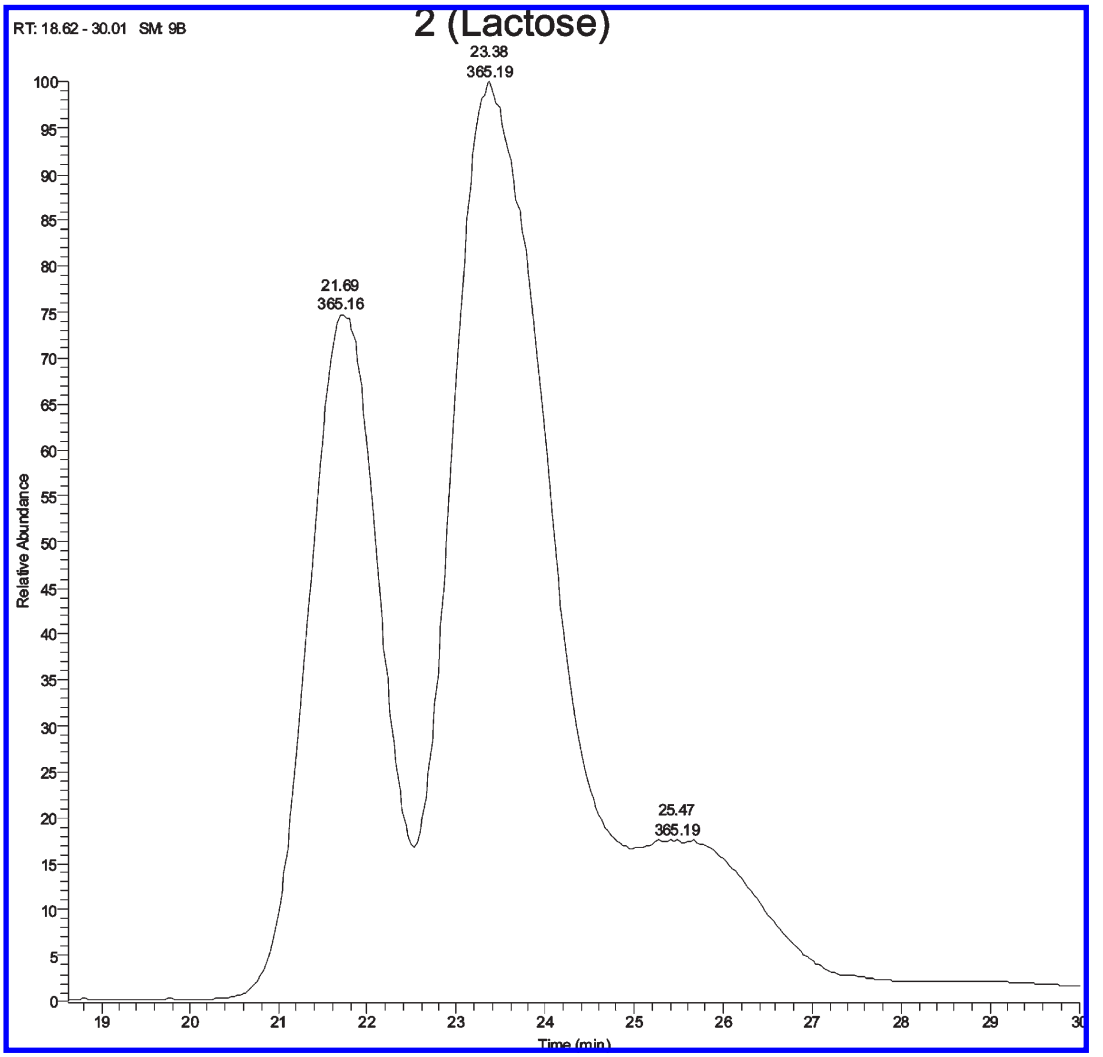

Figure 3. HPAEC-ESI-MS extracted ion chromatogram (*m*/*z* 365.4) of DP2 obtained with gradient 2.

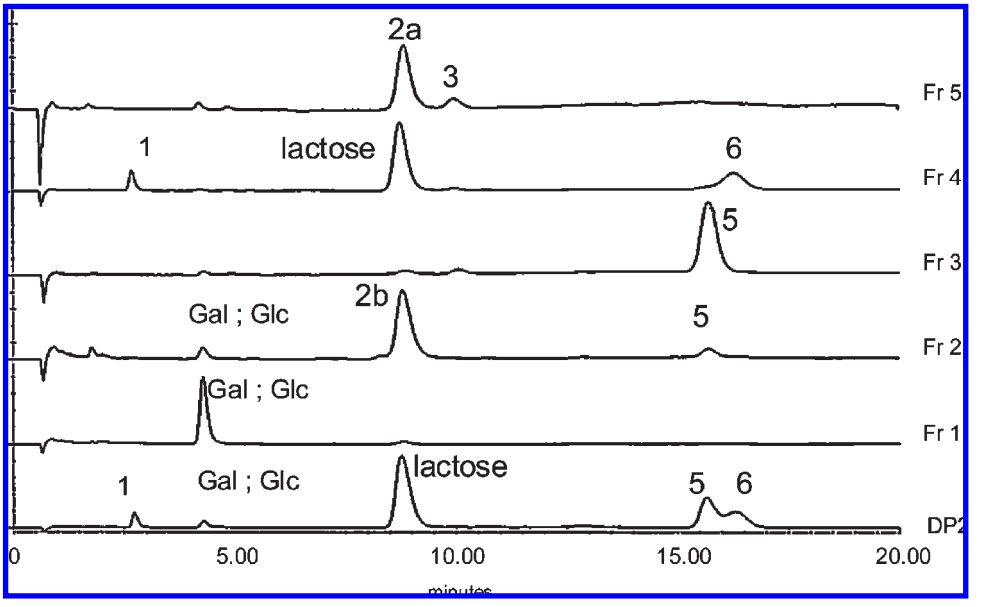

Figure 4. HPAEC-PAD chromatograms of the DP2 fraction of GOS and the HILIC fractions of DP2 (fractions 1−5).

HILIC separation of DP3 was carried out in a similar way, leading to four different fractions. The number of DP3 peaks in GOS is already significantly higher compared to DP2 (**Figures 1** and **2**). As a result, it is difficult to obtain HILIC fractions with

only one or two DP3 peaks due to coelution of peaks with HILIC. However, HILIC fractions containing the most abundant DP3 peaks were used for identification.

Identification. Methylation followed by GC-MS analysis was applied to GOS and DP fractions. **Table 2** shows the results of the methylation analysis of GOS and the DP fractions. It can be seen that the main structural elements in GOS are Gal-1 and 4-Gal-1, whereas for the reducing ends 4-Glc, 3-Glc, 6-Glc, and 2-Glc are present in almost equal amounts. This global view confirms partially the general structure of galacto-oligosaccharides in GOS as expected from the transgalactosylation of lactose, that is, Gal-(1→4)$_n$-Glc. Hereby it assumed that only linear structures are formed. The exception is the reducing ends, which are not only 4-Glc but also significant amounts of 3-Glc, 6-Glc, and 2-Glc. From the methylation analysis of the DP fractions it can be seen that this is caused by the low DPs, that is, DP2 and DP3, which show almost equal amounts of 2-Glc, 3-Glc, 4-Glc, and 6-Glc. Due to the relatively high amounts of DP2 and DP3 in GOS, that is, ~60 wt %, this is also reflected in GOS. For the higher DP fractions, that is, DP > 3, the main structural elements correspond to structures such as Gal-(1→4)$_n$-Glc, which are expected to be present in galacto-oligosaccharides synthesized by transgalactosylation of lactose. Note that fructose cannot be detected with this method and at least one compound, that is, lactulose, β-D-Gal-(1→4)-D-Fru, containing fructose is present, as will be described later. However, the contribution of fructose-containing compounds is expected to be small and should thus not have a large influence on the methylation GC-MS results.

Table 2. Relative Abundance of Structural Elements Present in GOS and DP Fractions As Determined by Methylation GC-MS

structural element	GOS	DP2	DP3	DP4	DP5	DP6	DP7	DP8
2-Glc	0.2	0.2	0.2	0.1	0.1			
3-Glc	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1
4-Glc	0.3	0.3	0.3	0.6	0.7	0.9	0.9	0.9
6-Glc	0.3	0.3	0.3	0.1	0.1			
Gal-1	0.7	0.7	0.9	0.9	1.0	1.0	1.0	1.0
Glc-1	0.3	0.3	0.1	0.1				
3-Gal-1					0.1	0.1		
4-Gal-1	0.9		0.9	1.0	0.9	0.9	1.0	1.0
6-Glc-1	0.1		0.1					
6-Gal-1								

Identification of individual DP2 and DP3 peaks in GOS was first carried out by analyzing commercially available reference compounds of relevant oligosaccharides. Although more than 25 reference compounds of oligosaccharides containing galactose and/or glucose were tested, only β-D-Gal-(1→4)-D-Glc (lactose, peak 2 in **Table 3**) and β-D-Gal-(1→4)-D-Gal (peak 4 in **Table 3**) could be identified in this way.

Next, HILIC fractions of DP2 and DP3 were analyzed by methylation analysis and NMR. Only fractions that contained one or two main components as observed by HPAEC-PAD analysis of these fractions were chosen. NMR data obtained were compared with reference data obtained from the literature, although comparison was severely hampered by differences in experimental conditions (*31*). Homemade software was developed to facilitate comparison with literature data. With this approach all eight DP2 peaks could be identified (**Table 3**).

From methylation analysis peak 1 could be identified as a compound containing Gal-(1↔1)-Glc. However, the ^{13}C NMR spectrum showed the presence of two compounds, one being β-D-Gal-(1↔1)-α-D-Glc on the basis of reference NMR data from the literature (*10*). The other compound could be identified as β-D-Gal-(1↔1)-β-D-Glc on the basis of the characteristic chemical shifts for β-configuration for the two anomeric C1 atoms at 100.50 and 101.09 ppm. Peak 2a could be assigned to β-D-Gal-(1→6)-β-D-Glc on the basis of methylation analysis and ^{13}C NMR data identical to data reported in the literature (*32*). The presence of β-D-Gal-(1→4)--Fru (peak 2b) and β--Gal-(1→3)-β--Gal (peak 3) could be confirmed by comparing ^{13}C NMR data of these peaks with data from Mayer et al. (*33*) and Messer et al. (*34*), respectively. The structures of peaks 5 and 6 could be assigned on the basis of methylation analysis. The structure of peak 5 was confirmed by comparing ^{13}C NMR data with the literature (*35*). For peak 6 no reference NMR data were available in the literature. However, chemical shifts at 82.00 and 82.57 ppm were observed, which are typical for glucose substituted at the C2 position. Moreover, the NMR spectrum showed great similarity with that of a trisaccharide containing β-D-Gal-(1→2)-D-Glc (*7*).

The identification of DP3 peaks was more difficult and less successful. However, the most abundant DP3 peaks could still be identified (**Table 3**). Peaks 7−10 could be assigned to compounds

Table 3. Identities and Quantities of DP2 and DP3 Compounds in GOS

peak	compound	wt % in DP2	wt % in DP2 GOS[a]
1	β-D-Gal-(1↔1)-α-D-Glc + β-D-Gal-(1↔1)-β-D-Glc	7	10
2	β-D-Gal-(1→4)-D-Glc (lactose)	27	
2a	β-D-Gal-(1→6)-D-Glc (*allo*-lactose)	15	21
2b	β-D-Gal-(1→4)-D-Fru (lactulose)	5	7
3	β-D-Gal-(1→3)-D-Gal	1	1
4	β-D-Gal-(1→4)-D-Gal	3	4
5	β-D-Gal-(1→3)-D-Glc	26	36
6	β-D-Gal-(1→2)-D-Glc	16	22

peak	compound	wt % in DP3 GOS
7 + 8	?[b]	6
9 + 10	?[c]	3
11	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc or β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc[d]	15
12	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc or β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc[e]	9
13 + 14	β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc + β-D-Gal-(1→4)-β-D-Gal-(1→4)-Fru[f]	45
15	β-D-Gal-(1→6)-[β-D-Gal-(1→2]-D-Glc	5
16	β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc	8
17	β-D-Gal-(1→4)-β-D-Gal-(1→2)-D-Glc	9

[a] Excluding lactose. [b] Total of four small peaks, all containing (1↔1) linkages in their structure. [c] Total of two small peaks, all containing (1↔1) linkages in their structure. [d] Coelution with one small unknown peak. [e] Coelution with two small unknown peaks. [f] Coeluting peaks.

containing (1↔1)-linkages on the basis of their short retention time with HPAEC (**Figure 1**). Peaks 16 and 17 could be identified on the basis of methylation analysis and comparison of the ^{13}C NMR data with those of Yanahira et al. (*7*). These compounds are the expected trisaccharides obtained by trans-galactosylation of β-D-Gal-(1→3)-D-Glc (peak 5) and β-D-Gal-(1→2)-D-Glc (peak 6) as could already be suggested on the basis of the patterns of peaks observed by HPAEC for DP2 and DP3 (**Figure 1**). Peak 13 could be identified as β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Glc primarily on the basis of methylation analysis and to some extent on NMR after comparison with the literature (*18*). Peak 13 coelutes with peak 14 in HPAEC, which could be assigned on the basis of interpretation of the ^{13}C NMR spectrum. ^{13}C NMR showed typical signals for β-D-Fru*p*, that is, 67.96 ppm (C5), 67.36 ppm (C3), 65.17 ppm (C1), and 64.30 ppm (C1), as well as signals for D-Fru*f*, indicating that fructose is at the reducing end. See, for comparison, the chemical shift observed for peak 2b, that is, 68.12 ppm (C5), 67.38 ppm (C3), 65.12 ppm (C1), and 64.33 ppm (C6). In addition, HPAEC analysis after hydrolysis showed only the presence of galactose but not glucose. The NMR spectrum showed great similarities with that of peak 2b, that is, β-D-Gal-(1→4)-D-Fru, especially when an additional β-D-Gal*p* is taken into account. Moreover, no signals typical for 1↔1, 1→2, or 1→3 linkages could be observed. Furthermore, the ^{13}C NMR spectrum was not similar to that of either β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Fru or β-D-Gal-(1→1)-[β-D-Gal-(1→4)]-D-Fru (*36*). Hence, the most likely structure for peak 14 is β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Fru. Peak 15 is another example of a peak for which no reference data were available in the literature and which could only be assigned by interpretation of the NMR spectrum. Comparison of NMR spectra showed that the NMR spectrum of peak 15 showed great similarity with those of peaks 6 and 2a. Typical signals were observed at 81.96 and 82.40 ppm, indicative of C2-subtituted glucose, and were also observed earlier for peak 6. This is supported by the fact that the isolated chemical shift for Glc-C2 (75.31 ppm) that was observed for peak 2a is not visible for peak 15. Furthermore, the absence of typical chemical shifts for nonsubstituted Glc-C6, as was found for peak 6 at 62.25 and 62.45 ppm, shows that the glucose in peak 15 is also C6-substituted. Hence, it was concluded that the most likely structure for peak 15 is β-D-Gal-(1→4)-[β-D-Gal-(1→2)]-D-Glc. For peaks 11 and 12 identification is complicated. Both peaks coelute in HPAEC with some smaller peaks and, more importantly, it was not possible to obtain clean NMR data from the HILIC fraction that could be used for identification. From methylation analysis of the HILIC fraction that contains peak 11 and 12, as well as some smaller peaks, it could be concluded that these peaks mostly contain the building blocks 4-Gal-1, Gal-1, and 6-Glc, whereas smaller peaks were visible for 6-Gal-1 and 4-Glc. Assuming linear structures, this leaves four possibilities, of which one was already assigned to peak 13. The three remaining possibilities are therefore mentioned as possible identities in **Table 3**. From the relative abundance of the different building blocks the most obvious and most abundant compound would be β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc, that is, *allo*-lactose (peak 2a) to which one Gal-(1→4) unit is added, although it cannot be said whether this is peak 11 or 12. The other, lower abundant, compound would then most likely be β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc, although the presence of β-D-Gal-(1→6)-β-D-Gal-(1→6)-D-Glc cannot be excluded. The last two structures suggest addition of Gal-(1→6) units, which is not impossible but less likely to take place during transgalactosylation of lactose.

From the results on the identification of individual DP2 and DP3 oligosaccharides it can be seen that the DP2 fraction of GOS contains six Gal-Glc compounds, two Gal-Gal compounds, and, interestingly, one Gal-Fru compound. Furthermore, most DP3 peaks had structures that can be explained by the elongation of DP2 compounds by the addition of Gal(1→4)-units.

Quantification. Quantifying oligosaccharides with HPAEC-PAD without reference materials is troublesome due to the strong differences in molar response factors. Due to the similarity in structure of galacto-oligosaccharides it was expected that the ionization efficiencies are very similar for these compounds. To demonstrate this, calibration curves of maltose oligosaccharides with different DPs and other di- and trisaccharides consisting of hexoses were analyzed by HPAEC-MS in the range of 10−250 μg/mL. The linearity of the calibration curves was generally good, that is, $r^2 > 0.998$. It was observed that the response strongly depends on the DP; however, the responses for different compounds with the same DP were rather similar (difference < 20%). Using a separate external standard for each DP, the total concentration of DP2, DP3, and DP4 was calculated in solutions containing SEC DP2, DP3, and DP4 fractions with known concentrations by adding all peak areas of peaks with the same DP. The actual concentrations of the SEC DP2, DP3, and DP4 solutions were 89, 93, and 201 μg/mL, respectively, whereas the calculated concentrations were 75, 95, and 183 μg/mL; that is, the calculated concentrations are close to the actual concentrations, that is, < 20%. Hence, it was concluded that for each DP a separate external standard should be used and that different compounds within a DP can be adequately quantified using the same external standard.

Next, quantification using HPAEC-MS was applied to determine the concentration of the individual DP2 and DP3 compounds in GOS (**Table 3**). For annotations of the different peaks see **Figures 1−4**. On the basis of the improved HPAEC separation and subsequent quantification of individual oligosaccharides a better estimate could be made for the DP2 content in GOS, that is, DP2 peaks excluding lactose (**Table 3**). It should be stressed that for higher DPs the number of peaks increases and, therefore, the chromatographic overlap increases, as can be seen in **Figure 2** and **Table 3** for DP3.

A commercial and complex prebiotic oligosaccharide mixture, GOS, was characterized in great detail using a combination of different analytical techniques. The integration of various sample preparation steps and different analytical techniques was successful for obtaining valuable information ranging from general characteristics to the identification and quantification of individual oligosaccharides. All DP2 oligosaccharides and the most abundant DP3 oligosaccharides could be identified and quantified in this way. Especially, the coupling of HPAEC to MS was found to be of added value and can be used to quantify individual oligosaccharides using a single external standard for each DP.

ABBREVIATIONS USED

SEC, size exclusion chromatography; HPAEC, high-performance anion-exchange chromatography; PAD, pulsed amperometric detection; MS, mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; GC-MS, gas chromatography−mass spectrometry; NMR, nuclear magnetic resonance; DP, degree of polymerization; ELSD, evaporative light scattering detector; GOS, galacto-oligosaccharides; ESI, electrospray ionization; LC, liquid chromatography; Gal, galactose; Glc, glucose.

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