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Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides

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ABSTRACT

Three different stationary phases (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridge hybrid (BEH) with trifunctionally bonded amide), operating at hydrophilic interaction liquid chromatographic (HILIC) mode, have been assayed and compared for the analysis of complex mixtures of galactooligosaccharides (GOS). Chromatographic methods have been optimized to obtain the best separation between two consecutive galactose containing standards and maltodextrins, measured on the basis of resolution. Influence of several factors such as chemical modifiers (formic acid, ammonium acetate and ammonium hydroxide), organic solvent and gradients of the mobile phases in the separation of oligosaccharides have been studied. The best results were achieved on the BEH amide stationary phase, using acetonitrile:water with 0.1% ammonium hydroxide as mobile phase, where the most of oligosaccharides were successfully resolved. Characteristic MS^2 fragmentation profiles of disaccharides containing galactose, glucose and/or fructose units with different linkages were evaluated and used for the characterization of di-, tri- and tetrasaccharides of three commercial prebiotic GOS mixtures (GOS-1, GOS-2 and GOS-3) by HILIC–MS^n. Similar qualitative and quantitative composition was observed for GOS-1 and GOS-3, whereas different linkages and abundances were detected for GOS-2. In general, (1 → 4) and (1 → 6) glycosidic linkages were the main structures found in GOS, although (1 → 2) and (1 → 3) linkages were also identified. Regarding molecular weight, up to pentasaccharides were detected in these samples, disaccharides being the most abundant carbohydrates.

1. Introduction

Galactooligosaccharides (GOS) are non-digestible neutral carbohydrates with the ability to manipulate the composition of colonic microflora in order to improve the gastrointestinal health [1,2]. These carbohydrates are enzymatically produced by transgalactosylation reactions of lactose catalyzed by β-galactosidases to give rise to galactose oligomers with a terminal glucose, with different glycosidic linkages and degrees of polymerization (DP). Depending on the enzymatic source used for their synthesis, the chemical structure of these oligosaccharides varies [3–5] and, consequently, their effect on gut microflora can change [6].

The characterization of GOS structures is a required and important task to understand their mechanism of action on human gut. However, structural analysis of GOS, that involves the determination of linkage position, monomeric composition and anomericity, is not straightforward considering the resulting complex mixtures, high number of isomers and scarce availability of standards.

In general, the analysis of oligosaccharides can be carried out either by spectroscopic, chromatographic, electrophoretic or spectrometric techniques depending on the required level of detail and the type of carbohydrate product [7]. Nuclear magnetic resonance (NMR) is a very useful technique for structural determination; however, a tedious purification step for each compound is required [8]. Chromatographic techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) usually coupled to mass spectrometry (MS), which provides qualitative and quantitative information of independent oligosaccharides, are the most widely used.

GC–MS is useful for the characterization and quantitation of low molecular weight carbohydrates (mono-, di- and trisaccharides) although a previous derivatization step is mandatory for their analysis [9,10].

Different operation modes of HPLC have been applied to the analysis of oligosaccharides. Low retention of underivatized carbohydrates is usually attained using reverse phase columns, whereas better separation can be achieved by high performance anion

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exchange chromatography (HPAEC) although complex profiles are obtained when families of oligosaccharides with different linkage variants are present [11].

Hydrophilic interaction liquid chromatography (HILIC) is gaining a great importance in the last years for the separation of polar compounds such as carbohydrates [12,13]. Partitioning of polar analytes between the bulk eluent and a water-rich layer partially immobilized on the stationary phase is the main retention mechanism described for HILIC [12], however, different functional groups can be present on the stationary phase giving rise to secondary interactions such as electrostatic [14,15]. Different stationary phases are currently used for this separation mode; silica particles or monolithic supports either modified with aminopropyl, diol, zwitterionic or amide groups and polymer based packing, among others, can be found [16].

Sensitive detection of oligosaccharides after HPLC analysis represents an additional difficulty for their analysis. The absence of chromophore and fluorophore groups avoids their direct detection by UV or fluorescence detectors, whereas pulse amperometric detection (PAD), when coupled to HPAEC, is a suitable tool for oligosaccharide analysis [17] and has been applied for several applications. Nevertheless, the use of mass spectrometric (MS) detectors coupled to HPLC systems has considerably enriched the field of oligosaccharide analysis, allowing the determination of their molecular weight [18]. Multi-stage mass spectrometry (MS^n) can also provide structural information; however, scarce studies have been still carried out about its utility for the characterization of neutral oligosaccharides [19,20]. Moreover, the addition of appropriate metals to HPLC mobile phases to form complexes with carbohydrates or their previous derivatization (peracetylation or permethylation) is usually required to facilitate the sequential identification of residues by MS [21].

Characterization of different GOS has been generally carried out by the combination of a great variety of analytical methodologies (methylation analysis followed by GC–MS, NMR, HPAEC–PAD–MS, ESI–MS) with previous fractionation of the oligosaccharides (yeast treatment, SEC, HILIC) [7,8,20]. HILIC–MS has been used for the analysis of GOS previously fractionated by cation exchange chromatography to determine their molecular weights [22]. On the other hand, Fu et al. [23] used a "click" maltose column made in their laboratory to separate GOS. A good resolution among the different degrees of polymerization was obtained, however, no separation of isomers was observed.

In this manuscript three different HILIC stationary phases have been assayed to obtain the best separation of oligosaccharides. HILIC–MS methods have been optimized and applied to the analysis of different and complex commercial GOS mixtures. Characterization of their structures has been accomplished by MS^n without any previous modification of carbohydrate structure.

2. Materials and methods

2.1. Standards

1,3-Galactobiose (α-Gal-[1 → 3]-Gal), 1,4-galactobiose (β-Gal-[1 → 4]-Gal), 1,6-galactobiose (β-Gal-[1 → 6]-Gal), galactotriose (α-Gal-[1 → 3]-β-Gal-[1 → 4]-Gal), galactotetraose (α-Gal-[1 → 3]-β-Gal-[1 → 4]-α-Gal-[1 → 3]-Gal) were acquired from Dextra Laboratories (Reading, UK), whereas lactose (β-Gal-[1 → 4]-Glc), maltose (α-Glc-[1 → 4]-Glc), maltotriose ((α-Glc-[1 → 4])$_2$-Glc), maltotetraose ((α-Glc-[1 → 4])$_3$-Glc), maltopentaose ((α-Glc-[1 → 4])$_4$-Glc), nigerose (α-Glc-[1 → 3]-Glc), raffinose (α-Gal-[1 → 6]-α-Glc-[1 → 2]-β-Fru) and stachyose (α-Gal-[1 → 6])$_2$-α-Glc-[1 → 2]-β-Fru) were obtained from Sigma (St. Louis, USA), and lactulose (β-Gal-[1 → 4]-Fru), melibiose (α-Gal-[1 → 6]-Glc), and verbascose ((α-Gal-[1 → 6])$_3$-α-Glc-[1 → 2]-β-Fru) were from Fluka (Madrid, Spain).

Table 1
Mobile phases used to optimize the chromatographic methods on a zwitterionic, a polyhydroxyethyl aspartamide and a BEH amide column for the separation of oligosaccharides.

Solvents	Modifiers	Concentration
MeOH:H_2O	Ammonium acetate	5 mM
	Ammonium acetate[a]	0.1; 3.5; 5; 6.5; 20 mM
MeCN:H_2O	Ammonium hydroxide	0.1%
	Formic acid	0.1%

[a] Present in aqueous phase with the exception of 5 mM, where the salt was contained in both solvents.

2.2. Samples

Vivinal-GOS® (GOS-1) was kindly provided by Friesland Foods Domo (Zwolle, The Netherlands), BiMuno (Clasado, Reading, UK) (GOS-2) and Yum-Yum GOS™ (Jarrow Formula, USA) (GOS-3) were acquired in local markets.

2.3. HILIC–MS

GOS analyses were performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments, UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto, CA, USA) provided with an electrospray ionization (ESI) source. Samples (20 μL) were injected using a Rheodyne 7725 valve. Three columns and different conditions were used for the analyses: (i) sulfoalkylbetaine zwitterionic stationary phase (ZIC®-HILIC column; 150 × 2.1 mm, 3.5 μm particle size, 200 Å pore size, SeQuant™, Umea, Sweden) at a flow rate of 0.2 mL min^{-1}; (ii) polyhydroxyethyl aspartamide stationary phase (PolyHydroxyethyl-A column; 100 × 2.1 mm; 3 μm particle size, 300 Å pore size, The Nest Group, Inc., Southborough, MA, USA) at a flow rate of 0.4 mL min^{-1} and (iii) ethylene bridge hybrid (BEH) with trifunctionally bonded amide phase (XBridge column; 150 × 4.6 mm; 3.5 μm particle size, 135 Å pore size, Waters, Hertfordshire, UK) at a flow rate of 0.4 mL min^{-1}. Different binary gradients consisting of acetonitrile (MeCN):water or methanol (MeOH):water, with the addition of different modifiers as indicated in Table 1, were assayed for the three columns and optimized. The temperature of elution was kept at 35 °C for all cases.

The electrospray ionization source was operated under positive polarity using the following MS parameters: nebulizing gas (N_2) pressure 276 kPa, nitrogen drying gas at a flow rate of 12 L min^{-1} and 300 °C and capillary voltage of 4000 V. Ions corresponding to mono-sodiated adducts $[M+Na]^+$ of the oligosaccharides under analysis were monitored in SIM mode using default variable fragmentor voltages at the following *m/z* values: 365.0 (disaccharides), 527.0 (trisaccharides), 689.0 (tetrasaccharides) and 851.0 (pentasaccharides). Data were processed using HPChem Station software version 10.02 (Hewlett-Packard, Palo Alto, CA, USA).

Quantitative analysis was performed in triplicate by the external standard method, using calibration curves in the range 9.6–400 ng for maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose. Correlation coefficients were obtained from these calibration curves. Reproducibility of the method was estimated on the basis of the intra-day and inter-day precision, calculated as the relative standard deviation (*RSD*) of retention times and concentrations of oligosaccharide standards obtained in $n = 5$ independent measurements. Limit of detection (*LOD*) and limit of quantitation (*LOQ*) were calculated as three and ten times, respectively, the

signal to noise ratio (*S/N*), where *N* is five times the standard deviation of the noise [24].

2.4. HILIC–MS^n

These experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system coupled to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections (20 μL) were carried out by a Finnigan Surveyor autosampler. All instruments (Thermo Fisher Scientific, San José, CA, USA), and data acquisition were managed by Xcalibur software (1.2 version; Thermo Fisher Scientific).

The mass spectrometer spray voltage was set at 4.5 kV and the heated capillary temperature at 290 °C. Nitrogen (99.5% purity) was used as sheath (0.9 L min^{-1}) and auxiliary (9 L min^{-1}) gas, and helium (99.9990% purity) as the collision gas in the collision induced dissociation (CID) experiments. Mass spectra were acquired in the positive ion mode.

Fragmentation behavior of the oligosaccharides was studied by infusing a solution of each oligosaccharide (10 μg mL^{-1} in MeCN:water, 60:40, v/v) at a flow rate of 10 μL min^{-1} using the syringe pump included in the instrument and mixing it with 100 μL min^{-1} of MeCN:water (60:40, v/v) both with 0.1% ammonium hydroxide by means of a zero-dead volume T-piece. Sheath and auxiliary gases were set at 0.6 and 6 L min^{-1}, respectively. CID experiments were carried out by isolating each $[M+Na]^+$ ion in the ion trap (isolation width 1.0 *m/z*), and subjecting them to a normalized collision energy (NCE%) selected to preserve a signal of the precursor ion in the order of 5%. The process was repeated up to two times by successive isolation (isolation width 1.0 *m/z*) of the generated ions corresponding to the loss of a monosaccharide unit (loss of 162 u).

Separation of GOS samples was performed on the BEH column following the elution gradient optimized in Section 3.1 that uses MeCN (solvent A): water (solvent B) both with 0.1% ammonium hydroxide at 35 °C. Optimal separation of isomeric oligosaccharides was obtained by changing solvent A from 80% to 50% in 31 min and, then, kept for 5 min. Initial conditions were recovered after 0.1 min and were kept for 15 min before the following injection.

Considering that two different LC systems were used, slight differences in oligosaccharide separations were only observed in two chromatographic peaks. Bearing in mind the fragmentation study realized with standards by infusion in Section 3.2, the following *m/z* (and NCE%) were used in the HILIC–MS^n analysis of the samples: 365.1 (29%) for disaccharides, 527.2 (31%) > 365.1 (29%) for trisaccharides, 689.2 (32%) > 527.2 (31%) > 365.1 (29%) for tetrasaccharides.

Identifications of GOS mixtures were tentative in all cases considering the absence of commercial standards.

3. Results and discussion

3.1. Optimization of HILIC methods

Optimization of HILIC methods was based on the chromatographic behavior of (i) a homologous series of maltodextrins (DP2–DP7) and (ii) oligosaccharide standards containing galactose units, to assess the separation among carbohydrates of both different molecular weights and/or isomeric composition.

Evaluation of the methods was carried out on the basis of the shortest retention times (t_R), the best peak symmetry, calculated as the ratio of the front to back widths (at 50% of the peak height) and the highest resolution (R_s, calculated as $2(t_{R2} - t_{R1})/(w_{b1} + w_{b2})$, where 1 and 2 refer to two consecutive eluting carbohydrates and w_b is the peak width at base); R_s values should be higher than 1.0 to get an appropriate separation and peak symmetry close to 1 to get a good symmetry of the peaks. In those cases where α and β isomers appeared as unresolved peaks, PeakFit software (v4.12; SeaSolve Software Inc.) was used for peak deconvolution.

First of all, the effect of different modifiers and organic solvents was assayed in the three HILIC columns using a gradient based on the method proposed by Sinclair et al. [22] with some modifications (the organic solvent (solvent A) changed from 80% to 50% in 40 min) unless otherwise stated.

3.1.1. Effect of formic acid

The effect of 0.1% formic acid added to both solvents (MeCN and water) as mobile phase for the separation of oligosaccharides on the three HILIC stationary phases was firstly assessed. In all cases, reducing carbohydrates showed split peaks corresponding to α and β isomers. This effect has been described by different authors who suggested the use of basic pH to avoid the mutarotation of carbohydrates [25,26]. The homologous series of maltodextrins were well resolved under these conditions in polyhydroxyethyl aspartamide, BEH amide and zwitterionic columns ($R_s > 1$). However, broad peaks with poor symmetry (higher than 1) were obtained in the three columns tested; as an example, in polyhydroxyethyl aspartamide column the maltotriose eluted having a w_b of 0.91 min and a symmetry of 1.57. However, the appearance of two peaks per reducing carbohydrate impaired the separation of isomers showing, thus, a bad resolution among galactose containing oligosaccharides in the three columns (data not shown). Therefore, formic acid was discarded for further analyses.

3.1.2. Effect of ammonium acetate

Ammonium acetate is a widely used salt for operation with HILIC columns due to its solubility at high percentages of organic solvents [27,28]. Separation of standard oligosaccharides using ammonium acetate 5 mM present in aqueous and organic mobile phase (H_2O and MeCN) was evaluated in the three columns with dissimilar results.

All tested oligosaccharides were very poorly resolved under these conditions ($R_s \leq 0.6$) in the zwitterionic column with retention times varying from 3.97 min of lactulose to 4.84 min of verbascose. Moreover, split peaks corresponding to α and β isomers were obtained for reducing carbohydrates, probably because the pH (4.75 in the aqueous phase) was not basic enough to avoid mutarotation of carbohydrates.

Separation of maltodextrins using the polyhydroxyethyl aspartamide column showed better resolution than the ZIC-HILIC column. However, broad peaks and low symmetry values were found in the former (i.e. $w_b = 1.38$ min and the symmetry 0.63 for maltose).

On the other hand, good resolution was achieved for the homologous series of maltodextrins using the BEH amide column with resolution values higher than 1.0 and t_R of 20.1 min for maltose and 34.2 min for maltoheptaose. However, similarly to the results obtained for the zwitterionic column, split peaks were found for reducing carbohydrates.

Effect of methanol as solvent A instead of acetonitrile was also evaluated under these conditions as suggested by Sinclair et al. [22] for the three columns. Although t_R of oligosaccharides were substantially shorter than those obtained with acetonitrile (i.e. t_R of maltose using methanol in BEH amide column was 7.5 min and 20.1 min using MeCN), resolution values among all tested carbohydrates were very low for BEH amide ($R_s < 0.85$) and zwitterionic columns ($R_s < 0.14$). Coelution of all carbohydrates in a single broad peak was observed for the polyhydroxyethyl aspartamide column. This behavior can be due to the protic nature of both methanol and water, which compete to solvate the stationary phase and provide strong hydrogen bonding interactions with each other [16].

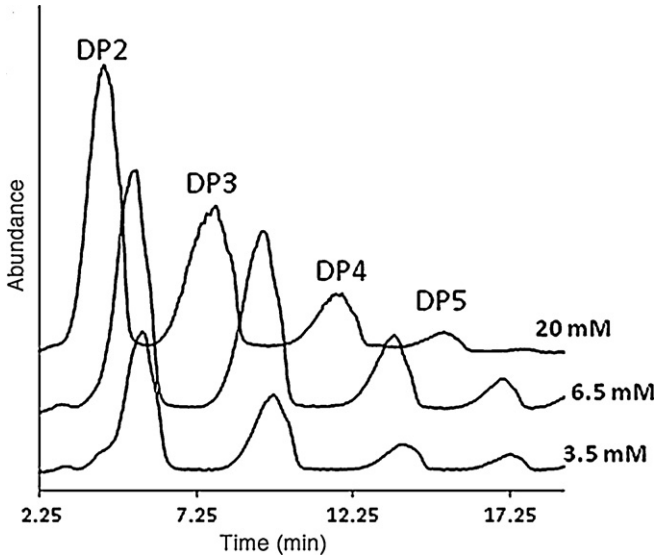

Fig. 1. HILIC profiles of maltodextrins (DP2–DP5) separated on a polyhydroxyethyl A column, using 3.5, 6.5 and 20 mM of ammonium acetate in the aqueous phase.

Therefore, the use of acetonitrile as mobile phase was selected for further studies.

As it was previously described by Alpert [12], HILIC retention is inversely proportional to the increase of salt concentration in the mobile phase. Therefore, four different concentrations (0.1, 3.5, 6.5 and 20 mM) of ammonium acetate only present in the aqueous phase were evaluated and, in consequence, the concentration of this salt increased as the water content rose. No substantial differences were detected among the different concentrations of salt for both zwitterionic and polyhydroxyethyl aspartamide columns. Fig. 1 shows the HILIC profile of maltodextrins obtained using the polyhydroxyethyl aspartamide column under these conditions. These profiles indicated that the order of elution of carbohydrates on these columns was not related to the salt content in the mobile phase. Likewise, no suppression of the MS signal was observed by increasing the salt concentration which could be explained by the high volatility of ammonium acetate. Therefore, an intermediate concentration of ammonium acetate (6.5 mM) in water mobile phase was selected. Similar results were observed by Strege [29] for the HILIC separation of polar compounds for drug discovery processes where only slight changes were detected between 0 and 3.3 mM buffer salt concentrations. Tolstikov and Fiehn [30] also used similar mobile phases for the analysis of polar compounds of plant origin in the polyhydroxyethyl aspartamide column; however, to the best of our knowledge, there are no data about the separation of different isomeric carbohydrates under these conditions in this stationary phase. Moreover, coelution of sucrose (DP2) and raffinose (DP3) was reported in the previous work, whereas oligosaccharides of different molecular weight could be separated under our optimized conditions (Fig. 1).

On the other hand, better resolution was obtained using a salt gradient than the elution method containing 5 mM ammonium acetate in both solvents, acetonitrile and water. Thus, resolution values were much higher using a salt gradient ($R_s \geq 1.6$) than those obtained using 5 mM ammonium acetate in both solvents ($R_s \leq 1.0$).

Different binary gradients using these mobile phases were assayed to optimize the separation of both maltodextrins and galactose containing oligosaccharides. For the zwitterionic column, the best results were obtained varying MeCN from 80% to 50% in 50 min. Although split peaks were obtained for reducing carbohydrates and their resolution (Table 2) was worse than that found using 0.1% formic acid. Carbohydrates without anomeric carbon (lactulose, raffinose, stachyose and verbascose) showed a single peak and a good resolution among them; however some of these peaks were not symmetric (Table 2). In general, separation of the standard oligosaccharides was carried out in function of increasing carbohydrate molecular weights, whereas the most retained isomeric carbohydrates were the oligosaccharides with $1 \rightarrow 6$ linkages.

Elution gradient was also optimized for polyhydroxyethyl aspartamide column and selected conditions were: solvent A kept at 80% for 3 min and changed to 50% for 40 min; under these experimental conditions, this stationary phase was unable to separate anomeric compounds and single peaks were detected in reducing carbohydrates. Similarly to the previous column, maltodextrins were eluted in the order of increasing molecular weight, with R_s values from 1.4 to 2.1 (Table 2), whereas among disaccharides, those with $1 \rightarrow 3$ and $1 \rightarrow 4$ glycosidic linkages were the first to elute followed by $(1 \rightarrow 6)$-linked carbohydrates. Although elution times ranged from 5.1 min of 1,4-galactobiose to 20.1 min of verbascose, broad peaks were obtained (i.e. 1,6-galactobiose: $w_b = 1.6$ min; galactotriose: $w_b = 1.8$ min; and so on) and resolution among them was poor (Table 2). Only peaks corresponding to (i) galactotriose and stachyose and (ii) galactotetraose and verbascose were well resolved, although only verbascose presented an acceptable symmetry (0.9).

Separation of oligosaccharide standards using BEH amide column using linear gradients of ammonium acetate at different concentrations was similar to that obtained under 5 mM ammonium acetate in both mobile phases (acetonitrile and water), contrary to the results obtained with polyhydroxyethyl aspartamide and zwitterionic columns where the salt gradient improved the separation of maltodextrins and galactose containing oligosaccharides as explained above. Moreover, splits peaks for reducing carbohydrates due to the separation of anomers were also detected using the BEH amide column (Table 2), showing a similar behavior than that found in the zwitterionic column.

3.1.3. *Effect of ammonium hydroxide*

To avoid the appearance of split peaks, 0.1% ammonium hydroxide was used in both mobile phases (MeCN and water). Although one single peak was obtained for each oligosaccharide, no satisfactory resolution was achieved under these conditions for the zwitterionic and polyhydroxyethyl aspartamide columns either for the separation of the maltodextrins or the galactose containing oligosaccharides (data not shown). However, these conditions resulted in a good resolution of maltodextrins on BEH amide column ($R_s \sim 4.8$). In this column, different binary gradients using MeCN and water as mobile phases containing both 0.1% ammonium hydroxide were assayed to optimize the separation of both maltodextrins and galactose containing oligosaccharides; the best results were obtained varying MeCN from 80% to 50% in 31 min, as previously reported by Brokl et al. [31] for the separation of fructooligosaccharides, gentiooligosaccharides and oligosaccharides from dextransucrase cellobiose acceptor reactions. Maltodextrins eluted within 34 min; t_R increasing with their molecular weight as consequence of the increase in hydrophilicity due to the increased number of hydroxyl groups. Wuhrer et al. [32] and Melmer et al. [33] reported a similar behavior of *N*-glycans in amide-based ligand columns. The galactose containing oligosaccharides eluted from 19.8 min of lactulose to 32.4 min of verbascose. Disaccharides with $1 \rightarrow 3$ and $1 \rightarrow 4$ linkages were the first to elute followed by carbohydrates with $1 \rightarrow 6$ glycosidic linkages. In general, resolution values were higher than 1, except for those between galactobiose $1 \rightarrow 4$ and $1 \rightarrow 3$; galactobiose $1 \rightarrow 3$ and lactose; and galactotriose and raffinose (Table 3). Therefore, BEH column under these elution conditions was selected for the analysis of commercial GOS mixtures.

Table 2
Retention time (t_R; min), resolution (R_s) and symmetry of standard carbohydrates analyzed using a zwitterionic, a polyhydroxyethyl aspartamide and a BEH amide column using acetonitrile and water containing 6.5 mM of ammonium acetate.

Column	Maltodextrins	t_R	R_s	Symmetry	Galactose containing oligosaccharides	t_R	R_s	Symmetry
Zwitterionic	Maltose 1	11.1		1.1	Lactulose	10.8		0.7
	Maltose 2	12.0	0.1	1.1	Galactobiose (α 1-3) 1	11.7	0.2	2.2
	Maltotriose 1	15.1	0.2	1.1	Galactobiose (β 1-4) 1	11.8	0.1	1.0
	Maltotriose 2	16.1	0.0	1.1	Lactose 1	12.0	0.0	2.1
	Maltotetraose 1	19.0	0.6	0.9	Lactose 2	12.9	0.6	1.4
	Maltotetraose 2	20.0	0.3	0.9	Galactobiose (β 1-4) 2	13.0	0.1	0.8
	Maltopentaose 1	22.5	0.2	1.0	Melibiose 1	13.4	0.3	1.6
	Maltopentaose 2	23.5	0.5	1.1	Galactobiose (α 1-3) 2	13.7	0.1	0.5
	Maltohexaose 1	25.0	0.8	1.1	Melibiose 2	14.3	0.3	1.0
	Maltohexaose 2	26.1	0.5	1.1	Galactobiose (β 1-6) 1	14.5	0.1	1.1
	Maltoheptaose 1	27.3	0.5	1.2	Galactobiose (β 1-6) 2	16.0	0.5	1.1
	Maltoheptaose 2	28.3	0.4	1.1	Raffinose	16.1	0.1	0.9
					Galactotriose 1	16.2	0.0	0.9
					Galactotriose 2	17.4	0.3	0.8
					Galactotetraose 1	20.5	0.1	2.7
					Stachyose	22.0	0.7	0.8
					Galactotetraose 2	22.0	0.0	0.8
					Verbascose	26.5	0.3	0.8
Polyhydroxyethyl aspartamide	Maltose	4.3		1.1	Galactobiose (β 1-4)	5.1		0.9
	Maltotriose	7.8	1.6	1.9	Galactobiose (α 1-3)	5.3	0.1	0.6
	Maltotetraose	11.9	1.7	2.1	Lactose	5.9	0.3	1.6
	Maltopentaose	15.1	1.6	1.1	Lactulose	6.5	0.3	1.0
	Maltohexaose	18.6	2.1	1.3	Galactobiose (β 1-6)	7.6	0.3	2.0
	Maltoheptaose	20.6	1.4	1.7	Melibiose	7.8	0.3	0.9
					Raffinose	11.0	0.7	1.3
					Galactotriose	11.5	0.2	1.2
					Stachyose	14.9	1.2	1.2
					Galactotetraose	14.9	0.0	0.8
					Verbascose	20.1	2.8	0.9
BEH amide	Maltose 1	21.4		1.3	Galactobiose (α 1-3) 1	20.8		0.6
	Maltose 2	21.8	0.6	0.6	Galactobiose (β 1-4) 1	21.4	0.5	0.4
	Maltotriose 1	25.7	6.2	2.0	Lactulose	21.5	0.1	1.0
	Maltotriose 2	26.0	0.5	0.9	Galactobiose (α 1-3) 2	22.0	0.6	0.7
	Maltotetraose 1	29.1	4.7	1.8	Galactobiose (β 1-4) 2	22.3	0.4	1.2
	Maltotetraose 2	29.4	0.4	0.3	Lactose 1	22.5	0.2	0.9
	Maltopentaose 1	31.8	5.3	0.0	Lactose 2	22.5	0.0	1.0
	Maltopentaose 2	31.9	0.2	0.3	Melibiose 1	23.4	1.8	0.9
	Maltohexaose 1	33.8	3.2	10.5	Melibiose 2	23.8	0.6	0.8
	Maltohexaose 2	33.9	0.3	0.6	Galactobiose (β 1-6) 1	24.4	0.7	1.1
					Galactobiose (β 1-6) 2	25.3	0.9	1.3
					Galactotriose 1	25.7	0.4	0.9
					Galactotriose 2	25.8	0.2	0.7
					Raffinose	26.5	1.0	1.0
					Galactotetraose 1	29.3	2.6	1.0
					Galactotetraose 2	30.0	0.6	0.8
					Stachyose	30.1	0.1	0.7
					Verbascose	33.7	3.5	1.3

Overall, the three tested columns provided substantial differences in selectivity, peak shape and, especially, in retention efficiency. This fact can be expected according to the different nature of the surface chemistry of the assayed stationary phases. In general terms, the best separation of GOS standards and maltodextrins was achieved using the BEH amide column which was selected for further analyses. Successful separations of monosaccharide and other small polar compounds have been previously performed on amide-silica HILIC columns [14,31,34]. The great retention efficiency observed for the GOS eluted on the BEH

Table 3
Retention time (t_R; min), resolution (R_s) and symmetry of standard carbohydrates analyzed with a BEH amide column using acetonitrile: water with 0.1% ammonium hydroxide as mobile phase.

Maltodextrins	t_R	R_s	Symmetry	Galactose containing oligosaccharides	t_R	R_s	Symmetry
Maltose	20.3		1.0	Lactulose	19.8		1.1
Maltotriose	24.7	6.1	1.0	Galactobiose (β 1-4)	20.6	1.2	1.0
Maltotetraose	28.2	6.0	1.0	Galactobiose (α 1-3)	21.0	0.5	1.1
Maltopentaose	30.9	5.1	0.9	Lactose	21.1	0.1	0.9
Maltohexaose	32.9	3.8	1.1	Melibiose	22.4	2.6	1.0
Maltoheptaose	34.5	3.3	1.2	Galactobiose (β 1-6)	23.2	1.4	1.0
				Raffinose	24.7	2.9	0.8
				Galactotriose	24.8	0.2	0.9
				Galactotetraose	28.5	6.5	1.0
				Stachyose	29.1	1.1	0.9
				Verbascose	32.4	9.5	0.9

Table 4
Relative abundances of characteristic *m/z* ratios of neutral losses from MS^2 of standard disaccharides.

Standard	Glycosidic linkage	Monomeric units	Neutral losses (*m/z* ion)						
			$C_6H_{10}O_5$ (203)	$C_4H_8O_4$ (245)	$C_3H_6O_3$ (275)	$C_2H_4O_2$ (305)	CH_4O_2 (317)	CH_2O (335)	H_2O (347)
α,α,-Trehalose	1 → 1	Glc, Glc	100.0	–	–	–	–	–	–
Kojibiose	1 → 2	Glc, Glc	–	93.2	–	–	–	–	–
1,3-Galactobiose	1 → 3	Gal, Gal	27.0	–	3.1	1.1	–	–	100.0
Nigerose	1 → 3	Glc, Glc	38.2	–	33.2	–	–	–	100.0
Lactose	1 → 4	Gal, Glc	27.1	2.2	3.2	100.0	1.7	1.6	72.0
1,4-Galactobiose	1 → 4	Gal, Gal	42.9	0.8	1.7	100.0	1.1	9.1	46.6
Lactulose	1 → 4	Gal, Fru	2.3	–	0.1	7.2	14.7	1.3	100.0
1,6-Galactobiose	1 → 6	Gal, Gal	9.6	11.0	46.2	100.0	–	1.1	17.0
Melibiose	1 → 6	Gal, Glc	39.7	2.4	16.0	100.0	–	1.4	-

Gal: galactose; Glc: glucose; Fru: fructose.

amide column can be due to the contribution of strong hydrogen-bonding effects between the amide group of the stationary phase and polar compounds containing hydroxyl groups [35], such as GOS. A similar behavior has recently been reported for the separation of estrogen metabolites on an amide-silica HILIC column [36]. Likewise, differences of properties in terms of column dimension and, especially, of particle properties (particle size, pore size and surface area) could also have an effect on retention of the GOS. Thus, the BEH amide column has the biggest surface area (185 m^2/g with a particle size of 3.5 μm and a pore size of 135 Å), while the sulfoalkylbetaine zwitterionic has a surface area of 135 m^2/g (3.5 μm particle size and 200 Å pore size) and the polyhydroxyethyl aspartamide has the lowest surface area (100 m^2/g with 3 μm particle size and 300 Å pore size). Therefore, the increased retention of the GOS on the BEH amide column might be also due to the increased surface area for analyte binding in addition to the functionality of the stationary phase [36].

3.2. Fragmentation of disaccharides by MS^n

Previous to the structural characterization of GOS samples, MS^2 fragmentation behavior of several standard disaccharides containing galactose, glucose and/or fructose units was evaluated (Table 4). The ion at *m/z* 365 corresponds to the sodium adduct of disaccharides and it was the precursor ion considered for MS^2 analyses. 1,3-Galactobiose spectrum was characterized by the high abundance of the *m/z* fragment 347 (corresponding to the loss of a molecule of water) followed by the loss of the monosaccharide unit (ion at *m/z* 203). Low intensities relative to the base peak were also detected for the ions at *m/z* 275 and 305 corresponding to the losses of $C_3H_6O_3$ and $C_2H_4O_2$, respectively. However, higher abundances of ion at *m/z* 275 were observed for nigerose, which could be attributed to the differences in the monosaccharide composition. Similar fragmentation profiles, but different relative ratios of the fragment ions had been previously observed by Zhang et al. [19] for disaccharides with the same linkage but different monosaccharide residues.

Analogous MS^2 fragmentation (prevalent fragments at *m/z* 305, 347 and 203 corresponding to the neutral losses of $C_2H_4O_2$, H_2O and the monosaccharide unit, respectively) was observed for lactose and 1,4-galactobiose. In contrast, lactulose (galactosyl-(1 → 4)-fructose) fragmentation showed different abundances for these characteristic ions.

1,6-Galactobiose and melibiose (both with 1 → 6 glycosidic linkage) showed a similar fragmentation characterized by abundances in decreasing order of ions at *m/z* 305, 275, 245 (corresponding to

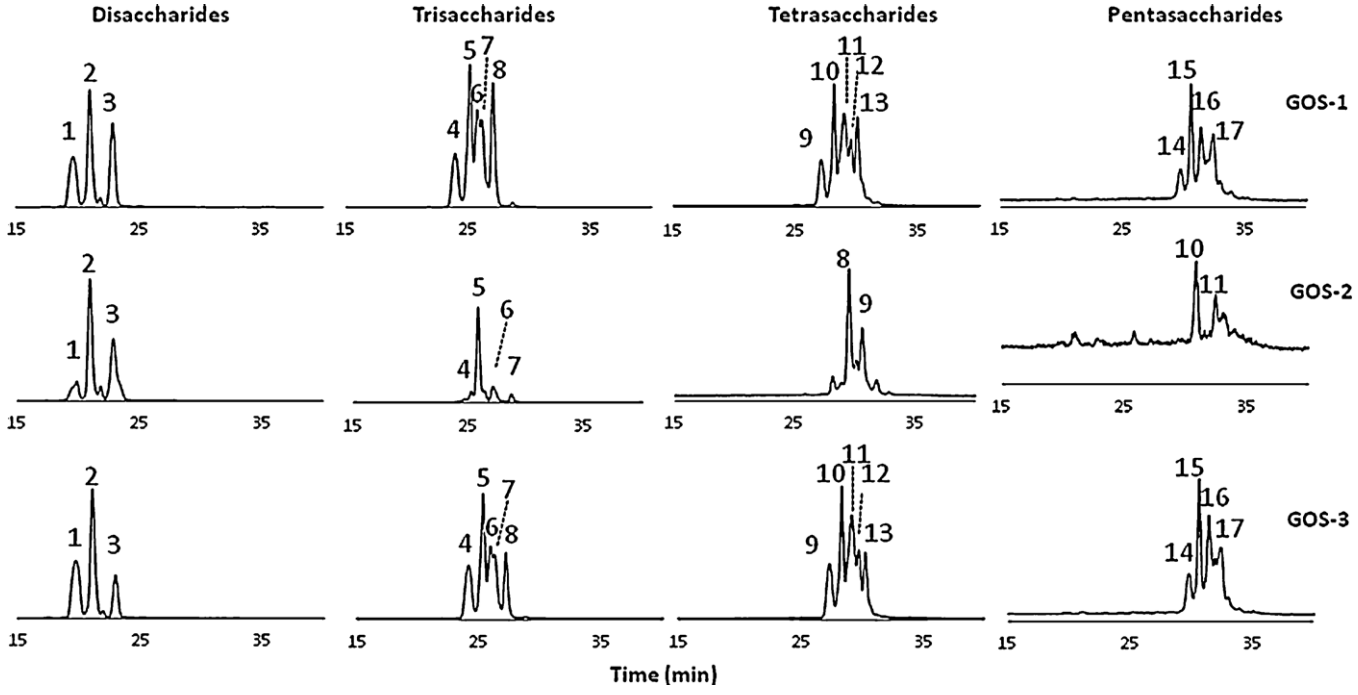

Fig. 2. HILIC profiles of commercial GOS separated on an ethylene bridge hybrid with trifunctionally bonded amide column Peak numbers are described in Tables 5–7.

Table 5
Relative abundance for characteristic *m/z* ratios of specific losses from MS^2 of disaccharides from commercial GOS separated in a BEH amide stationary phase.

Disaccharides									
MS^2									
Sample	Neutral loss Peak/*m/z*	$C_6H_{10}O_5$ 203	$C_4H_8O_4$ 245	$C_3H_6O_3$ 275	$C_2H_4O_2$ 305	CH_4O_2 317	CH_2O 335	H_2O 347	Tentative identification
GOS-1	1	6.8	0.3	45.1	0.3	-	-	100.0	Gal-(1 → 3)-Glc
	2	67.4	42.8	0.9	100.0	-	4.1	50.6	Gal-(1 → 4)-Glc and Gal-(1 → 2)-Glc
	3	3.4	6.1	25.3	100.0	0.6	1.9	3.7	Gal-(1 → 6)-Glc
GOS-2	1	4.0	-	12.8	3.8	4.3	–	100.0	Gal-(1 → 3)-Glc + lactulose
	2	16.7	7.4	0.4	100.0	2.7	5.9	52.3	Gal-(1 → 4)-
	3	2.1	6.7	28.0	100.0	0.2	1.9	4.7	Gal-(1 → 6)-Glc
GOS-3	1	7.7	-	38.9	0.8	1.6	0.4	100.0	Gal-(1 → 3)-Glc
	2	51.7	30.8	0.6	100.0	-	4.2	43.6	Gal-(1 → 4)- and Gal-(1 → 2)-Glc
	3	2.1	5.7	21.5	100.0	0.2	1.8	5.3	Gal-(1 → 6)-Glc

the loss of $C_4H_8O_4$) and 335 (corresponding to the loss of CH_2O). The main difference between fragmentations of these disaccharides was the higher abundance of the ion at *m/z* 203 corresponding to the monosaccharide for the melibiose and the abundance of the *m/z* ion 347 for 1,6-galactobiose. These results are in agreement with those found by Zhang et al. [19], who showed the characteristic fragmentation pattern of five different disaccharides, among them 1,3-galactobiose, maltose, and isomaltose, with 1 → 3, 1 → 4 and 1 → 6 linkages, respectively.

1,1 and 1,2-linked disaccharides with galactose units could not be acquired, but considering the similar fragmentation of 1 → 4 and 1 → 6 linkages with those shown by Zhang et al. [19], the reported fragmentation patterns of trehalose and 1,2-mannobiose were also used for the characterization of commercial GOS. In that

Table 6
Relative abundance for characteristic *m/z* ratios of specific losses from MS^2 and MS^3 of trisaccharides from commercial GOS separated on a BEH amide stationary phase.

Trisaccharides MS^2									
Sample	Neutral loss Peak/*m/z*	$C_6H_{12}O_6$ 347	$C_6H_{10}O_5$ 365	$C_4H_8O_4$ 407	$C_3H_6O_3$ 437	$C_2H_4O_2$ 467	CH_2O 497	H_2O 509	Identification
GOS-1	4	18.2	100.0	–	70.4	4.0	–	99.7	-Gal-(1 → 3)-Glc
	5a	3.2	100.0	66.2	5.3	3.2	–	5.3	-Gal-(1 → 2)-Glc
	5b	12.5	86.4	7.2	5.9	100.0	0.4	43.4	-Gal-(1 → 4)-Glc
	6	46.0	96.0	7.4	1.0	100.0	1.9	30.7	-Gal-(1 → 4)-Glc
	7	1.7	60.4	6.1	23.8	100.0	1.3	17.8	-Gal-(1 → 6)-Glc
	8	2.9	76.0	13.6	5.0	100.0	2.5	14.1	-Gal-(1 → 4)-Glc + -Gal-(1 → 2)-Glc
	4	9.6	89.9	3.8	12.5	100	–	88.3	-Gal-(1 → 6)-x
GOS-2	5	32.1	22.7	4.0	1.7	100	3.0	45.7	-Gal-(1 → 4)-x
	6	10.1	33.6	2.6	12.2	100	0.5	7.8	-Gal-(1 → 6)-x
	7	0.4	4.7	2.4	9.8	100	–	1.0	-Gal-(1 → 6)-x
	4	12.8	77.7	–	62.9	5.1	0.6	100.0	-Gal-(1 → 3)-Glc
	5a	6.0	90.8	100.0	0.8	1.9	–	8.8	-Gal-(1 → 2)-Glc
GOS-3	5b	8.8	99.1	6.0	5.0	100.0	2.2	37.3	-Gal-(1 → 4)-Glc
	6	34.8	100.0	2.2	2.2	58.9	–	35.3	-Gal-(1 → 4)-Glc + -Gal-(1 → 6)-Glc
	7	3.4	62.4	5.9	27.2	100.0	1.2	25.2	-Gal-(1 → 6)-Glc
	8	2.0	72.4	5.8	7.4	100.0	2.6	21.6	-Gal-(1 → 4)-Glc + -Gal-(1 → 6)-Glc
MS^3									
Sample	Neutral loss Peak/*m/z*	$C_6H_{10}O_5$ 203	$C_4H_8O_4$ 245	$C_3H_6O_3$ 275	$C_2H_4O_2$ 305	CH_4O_2 317	CH_2O 335	H_2O 347	Identification
GOS-1	4	4.5	–	100.0	–	–	–	88.1	Gal-(1 → 3)-
	5a	50.5	88.3	–	100.0	–	24.6	–	Gal-(1 → 4)- + x*-(1 → 2)-
	5b	17.2	–	–	100.0	1.0	9.4	26.0	Gal-(1 → 4)-
	6	–	14.6	15.2	100.0	–	6.5	22.6	Gal-(1 → 4)- + Gal-(1 → 6)-
	7	–	3.8	31.2	100.0	–	2.4	18.1	Gal-(1 → 6)-
	8	–	5.7	21.4	100.0	–	1.3	5.9	Gal-(1 → 6)-
GOS-2	4	–	10.7	–	100.0	11.8	5.8	20	Gal-(1 → 4)- and Gal-(1 → 2)-
	5	6.8	2.6	23.8	100.0	2.2	15.9	33.9	Gal-(1 → 6)-
	6	27.3	43.1	53.6	100.0	–	16.0	8.2	Gal-(1 → 6)-
	7	–	–	96.9	–	–	–	–	Gal-(1 → 3)-
GOS-3	4	–	–	100.0	7.4	–	–	50.4	Gal-(1 → 3)-
	5a	56.2	59.2	–	100.0	–	4.5	17.4	Gal-(1 → 4)- + x*-(1 → 2)-
	5b	5.4	9.0	–	100.0	1.2	5.5	29.7	Gal-(1 → 4)-
	6	23.1	30.9	6.8	100.0	14.1	–	18.5	Gal-(1 → 4)- + Gal-(1 → 2)-
	7	–	–	53.1	100.0	–	6.7	21.5	Gal-(1 → 6)-
	8	12.4	8.5	7.6	100.0	–	6.3	23.2	Gal-(1 → 6)-

*x: correspond to an unknown monosaccharide unit not previously described in the literature.

Table 7
Relative abundance for characteristic *m/z* ratios of specific losses from MS^2, and MS^3 of tetrasaccharides from commercial GOS separated on a BEH amide stationary phase.

Tetrasaccharides								
MS^2								
Sample	Neutral loss Peak/*m/z*	$C_6H_{12}O_6$ 509	$C_6H_{10}O_5$ 527	$C_4H_8O_4$ 569	$C_3H_6O_3$ 599	$C_2H_4O_2$ 629	H_2O 671	Identification
GOS-1	9	13.6	100.0	–	61.7	1.2	17.8	-(1 → 3)-
	10a	9.0	100.0	78.3	3.4	11.8	8.1	-(1 → 2)- + -(1 → 6)-
	10b	5.4	100.0	14.2	4.0	49.2	19.2	-(1 → 6)- + -(1 → 2)-
	11	5.1	100.0	3.7	6.2	20.5	6.2	-(1 → 6)-
	12	10.0	100.0	12.3	7.0	94.8	8.5	-(1 → 4)- + unkown
	13	6.4	84.8	4.1	–	100.0	12.3	-(1 → 4)-
GOS-2	8	70.5	42.9	2.7	3.5	100.0	24.3	-(1 → 4)- + -(1 → 6)-
	9	19.8	23.0	4.3	3.6	100.0	26.9	-(1 → 4)- + -(1 → 6)- + -(1 → 2)-
GOS-3	9	9.3	100.0	1.2	71.1	5.2	26.1	-(1 → 3)-
	10a	3.2	100.0	–	1.0	9.4	8.6	-(1 → 6)-
	10b	6.5	100.0	–	2.2	53.2	19.6	-(1 → 6)-
	11	6.8	100.0	–	–	38.0	6.0	-(1 → 4)-
	12	5.6	100.0	7.8	3.3	92.6	4.9	-(1 → 4)- + unkown
	13	3.0	98.1	3.3	1.9	100.0	13.7	-(1 → 4)- + unkown
MS^3								
Sample	Neutral loss Peak/*m/z*	$C_6H_{12}O_6$ 347	$C_6H_{10}O_5$ 365	$C_4H_8O_4$ 407	$C_3H_6O_3$ 437	$C_2H_4O_2$ 467	H_2O 509	Identification
GOS-1	9	4.5	35.9	–	100.0	10.0	31.0	-(1 → 3)-
	10a	–	100.0	37.3	–	45.3	–	-(1 → 4)- + -(1 → 2)-
	10b	8.2	77.4	11.0	4.1	100.0	19.0	-(1 → 6)- + -(1 → 4)-
	11	22.4	100.0	16.0	–	52.3	44.8	-(1 → 4)- + -(1 → 2)-
	12	5.4	48.5	–	17.0	100.0	10.0	-(1 → 4)- or -(1 → 6)-
	13	6.8	32.1	19.5	9.9	100.0	27.3	-(1 → 6)- + unknown
GOS-2	8	–	62.2	–	–	100.0	–	-(1 → 4)-
	9	–	20.0	–	–	100.0	–	-(1 → 4)-
GOS-3	9	6.7	50.2	–	100.0	1.2	26.0	-(1 → 3)-
	10a	–	100.0	30.3	–	75.8	18.0	Unknown
	10b	9.6	91.2	18.0	7.4	100.0	21.4	-(1 → 6)- + -(1 → 4)- +-(1 → 2)-
	11	–	100.0	4.4	15.9	63.0	32.5	-(1 → 4)- + -(1 → 6)-
	12	–	24.9	12.0	–	100.0	13.9	-(1 → 4)-
	13	–	100.0	14.2	–	11.0	11.7	Unknown

work, Zhang et al. [19] described that the MS^2 fragmentation of 1,1-linked disaccharide was dominated by the *m/z* ion at 203, although it was also detected that the very minor presence of the *m/z* ion at 305. Nevertheless, the characteristic fragmentation pattern of 1,2-linked disaccharides gave rise to the main neutral loss of $C_4H_8O_4$ (*m/z* ion at 245), followed by the ions in decreasing order of abundance at *m/z* 203, 347, 275 and 305.

3.3. Characterization of commercial GOS by HILIC–MS and HILIC–MS^n

Fig. 2 shows the SIM profiles of the three commercial GOS mixtures by HILIC–MS using the BEH column. Di-, tri-, tetra- and pentasaccharides were observed in all samples, whereas traces of hexasaccharides were detected in GOS-1 and GOS-3 (data not shown).

Three main peaks were clearly distinguished for disaccharides of GOS-1. HILIC–MS^2 analyses (Table 5) using *m/z* 365 as precursor ion, showed relative high intensities of fragments at *m/z* 347, 275, 203 for peak 1 which could correspond to a disaccharide with 1 → 3 linkage. However, relative abundances of these *m/z* fragments are different to those observed for 1,3-galactobiose which could be attributed to a different monomeric composition, more similar to that of nigerose (Table 4). It has been reported that galactosyl-(1 → 3)-glucose (26 wt%) is more abundant than the 1,3-galactobiose (1 wt%) in Vivinal-GOS® [7]. Therefore, this peak could be attributed to the first compound or a mixture of both. Peak 2 was the most abundant disaccharide of GOS-1 and showed a MS^2 fragmentation pattern different to those of commercial standards, probably due to the co-elution of different compounds. The most abundant fragments were *m/z* 305, 203 and 347 characteristic of 1 → 4 linked disaccharides and could correspond to 1,4-galactobiose. However, high relative abundances of ion *m/z* 245 distinctive of 1 → 2 linkages can be also observed. Therefore, this peak could be a mixture of (1 → 4)- and (1 → 2)- linked disaccharides. Coulier et al. [7] reported the presence of lactose, 1,4-galactobiose and galactosyl-(1 → 2)-glucose in Vivinal-GOS®. Therefore, peak 2 could be a mixture of these three disaccharides. Peak 3 could clearly correspond to a (1 → 6)- linked disaccharide considering the relative abundances of *m/z* ions at 305, 275 and 245 and could be assigned to allolactose (galactosyl-(1 → 6)-glucose) which was previously identified by Coulier et al. [7] following isolation, methylation and NMR analyses in Vivinal-GOS®.

Regarding trisaccharides of GOS-1, five peaks were observed (Fig. 2), however, resolution among them was not completely achieved which could difficult mass interpretation. MS^2 and MS^3 fragmentations were carried out using the ions *m/z* 527 and 365 as precursor ions, respectively. HILIC–MS^2 and HILIC–MS^3 analyses of peak 4 revealed a characteristic fragmentation of 1 → 3 linkages, similar to that observed for peak 1, as the main ion fragments corresponded to the neutral losses of $C_3H_6O_3$ (*m/z* fragments 437 and 275, in MS^2 and MS^3 spectra, respectively) and H_2O (*m/z* fragments 509 and 347, in MS^2 and MS^3 spectra, respectively) (Table 6). Therefore, this peak could tentatively be assigned to Gal-(1 → 3)-Gal-(1 → 3)-Glc, although mixtures with other trisaccharides with different monosaccharide compositions could not be discarded.

Table 8
Relative percentages of quantified and identified oligosaccharides using a BEH amide stationary phase in commercial GOS.

Sample	DP	Peak number	%	Identification
GOS-1	DP2	1	15.11 (0.07)[a]	Gal-(1 → 3)-Glc
		2	22.20 (0.18)	Gal-(1 → 4)-Glc + Gal-(1 → 2)-Glc
		3	17.07 (0.16)	Gal-(1 → 6)-Glc
	DP3	4	3.86 (0.02)	Gal-(1 → 3)-Gal-(1 → 3)-Glc
		5a + 5b	8.55 (0.07)	Gal-(1 → 4)-Gal-(1 → 2)-Glc + x*-(1 → 2)-Gal-(1 → 2)-Glc + Gal-(1 → 4)-Gal-(1 → 4)-Glc
		6	5.05 (0.12)	Gal-(1 → 4)-Gal-(1 → 4)-Glc + Gal-(1 → 6)-Gal-(1 → 4)-Glc
		7	5.00 (0.11)	Gal-(1 → 6)-Gal-(1 → 6)-Glc
		8	6.38 (0.04)	Gal-(1 → 6)-Gal-(1 → 4)-Glc + Gal-(1 → 6)-Gal-(1 → 2)-Glc
	DP4	9	1.25 (0.02)	x-(1 → 3)-x-(1 → 3)-x-(1 → 3)-x
		10a + 10b	2.58 (0.04)	x-(1 → 6)-x-(1 → 4)-x-(1 → y)-x + x-(1 → 6)-x-(1 → 2)-x-(1 → y)-x + x-(1 → 6)-x-(1 → 6)-x-(1 → 4)-x + x-(1 → 6)-x-(1 → 4)-x-(1 → 4)-x
		11	3.66 (0.04)	x-(1 → 6)-x-(1 → 4)-x-(1 → y)-x + x-(1 → 6)-x-(1 → 2)-x-(1 → y)-x
		12	1.44 (0.03)	x-(1 → 4)-x-(1 → 4)-x-(1 → 6)-x or x-(1 → y)-x-(1 → 6)-x-(1 → 6)-x
		13	2.24 (0.07)	x-(1 → 4)-x-(1 → 6)-x-(1 → y)-x + x-(1 → 4)-x-(1 → y)-x-(1 → y)-x
	DP5	14	1.13 (0.02)	Unknown
		15	1.82 (0.04)	Unknown
		16	1.11 (0.03)	Unknown
		17	1.56 (0.02)	Unknown
GOS-2	DP2	1	9.26 (0.18)	Gal-(1 → 3)-Glc + lactulose
		2	37.89 (1.65)	Gal-(1 → 4)-Glc
		3	29.17 (0.39)	Gal-(1 → 6)-Glc
	DP3	4	1.53 (0.35)	Gal-(1 → 4)-Gal-(1 → 6)-x + Gal-(1 → 2)-Gal-(1 → 6)-x
		5	17.62 (0.24)	Gal-(1 → 6)-Gal-(1 → 4)-x
		6	2.99 (0.11)	Gal-(1 → 6)-Gal-(1 → 6)-x
		7	0.34 (0.18)	Gal-(1 → 3)-Gal-(1 → 6)-x
	DP4	8	1.02 (0.06)	x-(1 → 4)-x-(1 → 4)-x-(1 → y*)-x + x-(1 → 6)-x-(1 → 4)-x-(1 → y*)-x
		9	0.50 (0.02)	x-(1 → 4)-x-(1 → 4)-x-(1 → y*)-x + x-(1 → 6)-x-(1 → 4)-x-(1 → y*)-x + x-(1 → 6)-x-(1 → 4)-x-(1 → y*)-x
	DP5	10	tr	Unknown
		11	tr	Unknown
GOS-3	DP2	1	18.88 (0.25)	Gal-(1 → 3)-Glc
		2	26.11 (0.48)	Gal-(1 → 4)- + Gal-(1 → 2)- Glc
		3	8.34 (0.04)	Gal-(1 → 6)-Glc
	DP3	4	5.61 (0.05)	Gal-(1 → 3)-Gal-(1 → 3)-Glc
		5a + 5b	10.20 (0.08)	Gal-(1 → 4)-Gal-(1 → 2)-Glc + x*-(1 → 2)-Gal-(1 → 2)-Glc + Gal-(1 → 4)-Gal-(1 → 4)-Glc
		6	4.96 (0.15)	Gal-(1 → 4)-Gal-(1 → 4)-Glc + Gal-(1 → 4)-Gal-(1 → 2)-Glc + Gal-(1 → 4)-Gal-(1 → 6)-Glc + Gal-(1 → 2)-Gal-(1 → 6)-Glc
		7	4.30 (0.09)	Gal-(1 → 6)-Gal-(1 → 6)-Glc
		8	3.80 (0.05)	Gal-(1 → 6)-Gal-(1 → 4)-Glc + Gal-(1 → 6)-Gal-(1 → 6)-Glc
	DP4	9	1.66 (0.09)	x-(1 → 3)-x-(1 → 3)-x-(1 → 4)-x
		10a + 10b	3.09 (0.19)	x-(1 → 6)-x-(1 → y)-x-(1 → y)-x + x-(1 → 6)-x-(1 → 6)-x-(1 → y)-x + x-(1 → 6)-x-(1 → 4)-x-(1 → y)-x + x-(1 → 6)-x-(1 → 2)-x-(1 → y)-x
		11	4.18 (0.17)	x-(1 → 4)-x-(1 → 4)-x-(1 → 6)-x + x-(1 → 4)-x-(1 → 6)-x-(1 → 6)-x
		12	1.34 (0.12)	x-(1 → 4)-x-(1 → 4)-x-(1 → y)-x + x-(1 → y)-x-(1 → 4)-x-(1 → y)-x
		13	1.38 (0.12)	x-(1 → 4)-x-(1 → y)-x-(1 → 6)-x + x-(1 → y)-x-(1 → y)-x-(1 → 6)-x + x-(1 → 4)-x-(1 → y)-x-(1 → 3)-x + x-(1 → y)-x-(1 → y)-x-(1 → 3)-x
	DP5	14	1.13 (0.04)	Unknown
		15	1.83 (0.09)	Unknown
		16	1.73 (0.06)	Unknown
		17	1.45 (0.02)	Unknown

*x: unknown monosaccharide unit.
*y: an unknown bond.
tr: traces.
[a] Standard deviation ($n = 3$).

Two compounds can be clearly distinguished by HILIC–MS^2 of peak 5. First of them, peak 5a, showed a m/z fragmentation pattern characteristic of (1 → 2)-linked carbohydrates [19] differing from 2α-mannobiose in the relative abundance of the neutral loss of monomeric units: m/z 365 for the MS^2 fragmentation of the trisaccharide, and m/z 203 for the MS^3 fragmentation of the disaccharide, being this loss more abundant in the first case (Table 6). HILIC–MS^3 of this peak revealed a similar fragmentation profile to peak 2 which could indicate the presence of a mixture of two compounds with 1 → 2 and 1 → 4 glycosidic linkages. Gal-(1 → 4)-Gal-(1 → 2)-Glc has been previously identified in Vivinal-GOS® [7], however, the presence of x-(1 → 2)-Gal-(1 → 2)-Glc has not been previously reported. HILIC–MS^2 and HILIC–MS^3 analyses of peak 5b seem to indicate the presence of 1 → 4 glycosidic linkages with the characteristic MS^2 losses of $C_2H_4O_2$, $C_6H_{10}O_5$, H_2O and $C_6H_{12}O_6$, and MS^3 losses of $C_2H_4O_2$, H_2O, $C_6H_{10}O_5$ and CH_2O, which is indicative of the presence of Gal-(1 → 4)-Gal-(1 → 4)-Glc. Peak 6 could be tentatively assigned to Gal-(1→6)-Gal-(1 → 4)-Glc considering the MS^2 (losses of $C_6H_{12}O_6$, $C_2H_4O_2$ and H_2O) and MS^3 (losses of $C_2H_4O_2$, $C_3H_6O_3$ and $C_4H_8O_4$) fragmentations although contribution of Gal-(1 → 4)- cannot be discarded taking into account the relative ratios of the fragment ions in MS^3. Peak 7 showed the characteristic pattern of 1 → 6 glycosidic linkages for both MS^2 and MS^3 fragmentations and could correspond to Gal-(1 → 6)-Gal-(1 → 6)-Glc. Finally, MS^3 of peak 8 clearly revealed the presence of 1 → 6 glycosidic linkage (losses of $C_2H_4O_2$, $C_3H_6O_3$ and $C_4H_8O_4$), however MS^2 was more confusing, considering the fragment at m/z 467, the low abundance of m/z 437 and the relatively high intensity of m/z 407. This profile is similar to that detected for peak 2 and could be assigned to a mixture of 1 → 2 and 1 → 4 linkages.

Five peaks corresponding to tetrasaccharides were observed in GOS-1 by HILIC–MS (Fig. 2). Fragments at m/z 689 and 527 were

used as precursor ions of MS^2 and MS^3, respectively. Fragment at *m/z* 365 was also used as a precursor ion of MS^4, although detected ions had much lower abundances (data not shown). Characterization of these peaks was more complex considering the low abundances and the existence of multiple coelutions. Only some linkages could be tentatively assigned as indicated in Table 7.

A similar reasoning was followed for the characterization of di-, tri- and tetrasaccharides of GOS-2 and GOS-3. These data are shown in Tables 5–7. In general, GOS-3 showed a similar qualitative composition to GOS-1, however, notable differences were observed for GOS-2 which exhibited a lower diversity of glycosidic linkages. This fact is supported by the high similarity of the chromatographic profiles of GOS-1 and GOS-3 in oligosaccharide retention times and peak shapes, while the HILIC profile of GOS-2 exhibited some differences in terms of retention times and, especially, in peak abundances (Fig. 2), as it will be discussed in Section 3.4.

Regarding GOS-2 disaccharides (Table 5), in peak 1 co-eluted two different carbohydrates, probably Gal-(1 → 3)-Glc characterized by the fragment at *m/z* 275 and lactulose which showed high contribution of *m/z* 347 and low of *m/z* 305 and 317 (Table 5). The presence of 1 → 4 glycosidic linkage could be easily detected in peak 2 of GOS-2, whereas the contribution of 1 → 2 linkage (fragment at *m/z* 245) was smaller than those of GOS-1 and GOS-3. Peak 3 was identified as Gal-(1 → 6)-Glc, likewise in the other two samples. The main trisaccharide (peak 5, Table 6) was assigned to Gal-(1 → 6)-Gal-(1 → 4)-x, whereas peak 4 could be characterized by a mixture of two compounds (Gal-(1 → 4)-Gal-(1 → 6)-x and Gal-(1 → 2)-Gal-(1 → 6)-x). Peaks 6 and 7 showed the typical MS^2 fragmentation of (1 → 6) linkages, MS^3 spectra being characteristic of (1 → 6) and (1 → 3), respectively. Tetrasaccharides showed very low abundances and mainly the presence of -(1 → 4)- and -(1 → 6)- could be hypothesized (Table 7).

3.4. Quantitation of GOS by HILIC–MS

Quantitative analysis was carried out following the external standard method using the homologous series of maltodextrins. Limit of detection (*LOD*) showed values of 0.04–0.08 ng injected; whereas limit of quantitation (*LOQ*) was 0.14–0.28 ng injected. Intra- and inter-day reproducibility was also evaluated, relative standard deviation being lower than 10% for the different standards analyzed.

Table 8 shows quantitative data for GOS mixtures. Disaccharides were the main carbohydrates present in GOS samples (54%, 76% and 53% for GOS-1, GOS-2 and GOS-3, respectively); lactose (quantified together with Gal-(1 → 2)-Glc in GOS-1 and GOS-3) being the most abundant. Regarding trisaccharides, similar percentages were observed for GOS-1 and GOS-3 (∼29%), while GOS-2 had lower percentages (22.5%). Likewise, tetrasaccharides of GOS-2 only constituted the 1.5% of its composition, whereas levels of 11–12% were found in GOS-1 and GOS-3. Only traces of pentasaccharides could be detected in GOS-2. Therefore, yields of oligosaccharides in GOS-1 and GOS-3 were higher than those found in GOS-2, probably due to the manufacturing conditions used to obtain these products [5].

4. Conclusions

The results presented in this work show the usefulness of HILIC–MS^n to separate and tentatively characterize complex mixtures of GOS without a previous fractionation, enrichment or derivatization step. The three studied silica-based HILIC columns exhibited substantial differences in peak shape, retention and selectivity which could be mainly attributed to the nature of the surface chemistry of the assayed stationary phases (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridge hybrid (BEH) with trifunctionally-bonded amide). Likewise, differences in the dimension of columns and, especially, particle properties (particle size, pore size and surface area) might also contribute to the retention of GOS. In this context, polar compounds possessing a high number of hydroxyl groups such as GOS were efficiently retained and separated on the BEH amide stationary phase using acetonitrile:water with 0.1% ammonium hydroxide as mobile phase.

The characterization of prebiotic GOS is of paramount importance for the elucidation of the structure–bioactivity relationship with respect to the effect of these carbohydrates on the human gastrointestinal health. MS^n characterization of GOS (in terms of monosaccharide composition, degree of polymerization and glycosidic linkages) should be considered tentative, taking into account the lack of standards. However, it requires much less handling, is less tedious and time consuming than the combination of complex techniques (isolation of each compound by fractionation methods and the subsequent analysis by NMR and methylation procedures) traditionally proposed in the literature.

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