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1 # HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COUPLED TO
2 MASS SPECTROMETRY FOR THE CHARACTERIZATION OF PREBIOTIC
3 GALACTOOLIGOSACCHARIDES

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19 **Abstract**

20 Three different stationary phases (sulfoalkylbetaine zwitterionic, 21 polyhydroxyethyl aspartamide and ethylene bridge hybrid (BEH) with trifunctionally-22 bonded amide), operating at hydrophilic interaction liquid chromatographic (HILIC) 23 mode, have been assayed and compared for the analysis of complex mixtures of 24 galactooligosaccharides (GOS). Chromatographic methods have been optimized to 25 obtain the best separation between two consecutive galactose containing standards and 26 maltodextrins, measured on the basis of resolution. Influence of several factors such as 27 chemical modifiers (formic acid, ammonium acetate and ammonium hydroxide), 28 organic solvent and gradients of the mobile phases in the separation of oligosaccharides 29 have been studied. The best results were achieved on the BEH amide stationary phase, 30 using acetonitrile: water with 0.1% ammonium hydroxide as mobile phase, where the 31 most of oligosaccharides were successfully resolved.

32 Characteristic MS^2 fragmentation profiles of disaccharides containing galactose, 33 glucose and/or fructose units with different linkages were evaluated and used for the 34 characterization of di-, tri- and tetrasaccharides of three commercial prebiotic GOS 35 mixtures (GOS-1, GOS-2 and GOS-3) by HILIC-MS^n. Similar qualitative and 36 quantitative composition was observed for GOS-1 and GOS-3, whereas different 37 linkages and abundances were detected for GOS-2. In general, (1→4) and (1→6) 38 glycosidic linkages were the main structures found in GOS, although (1→2) and (1→3) 39 linkages were also identified. Regarding molecular weight, up to pentasaccharides were 40 detected in these samples, disaccharides being the most abundant carbohydrates.

41

42 **Keywords**: hydrophilic interaction liquid chromatography (HILIC),
43 galactooligosaccharides (GOS), multi-stage mass spectrometry (MS^n), glycosidic
44 linkages

45

46 **1. Introduction**

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

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

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

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

72 Different operation modes of HPLC have been applied to the analysis of
73 oligosaccharides. Low retention of underivatized carbohydrates is usually attained using
74 reverse phase columns, whereas better separation can be achieved by high performance
75 anion exchange chromatography (HPAEC) although complex profiles are obtained
76 when families of oligosaccharides with different linkage variants are present [11].

77 Hydrophilic interaction liquid chromatography (HILIC) is gaining a great
78 importance in the last years for the separation of polar compounds such as
79 carbohydrates [12,13]. Partitioning of polar analytes between the bulk eluent and a
80 water-rich layer partially immobilized on the stationary phase is the main retention
81 mechanism described for HILIC [12], however, different functional groups can be
82 present on the stationary phase giving rise to secondary interactions such as electrostatic
83 [14,15]. Different stationary phases are currently used for this separation mode; silica
84 particles or monolithic supports (Ikegami et al., 2008) either modified with
85 aminopropyl, diol, zwitterionic or amide groups and polymer based packing, among
86 others, can be found [16].

87 Sensitive detection of oligosaccharides after HPLC analysis represents an
88 additional difficulty for their analysis. The absence of chromophore and fluorophore
89 groups avoids their direct detection by UV or fluorescence detectors, whereas pulse
90 amperometric detection (PAD), when coupled to HPAEC, is a suitable tool for
91 oligosaccharide analysis [17] and has been applied for several applications.
92 Nevertheless, the use of mass spectrometric (MS) detectors coupled to HPLC systems
93 has considerably enriched the field of oligosaccharide analysis, allowing the

94 determination of their molecular weight [18]. Multi-stage mass spectrometry (MS^n) can
95 also provide structural information; however, scarce studies have been still carried out
96 about its utility for the characterization of neutral oligosaccharides [19,20]. Moreover,
97 the addition of appropriate metals to HPLC mobile phases to form complexes with
98 carbohydrates or their previous derivatization (peracetylation or permethylation) is
99 usually required to facilitate the sequential identification of residues by MS [21].
100 Characterization of different GOS has been generally carried out by the
101 combination of a great variety of analytical methodologies (methylation analysis
102 followed by GC-MS, NMR, HPAEC-PAD-MS, ESI-MS) with previous fractionation of
103 the oligosaccharides (yeast treatment, SEC, HILIC) [7,8,20]. HILIC-MS has been used
104 for the analysis of GOS previously fractionated by cation exchange chromatography to
105 determine their molecular weights [22]. On the other hand, Fu et al. [23] used a "click"
106 maltose column made in their laboratory to separate GOS. A good resolution among the
107 different degrees of polymerization was obtained, however, no separation of isomers
108 was observed.
109 In this manuscript three different HILIC stationary phases have been assayed to
110 obtain the best separation of oligosaccharides. HILIC-MS methods have been optimized
111 and applied to the analysis of different and complex commercial GOS mixtures.
112 Characterization of their structures has been accomplished by MS^n without any previous
113 modification of carbohydrate structure.
114

115 **2. Materials and methods**

116 2.1. Standards

117 1,3-galactobiose (α-Gal-[1→3]-Gal), 1,4-galactobiose (β-Gal-[1→4]-Gal), 1,6-
118 galactobiose (β-Gal-[1→6]-Gal), galactotriose (α-Gal-[1→3]-β-Gal-[1→4]-Gal),

119 galactotetraose (α-Gal-[1→3]-β-Gal-[1→4]-α-Gal-[1→3]-Gal) were acquired from
120 Dextra Laboratories (Reading, UK), whereas lactose (β-Gal-[1→4]-Glc), maltose (α-
121 Glc-[1→4]-Glc), maltotriose ((α-Glc-[1→4])$_2$-Glc), maltotetraose ((α-Glc-[1→4])$_3$-
122 Glc), maltopentaose ((α-Glc-[1→4])$_4$-Glc), nigerose (α-Glc-[1→3]-Glc), raffinose (α-
123 Gal-[1→6]-α-Glc-[1→2]-β-Fru) and stachyose (α-Gal-[1→6])$_2$-α-Glc-[1→2]-β-Fru)
124 were obtained from Sigma (St. Louis, US), and lactulose (β-Gal-[1→4]-Fru), melibiose
125 (α-Gal-[1→6]-Glc), and verbascose ((α-Gal-[1→6])$_3$-α-Glc-[1→2]-β-Fru) from Fluka
126 (Madrid, Spain).
127
128 2.2. Samples
129 Vivinal-GOS® (GOS-1) was kindly provided by Friesland Foods Domo (Zwolle,
130 The Netherlands), BiMuno (Clasado, Reading, UK) (GOS-2) and Yum-Yum GOS™
131 (Jarrow Formula, USA) (GOS-3) were acquired in local markets.
132
133 2.3. HILIC-MS
134 GOS analyses were performed on an Agilent 1200 series HPLC system
135 (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments,
136 UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto,
137 CA, USA) provided with an electrospray ionization (ESI) source. Samples (20 µL) were
138 injected using a Rheodyne 7725 valve. Three columns and different conditions were
139 used for the analyses: (i) Sulfoalkylbetaine zwitterionic stationary phase (ZIC®-HILIC
140 column; 150 x 2.1 mm, 3.5 µm particle size, 200 Å pore size, SeQuant™, Umea,
141 Sweden) at a flow rate of 0.2 mL min^{-1}; (ii) Polyhydroxyethyl aspartamide stationary
142 phase (PolyHydroxyethyl-A column; 100 x 2.1 mm; 3 µm particle size, 300 Å pore size,
143 The Nest Group, Inc., Southborough, MA) at a flow rate of 0.4 mL min^{-1} and (iii)

144 Ethylene bridge hybrid (BEH) with trifunctionally-bonded amide phase (XBridge
145 column; 150 x 4.6 mm; 3.5 µm particle size, 135 Å pore size, Waters, Hertfordshire,
146 UK) at a flow rate of 0.4 mL min^{-1}. Different binary gradients consisting of acetonitrile
147 (MeCN) : water or methanol (MeOH) : water, with addition of different modifiers as
148 indicated in **Table 1**, were assayed for the three columns and optimized. The
149 temperature of elution was kept at 35 ºC for all cases.

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

158 Quantitative analysis was performed in triplicate by the external standard
159 method, using calibration curves in the range 9.6-400 ng for maltose, maltotriose,
160 maltotetraose, maltopentaose, and maltohexaose. Correlation coefficients were obtained
161 from these calibration curves. Reproducibility of the method was estimated on the basis
162 of the intra-day and inter-day precision, calculated as the relative standard deviation
163 (*RSD*) of retention times and concentrations of oligosaccharide standards obtained in *n*
164 = 5 independent measurements. Limit of detection (*LOD*) and limit of quantitation
165 (*LOQ*) were calculated as three and ten times, respectively, the signal to noise ratio
166 (*S/N*), where *N* is five times the standard deviation of the noise [24].

167

168 2.4. HILIC-MS^n

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

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

180 Fragmentation behaviour of the oligosaccharides was studied by infusing a
181 solution of each oligosaccharide (10 µg mL^{-1} in MeCN : water, 60:40, v/v) at a flow rate
182 of 10 µL min^{-1} using the syringe pump included in the instrument and mixing it with
183 100 µL min^{-1} of MeCN : water (60:40, v/v) both with 0.1% ammonium hydroxide by
184 means a zero-dead volume T-piece. Sheath and auxiliary gases were set at 0.6 and 6 L
185 min^{-1}, respectively. CID experiments were carried out by isolating each $[M+Na]^+$ ion in
186 the ion trap (isolation width 1.0 *m/z*), and subjecting them to a normalized collision
187 energy (NCE%) selected to preserve a signal of the precursor ion in the order of 5%.
188 The process was repeated up to two times by successive isolation (isolation width 1.0
189 m/z) of the generated ions corresponding to the loss of a monosaccharide unit (loss of
190 162 u).

191 Separation of GOS samples were performed on the BEH column following the
192 elution gradient optimized in Section 3.1 that uses MeCN (solvent A) : water (solvent

193 B) both with 0.1% ammonium hydroxide at 35 ºC. Optimal separation of isomeric
194 oligosaccharides was obtained by changing solvent A from 80% to 50% in 31 min and,
195 then, kept for 5 min. Initial conditions were recovered after 0.1 min and were kept for
196 15 min before the following injection.

197 Considering that two different LC systems were used, slight differences in
198 oligosaccharide separations were only observed in two chromatographic peaks. Bearing
199 in mind the fragmentation study realized with standards by infusion in Section 3.2, the
200 following *m/z* (and NCE%) were used in the HILIC-MS^n analysis of the samples: 365.1
201 (29%) for disaccharides, 527.2 (31%) > 365.1 (29%) for trisaccharides, 689.2 (32%) >
202 527.2 (31%) > 365.1 (29%) for tetrasaccharides.

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

205

206 **3. Results and discussion**

207

208 *3.1. Optimization of HILIC methods*

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

213 Evaluation of the methods was carried out on the basis of the shortest retention
214 times (t_R), the best peak symmetry, calculated as the ratio of the front to back widths (at
215 50% of the peak height) and the highest resolution (R_s, calculated as $2(t_{R2}$-
216 $t_{R1})/(w_{b1}+w_{b2})$, where 1 and 2 refer to two consecutive eluting carbohydrates and w_b is
217 the peak width at base); R_s values should be higher than 1.0 to get an appropriate

218 separation and peak symmetry close to 1 to get a good symmetry of the peaks. In those
219 cases where α and β isomers appeared as unresolved peaks, PeakFit software (v4.12;
220 SeaSolve Software Inc.) was used for peak deconvolution.

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

225

226 *Effect of formic acid*

227 The effect of 0.1% formic acid added to both solvents (MeCN and water) as
228 mobile phase for separation of oligosaccharides on the three HILIC stationary phases
229 was firstly assessed. In all cases, reducing carbohydrates showed split peaks
230 corresponding to α and β isomers. This effect has been described by different authors
231 who suggested the use of basic pH to avoid the mutarotation of carbohydrates [25,26].
232 The homologous series of maltodextrins were well resolved under these conditions in
233 both polyhydroxyethyl aspartamide column and BEH amide columns (R_s>1). However,
234 broad peaks with poor symmetry (higher than 1) were obtained in polyhydroxyethyl
235 aspartamide column; as an example, maltotriose eluted having a w_b of 0.91 min and a
236 symmetry of 1.57, whereas no separation was achieved in the zwitterionic column.
237 However, the appearance of two peaks per reducing carbohydrate impaired the
238 separation of isomers showing, thus, a bad resolution among galactose containing
239 oligosaccharides in the three columns (data not shown). Therefore, formic acid was
240 discarded for further analyses.

241

242

243 *Effect of ammonium acetate*

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

248 All tested oligosaccharides were very poorly resolved under these conditions ($R_s \leq$
249 0.6) in the zwitterionic column with retention times varying from 3.97 min of lactulose
250 to 4.84 min of verbascose. Moreover, split peaks corresponding to α and β isomers were
251 obtained for reducing carbohydrates, probably because the pH (4.75 in the aqueous
252 phase) was not basic enough to avoid mutarotation of carbohydrates.

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

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

262 Effect of methanol as solvent A instead of acetonitrile was also evaluated under
263 these conditions as suggested by Sinclair et al. [22] for the three columns. Although t_R
264 of oligosaccharides were substantially shorter than those obtained with acetonitrile, (i.e
265 t_R of maltose using methanol in BEH amide column was 7.5 min and 20.1 min using
266 MeCN), resolution values among all tested carbohydrates were very low for BEH amide
267 (R_s<0.85) and zwitterionic columns (R_s < 0.14). Coelution of all carbohydrates in a

268 single broad peak was observed for the polyhydroxyethyl aspartamide column. This
269 behaviour can be due to the protic nature of both methanol and water, which compete to
270 solvate the stationary phase and provide strong hydrogen bonding interactions with each
271 other [16]. Therefore, the use of acetonitrile as mobile phase was selected for further
272 studies.

273 As it was previously described by Alpert [12], HILIC retention is inversely
274 proportional to the increase of salt concentration in the mobile phase. Therefore, four
275 different concentrations (0.1, 3.5, 6.5 and 20 mM) of ammonium acetate only present in
276 the aqueous phase were evaluated and, in consequence, the concentration of this salt
277 increased as the water content rose. No substantial differences were detected among the
278 different concentrations of salt for both zwitterionic and polyhydroxyethyl aspartamide
279 columns. Figure 1 shows the HILIC profile of maltodextrins obtained using the
280 polyhydroxyethyl aspartamide column under these conditions. These profiles indicated
281 that the order of elution of carbohydrates on these columns was not related to the salt
282 content in the mobile phase. Likewise, no suppression of the MS signal was observed
283 by increasing the salt concentration which could be explained by the high volatility of
284 ammonium acetate. Therefore, an intermediate concentration of ammonium acetate (6.5
285 mM) in water mobile phase was selected. Similar results were observed by Strege [30]
286 for the HILIC separation of polar compounds for drug discovery processes where only
287 slight changes were detected between 0 and 3.3 mM buffer salt concentrations.
288 Tolstikov and Fiehn [31] also used similar mobile phases for the analysis of polar
289 compounds of plant origin in the polyhydroxyethyl aspartamide column; however, to
290 the best of our knowledge, there are not data about the separation of different isomeric
291 carbohydrates under these conditions in this stationary phase. Moreover, coelution of
292 sucrose (DP2) and raffinose (DP3) was reported in the previous work, whereas

293 oligosaccharides of different molecular weight could be separated under our optimised
294 conditions (**Figure 1**).

295 On the other hand, better resolution was obtained using a salt gradient than the
296 elution method containing 5 mM ammonium acetate in both solvents, acetonitrile and
297 water. Thus, resolution values were much higher using a salt gradient (R_s ≥1.6) than
298 those obtained using 5mM ammonium acetate in both solvents (R_s≤1.0).

299 Different binary gradients using these mobile phases were assayed to optimise
300 the separation of both maltodextrins and galactose containing oligosaccharides. For the
301 zwitterionic column, the best results were obtained varying MeCN from 80% to 50% in
302 50 min. Although split peaks were obtained for reducing carbohydrates their resolution
303 (**Table 2**) was slightly better than that found using formic acid 0.1%. Carbohydrates
304 without anomeric carbon (lactulose, raffinose, stachyose and verbascose) showed a
305 single peak and a good resolution among them; however some of these peaks were not
306 symmetric (**Table 2**). In general, separation of the standard oligosaccharides was carried
307 out in function of increasing carbohydrate molecular weights, whereas the most retained
308 isomeric carbohydrates were the oligosaccharides with 1→6 linkages.

309 Elution gradient was also optimized for polyhydroxyethyl aspartamide column
310 and selected conditions were: solvent A kept at 80% for 3 min and changed to 50% for
311 40 min; under these experimental conditions, this stationary phase was unable to
312 separate anomeric compounds and single peaks were detected in reducing
313 carbohydrates. Similarly to the previous column, maltodextrins were eluted in the order
314 of increasing molecular weight, with R_s values from 1.4 to 2.1 (**Table 2**), whereas
315 among disaccharides, those with 1→3 and 1→4 glycosidic linkages were the first to
316 elute followed by (1→6)-linked carbohydrates. Although elution times ranged from 5.1
317 min of 1,4-galactobiose to 20.1 min of verbascose, broad peaks were obtained (i.e. 1,6-

318 galactobiose: w_b= 1.6 min; galactotriose: w_b= 1.8 min; and so on) and resolution among
319 them was poor (**Table 2**). Only peaks corresponding to i) galactotriose and stachyose
320 and ii) galactotetraose and verbascose were well resolved, although only verbascose
321 presented an acceptable symmetry (0.9).

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

331

332 *Effect of ammonium hydroxyde*

333 To avoid the appearance of split peaks, 0.1% ammonium hydroxide was used in
334 both mobile phases (MeCN and water). Although one single peak was obtained for each
335 oligosaccharide, no satisfactory resolution was achieved under these conditions for the
336 zwitterionic and polyhydroxyethyl aspartamide columns either for the separation of the
337 maltodextrins or the galactose containing oligosaccharides (data not shown). However,
338 these conditions resulted in a good resolution of maltodextrins on BEH amide column
339 (R_s~ 4.8). In this column, different binary gradients using MeCN and water as mobile
340 phases containing both 0.1% ammonium hydroxide were assayed to optimise the
341 separation of both maltodextrins and galactose containing oligosaccharides; the best
342 results were obtained varying MeCN from 80% to 50% in 31 min, as previously

343 reported by Brokl et al. [32] for the separation of fructooligosaccharides,
344 gentiooligosaccharides and oligosaccharides from dextransucrase cellobiose acceptor
345 reactions. Maltodextrins eluted within 34 min; t_R increasing with their molecular weight
346 as consequence of the increase in hydrophilicity due to the increased number of
347 hydroxyl groups. Wuhrer et al. [33] and Melmer et al. [34] reported a similar behaviour
348 of *N*-glycans in amide-based ligand columns. The galactose containing oligosaccharides
349 eluted from 19.8 min of lactulose to 32.4 min of verbascose. Disaccharides with 1→3
350 and 1→4 linkages were the first to elute followed by carbohydrates with 1→6
351 glycosidic linkages. In general, resolution values were higher than 1, except for those
352 between galactobiose 1→4 and 1→3; galactobiose 1→3 and lactose; and galactotriose
353 and raffinose (**Table 3**). Therefore, BEH column under these elution conditions was
354 selected for the analysis of commercial GOS mixtures.
355
356 Overall, the three tested columns provided substantial differences in selectivity,
357 peak shape and, especially, in retention efficiency. This fact can be expected according
358 to the different nature of the surface chemistry of the assayed stationary phases. In
359 general terms, the best separation of GOS standards and maltodextrins was achieved
360 using the BEH amide column which was selected for further analyses. Successful
361 separations of monosaccharide and other small polar compounds have been previously
362 performed on amide-silica HILIC columns [14,32,35]. The great retention efficiency
363 observed for the GOS eluted on the BEH amide column can be due to the contribution
364 of strong hydrogen-bonding effects between the amide group of the stationary phase
365 and polar compounds containing hydroxyl groups [36], such as GOS. A similar
366 behaviour has recently been reported for the separation of estrogen metabolites on an
367 amide-silica HILIC column [37]. Likewise, differences of properties in terms of column

368 dimension and, especially, of particle properties (particle size, pore size and surface
369 area) could also have an effect on retention of the GOS. Thus, the BEH amide column
370 has the biggest surface area (185 m^2/g with a particle size of 3.5 µm and a pore size of
371 135 Å), whilst the sulfoalkylbetaine zwitterionic has a surface area of 135 m^2/g (3.5 µm
372 particle size and 200 Å pore size) and the polyhydroxyethyl aspartamide has the lowest
373 surface area (100 m^2/g with 3 µm particle size and 300 Å pore size). Therefore, the
374 increased retention of the GOS on the BEH amide column might be also due to the
375 increased surface area for analyte binding in addition to the functionality of the
376 stationary phase [37].

377

378 *3.2. Fragmentation of disaccharides by MS^n*

379 Previous to the structural characterization of GOS samples, MS^2 fragmentation
380 behaviour of several standard disaccharides containing galactose, glucose and/or
381 fructose units was evaluated (**Table 4**). The ion at *m/z* 365 corresponds to the sodium
382 adduct of disaccharides and it was the precursor ion considered for MS^2 analyses. 1,3-
383 galactobiose spectrum was characterized by the high abundance of the *m/z* fragment 347
384 (corresponding to the loss of a molecule of water) followed by the loss of the
385 monosaccharide unit (ion at *m/z* 203). Low intensities relative to the base peak were
386 also detected for the ions at *m/z* 275 and 305 corresponding to the losses of $C_3H_6O_3$ and
387 $C_2H_4O_2$, respectively. However, higher abundances of ion at *m/z* 275 were observed for
388 nigerose, which could be attributed to the differences in the monosaccharide
389 composition. Similar fragmentation profiles, but different relative ratios of the fragment
390 ions had been previously observed by Zhang et al. [19] for disaccharides with the same
391 linkage but different monosaccharide residues.

392 Analogous MS^2 fragmentation (prevalent fragments at *m/z* 305, 347 and 203
393 corresponding to the neutral losses of $C_2H_4O_2$, H_2O and the monosaccharide unit,
394 respectively), was observed for lactose and 1,4-galactobiose. In contrast, lactulose
395 (galactosyl-(1→4)-fructose) fragmentation showed different abundances for these
396 characteristic ions.
397 1,6-galactobiose and melibiose (both with 1→6 glycosidic linkage) showed a
398 similar fragmentation characterized by abundances in decreasing order of ions at *m/z*
399 305, 275, 245 (corresponding to the loss of $C_4H_8O_4$) and 335 (corresponding to the loss
400 of CH_2O). The main difference between fragmentations of these disaccharides was the
401 higher abundance of the ion at *m/z* 203 corresponding to the monosaccharide for the
402 melibiose and the abundance of the *m/z* ion 347 for 1,6-galactobiose. These results are
403 in agreement with those found by Zhang et al. [19], who showed the characteristic
404 fragmentation pattern of five different disaccharides, among them 1,3-galactobiose,
405 maltose, and isomaltose, with 1→3, 1→4 and 1→6 linkages, respectively.
406 1,1 and 1,2-linked disaccharides with galactose units could not be acquired, but
407 considering the similar fragmentation of 1→4 and 1→6 linkages with those shown by
408 Zhang et al. [19], the reported fragmentation patterns of trehalose and 1,2-mannobiose
409 were also used for the characterization of commercial GOS. In that work, Zhang et al.
410 [19] described that the MS^2 fragmentation of 1,1-linked disaccharide was dominated by
411 the *m/z* ion at 203, although it was also detected the very minor presence of the *m/z* ion
412 at 305. Nevertheless, the characteristic fragmentation pattern of 1,2-linked disaccharides
413 gave rise to the main neutral loss of $C_4H_8O_4$ (*m/z* ion at 245), followed by the ions in
414 decreasing order of abundance at *m/z* 203, 347, 275 and 305.
415
416 *3.3. Characterization of commercial GOS by HILIC-MS and HILIC-MS^n*

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

421 Three main peaks were clearly distinguished for disaccharides of GOS-1. HILIC-
422 MS^2 analyses (**Table 5**) using *m/z* 365 as precursor ion, showed relative high intensities
423 of fragments at *m/z* 347, 275, 203 for peak 1 which could correspond to a disaccharide
424 with 1→3 linkage. However, relative abundances of these *m/z* fragments are different to
425 those observed for 1,3-galactobiose which could be attributed to a different monomeric
426 composition, more similar to that of nigerose (**Table 4**). It has been reported that
427 galactosyl-(1→3)-glucose (26% wt) is more abundant than the 1,3-galactobiose (1% wt)
428 in Vivinal-GOS® [7]. Therefore, this peak could be attributed to the first compound or a
429 mixture of both. Peak 2 was the most abundant disaccharide of GOS-1 and showed a
430 MS^2 fragmentation pattern different to those of commercial standards, probably due to
431 the co-elution of different compounds. The most abundant fragments were *m/z* 305, 203
432 and 347 characteristic of 1→4 linked disaccharides and could correspond to 1,4-
433 galactobiose. However, high relative abundances of ion *m/z* 245 distinctive of 1→2
434 linkages can be also observed. Therefore, this peak could be a mixture of (1→4)- and
435 (1→2)- linked disaccharides. Coulier et al. (2009) reported the presence of lactose, 1,4-
436 galactobiose and galactosyl-(1→2)-glucose in Vivinal-GOS®. Therefore, peak 2 could
437 be a mixture of these three disaccharides. Peak 3 could clearly correspond to a (1→6)-
438 linked disaccharide considering the relative abundances of *m/z* ions at 305, 275 and 245
439 and could be assigned to allolactose (galactosyl-(1→6)-glucose) which was previously
440 identified by Coulier et al. (2009) following isolation, methylation and NMR analyses
441 in Vivinal-GOS®.

442 Regarding trisaccharides of GOS-1, five peaks were observed (**Figure 2**), however,
443 resolution among them was not completely achieved which could difficult mass
444 interpretation. MS^2 and MS^3 fragmentations were carried out using the ions *m/z* 527 and
445 365 as precursor ions, respectively. HILIC-MS^2 and HILIC-MS^3 analyses of peak 4
446 revealed a characteristic fragmentation of 1→3 linkages, similar to that observed for
447 peak 1, as the main ion fragments corresponded to the neutral losses of $C_3H_6O_3$ (*m/z*
448 fragments 437 and 275, in MS^2 and MS^3 spectra, respectively) and H_2O (*m/z* fragments
449 509 and 347, in MS^2 and MS^3 spectra, respectively) (**Table 6**). Therefore, this peak
450 could tentatively be assigned to Gal-(1→3)-Gal-(1→3)-Glc, although mixtures with
451 other trisaccharides with different monosaccharide composition could not be discarded.
452 Two compounds can be clearly distinguished by HILIC-MS^2 of peak 5. First of them,
453 peak 5a, showed a *m/z* fragmentation pattern characteristic of (1→2)-linked
454 carbohydrates [19] differing from 2α-mannobiose in the relative abundance of the
455 neutral loss of monomeric units: *m/z* 365 for the MS^2 fragmentation of the trisaccharide,
456 and *m/z* 203 for the MS^3 fragmentation of the disaccharide, being this loss more
457 abundant in the first case (**Table 6**). HILIC-MS^3 of this peak revealed a similar
458 fragmentation profile to peak 2 which could indicate the presence of a mixture of two
459 compounds with 1→2 and 1→4 glycosidic linkages. Gal-(1→4)-Gal-(1→2)-Glc has
460 been previously identified in Vivinal-GOS® [7], however, the presence of x-(1→2)-Gal-
461 (1→2)-Glc has not been previously reported. HILIC-MS^2 and HILIC-MS^3 analyses of
462 peak 5b seem to indicate the presence of 1→4 glycosidic linkages with the
463 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
464 $C_2H_4O_2$, H_2O, $C_6H_{10}O_5$ and CH_2O, which is indicative of the presence of Gal-(1→4)-
465 Gal-(1→4)-Glc. Peak 6 could be tentatively assigned to Gal-(1→6)-Gal-(1→4)-Glc
466 considering the MS^2 (losses of $C_6H_{12}O_6$, $C_2H_4O_2$ and H_2O) and MS^3 (losses of $C_2H_4O_2$,

467 $C_3H_6O_3$ and $C_4H_8O_4$) fragmentations although contribution of Gal-(1→4)- can not be
468 discarded taking into account the relative ratios of the fragment ions in MS^3. Peak 7
469 showed the characteristic patter of 1→6 glycosidic linkages for both MS^2 and MS^3
470 fragmentations and could correspond to Gal-(1→6)-Gal-(1→6)-Glc. Finally, MS^3 of
471 peak 8 clearly revealed the presence of 1→6 glycosidic linkage (losses of $C_2H_4O_2$,
472 $C_3H_6O_3$ and $C_4H_8O_4$), however MS^2 was more confusing, considering the fragment at
473 *m/z* 467, the low abundance of *m/z* 437 and the relatively high intensity of *m/z* 407. This
474 profile is similar to that detected for peak 2 and could be assigned to a mixture of 1→2
475 and 1→4 linkages.

476 Five peaks corresponding to tetrasaccharides were observed in GOS-1 by HILIC-
477 MS (**Figure 2**). Fragments at *m/z* 689 and 527 were used as precursor ions of MS^2 and
478 MS^3, respectively. Fragment at *m/z* 365 was also used as a precursor ion of MS^4,
479 although detected ions had much lower abundances (data not shown). Characterization
480 of these peaks was more complex considering the low abundances and the existence of
481 multiple coelutions. Only some linkages could be tentatively assigned as indicated in
482 **Table 7**.

483 A similar reasoning was followed for the characterization of di-, tri- and
484 tetrasaccharides of GOS-2 and GOS-3. These data are shown in **Tables 5**, **6** and **7**. In
485 general, GOS-3 showed a similar qualitative composition to GOS-1, however, notable
486 differences were observed for GOS-2 which exhibited a lower diversity of glycosidic
487 linkages. This fact is supported by the high similarity of the chromatographic profiles of
488 GOS-1 and GOS-3 in oligosaccharide retention times and peak shapes, whilst the
489 HILIC profile of GOS-2 exhibited some differences in terms of retention times and,
490 especially, in peak abundances (**Figure 2**), as it will be discussed in section 3.4.

491 Regarding GOS-2 disaccharides (**Table 5**), in peak 1 co-eluted two different
492 carbohydrates, probably Gal-(1→3)-Glc characterized by the fragment at *m/z* 275 and
493 lactulose which showed high contribution of *m/z* 347 and low of *m/z* 305 and 317
494 (**Table 5**). Presence of 1→4 glycosidic linkage could be easily detected in peak 2 of
495 GOS-2, whereas the contribution of 1→2 linkage (fragment at *m/z* 245) was smaller
496 than those of GOS-1 and GOS-3. Peak 3 was identified as Gal-(1→6)-Glc, likewise in
497 the other two samples. The main trisaccharide (peak 5, **Table 6**) was assigned to Gal-
498 (1→6)-Gal-(1→4)-x, whereas peak 4 could be characterized by a mixture of two
499 compounds (Gal-(1→4)-Gal-(1→6)-x and Gal-(1→2)-Gal-(1→6)-x). Peaks 6 and 7
500 showed the typical MS^2 fragmentation of (1→6) linkages, MS^3 spectra being
501 characteristic of (1→6) and (1→3), respectively. Tetrasaccharides showed very low
502 abundances and mainly presence of -(1→4)- and -(1→6)- could be hypothesized (**Table
503 7**).
504
505 *3.4. Quantitation of GOS by HILIC-MS*
506 Quantitative analysis was carried out following the external standard method
507 using the homologous series of maltodextrins. Limit of detection (*LOD*) showed values
508 of 0.04-0.08 ng injected; whereas limit of quantitation (*LOQ*) was 0.14-0.28 ng injected.
509 Intra- and inter-day reproducibility was also evaluated, relative standard deviation being
510 lower than 10 % for the different standards analyzed.
511 **Table 8** shows quantitative data for GOS mixtures. Disaccharides were the main
512 carbohydrates present in GOS samples (54, 76 and 53% for GOS-1, GOS-2 and GOS-3,
513 respectively); lactose (quantified together with Gal-(1→2)-Glc in GOS-1 and GOS-3)
514 being the most abundant. Regarding trisaccharides, similar percentages were observed
515 for GOS-1 and GOS-3 (~29 %), whilst GOS-2 had lower percentages (22.5%).

516 Likewise, tetrasaccharides of GOS-2 only constituted the 1.5 % of its composition,
517 whereas levels of 11-12% were found in GOS-1 and GOS-3. Only traces of
518 pentasaccharides could be detected in GOS-2. Therefore, yields of oligosaccharides in
519 GOS-1 and GOS-3 were higher than those found in GOS-2, probably due to the
520 manufacturing conditions used to obtain these products [5].

521

522 4. Conclusions

523 The results presented in this work show the usefulness of HILIC-MS^n to separate
524 and tentatively characterize complex mixtures of GOS without a previous fractionation,
525 enrichment or derivatization step. The three studied silica-based HILIC columns
526 exhibited substantial differences in peak shape, retention and selectivity which could be
527 mainly attributed to the nature of the surface chemistry of the assayed stationary phases
528 (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridge
529 hybrid (BEH) with trifunctionally-bonded amide). Likewise, differences in the
530 dimension of columns and, especially, particle properties (particle size, pore size and
531 surface area) might also contribute to the retention of GOS. In this context, polar
532 compounds possessing a high number of hydroxyl groups such as GOS were efficiently
533 retained and separated on the BEH amide stationary phase using acetonitrile: water with
534 0.1% ammonium hydroxide as mobile phase.

535 The characterization of prebiotic GOS is of paramount importance for the
536 elucidation of the structure-bioactivity relationship with respect to the effect of these
537 carbohydrates on the human gastrointestinal health. MS^n characterization of GOS (in
538 terms of monosaccharide composition, degree of polymerization and glycosidic
539 linkages) should be considered tentative, taking into account the lack of standards.

540 However, it requires much less handling, is less tedious and time consuming than the

541 combination of complex techniques (isolation of each compound by fractionation

542 methods and the subsequent analysis by NMR and methylation procedures) traditionally

543 proposed in the literature.

544

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