Separation of di- and trisaccharide mixtures by comprehensive two-dimensional liquid chromatography. Application to prebiotic oligosaccharides

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18 **Abstract**

19 Carbohydrates are one of the most important ingredients in foods. They are normally
20 present as complex mixtures with different glycosidic linkages, monomeric units and
21 degrees of polymerization. This structural heterogeneity impairs their comprehensive
22 characterization and requires the use of analytical techniques with high resolving power
23 and sensitivity. The use of chromatographic techniques, especially liquid chromatography
24 (LC), has been extremely helpful for the analysis of carbohydrates. However, in many
25 cases, the use of monodimensional LC is not enough to resolve these complex mixtures;
26 then, the use of techniques with a higher resolving power, as multidimensional LC, could
27 be a good alternative. To the best of our knowledge, our findings are pioneer in applying
28 online LC×LC for the analysis of carbohydrate mixtures. For this purpose, different
29 conditions such as stationary phases (BEH amide, C_{18} and PGC columns) and
30 chromatographic conditions for the separation of di- and trisaccharide mixtures were
31 optimized. The BEH amide × C_{18} combination was selected for the LC×LC analysis of
32 carbohydrate standards with different degree of polymerization, linkages and monomeric
33 units. In order to allow their proper UV detection, carbohydrates were previously
34 derivatized using *p*-aminobenzoic ethyl ester. This method also resulted to be successful
35 for the separation of commercial prebiotic mixtures of galacto-oligosaccharides and
36 gentio-oligosaccharides. This is the first time that LC×LC has been applied for the
37 separation of bioactive carbohydrate mixtures and it could be considered as a powerful
38 analytical technique for the characterization of other oligosaccharide complex mixtures.

39

40 **Keywords:** comprehensive two-dimensional liquid chromatography (LC×LC),
41 disaccharides, trisaccharides, prebiotic, glycosidic linkages.

42 **1. Introduction**

43 Carbohydrates are one of the most important food constituents, usually present as
44 complex mixtures of isomeric molecules of different degrees of polymerization which
45 only differ in the configuration of their hydroxyl groups and in the position of their
46 glycosidic linkages. The primary role of food carbohydrates is to provide energy to all
47 cells in the body and dietary fiber [1]. Within dietary fiber, non-digestible carbohydrates,
48 which are not absorbed in the small intestine and, therefore, move down to be fermented
49 in the large intestine, have drawn attention because their beneficial impact on human
50 health. Concretely, low glycemic index foods, whose intake is related to a reduced risk of
51 common Western chronic diseases associated with central obesity and insulin resistance
52 [2,3], are characterized by the presence of non-digestible or slowly absorbed
53 carbohydrates instead of free sugars. Those non-digestible carbohydrates having the
54 ability to be “selectively utilized by living host microorganisms (usually, in the gut
55 microbiota ecosystem) conferring a health benefit” are defined as prebiotics [4]. Among
56 the carbohydrates with a well-recognized prebiotic status, galactooligosaccharides (GOS)
57 are by far the most complex structurally. They may comprise a wide mixture of
58 oligosaccharides that can vary from 1 to 8 galactose units and a terminal glucose linked
59 by a great diversity of β-glycosidic linkages, mainly (1→3), (1→4) and/or (1→6). The
60 presence of (1↔1) and (1→2) linkages has also been reported for commercial dietary
61 GOS [5]. Similarly, other potential prebiotic carbohydrates such as
62 gentiooligosaccharides (GEOS), xylooligosaccharides, isomaltooligosaccharides, etc. are
63 also complex structures. For this reason, the analysis and structural characterization of
64 prebiotic carbohydrates, which is required to fully understand their functionality and
65 correlation with the chemical structure, is a challenging task.

66 Nowadays, high performance liquid chromatography (LC), combined with pulsed
67 amperometric, refractive index, ultraviolet and fluorescence detectors or mass
68 spectrometry, is the most used chromatographic technique for the analysis of complex
69 mixtures of oligosaccharides. This is due to the huge development during the last decades
70 of a wide range of new support materials and/or stationary phases operating under
71 different separation modes [6]. Derivatization procedures of oligosaccharides before their
72 LC separation have also been suggested to improve their chromatographic properties [7,
73 8]. Nevertheless, there are still important limitations for the efficient separation of
74 complex oligosaccharide mixtures when several structural features, such as degree of
75 polymerization, linkage pattern, monomeric composition and/or isomerism, differ. In this
76 context, the application of multidimensional liquid chromatography and, particularly, the
77 use of on-line comprehensive two-dimensional LC (LC×LC) could be a very powerful
78 and promising alternative to overcome the drawbacks on the capability of separating
79 complex oligosaccharide mixtures by the currently available monodimensional LC
80 (1DLC) methods. Nevertheless, although the applications developed by on-line LC×LC
81 devoted to food and natural products are increasing [9-11], to the best of our knowledge
82 there is a remarkable lack of comprehensive LC applications for carbohydrates analysis.
83 Difficulties in finding the correct columns combinations to attain good degrees of
84 orthogonality between dimensions, solvent mismatch problems during fraction transfer
85 in on-line methods, as well as complications related to detection are partly responsible
86 for this absence of LC×LC methods.
87 Therefore, considering these analytical challenges, the current work aims to develop a
88 novel LC×LC method for the efficient separation of di- and trisaccharide mixtures with
89 different glycosidic linkages and monomeric composition. A thorough optimization by
90 using a wide range of carbohydrate standards was previously accomplished by 1DLC to

91 select the most appropriate stationary phase combination for the LC×LC separation as
92 well as the derivatization procedure to facilitate their UV-based detection. The optimized
93 method was applied to the separation of different prebiotic oligosaccharide mixtures,
94 dominated by di- and trisaccharides, such as three commercial dietary GOS differing in
95 the predominant β-glycosidic linkage and a sample of GEOS. This latter product is a
96 novel functional oligosaccharide mixture composed of glucose units mainly linked
97 through β(1→6) glycosidic bonds.

98

99 **2. Materials and methods**

100 **2.1. Standards and oligosaccharide mixtures.**

101 1,3-Galactobiose (α-Gal-[1→3]-Gal), 1,4-galactobiose (β-Gal-[1→4]-Gal), 1,6-
102 galactobiose (β-Gal-[1→6]-Gal), galactotriose (α-Gal-[1→3]-β-Gal-[1→4]-Gal) were
103 acquired from Dextra Laboratories (Reading, UK), whereas kojibiose (α-Glc-[1→2]-
104 Glc), kojitriose (α-Glc-[1→2]$_2$-Glc), lactose (β-Gal-[1→4]-Glc), laminaribiose (β-Glc-
105 [1→3]-Glc), laminaritriose (β-Glc-[1→3]$_2$-Glc), isomaltose (α-Glc-[1→6]-Glc),
106 isomaltotriose (α-Glc-[1→6]$_2$-Glc), maltose (α-Glc-[1→4]-Glc), maltotriose (α-Glc-
107 [1→4])$_2$-Glc), cellobiose (β-Glc-[1→4]-Glc), were obtained from Sigma (St. Louis,
108 USA), 3'-Galactosyl lactose (β-Gal-[1→3]-β-Gal-[1→4]-Glc), 4'-Galactosyl lactose (β-
109 Gal-[1→4]-β-Gal-[1→4]-Glc) and 6'-Galactosyl lactose (β-Gal-[1→6]-β-Gal-[1→4]-
110 Glc) were acquired from Carbosynth (Berkshire, UK). *p*-Aminobenzoic ethyl ester
111 (ABBE) was obtained from Sigma (St. Louis, USA).

112 Vivinal-GOS (GOS1) was kindly provided by Friesland Foods Domo (Zwolle, The
113 Netherlands); BiMuno (Clasado, Reading, UK) (GOS2) and PromoVita (Dairy Crest,
114 Esher, UK) (GOS3) were acquired in local markets. Gentio-oligosaccharides (GEOS)
115 were acquired from Wako Chemicals GmbH (Neuss, Germany).

Commercial standards and commercial oligosaccharide mixtures were dissolved in the corresponding HPLC mobile phase at a concentration of 0.1 mg mL^{-1} and filtered through a nylon FH membrane (0.22 µm) (Millipore, Bedford, MA, USA) before injection. All HPLC analyses were carried out in duplicate.

2.2. Derivatization procedure.

The absence of chromophore groups in carbohydrates, which hinder their direct detection by UV detectors, was solved by a previously developed derivatization procedure with *p*-aminobenzoic ethyl ester (ABBE) [12]. Briefly, the ABEE reagent was firstly prepared by mixing 165 mg ABEE, 35 mg sodium cyanoborohydride, 41 µL acetic acid, and 0.35 mL warm methanol. Then, 40 µL of ABEE reagent was added to 10 µL sample (dissolved in water) and kept at 80ºC for 1h. After cooling, 0.2 mL of H_20 and 0.2 mL of chloroform were added. The sample was then centrifuged for 1 min and the upper aqueous layer recovered and used for analysis. These derivatives were stable at least for 1 week (RSD 3.4%).

2.3. One-dimensional (1DLC) analysis of carbohydrates

2.3.1. Instrumentation

Conventional LC analyses were carried out on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) equipped with an oven (Kariba Instruments, UK) and a UV detector (Agilent) with a wavelength set at 304 nm.

2.3.2. Separation conditions.

Three different columns based on different LC operation modes were evaluated. These columns were an ethylene bridge hybrid with trifunctionally-bonded amide phase (BEH

141 amide column) (150×4.6 mm; 3.5 µm) from Waters (Hertfordshire, UK), a C_{18} column
142 (Zorbax Eclipse XDB) (50×4.6 mm; 1.8 µm) from Agilent Technologies (Waldbronn,
143 Germany) and a Hypercarb porous graphitic carbon (PGC) column (150×2.1 mm; 3 µm)
144 from Thermo Scientific (Waltham, MA, USA). Different gradients of ACN and water in
145 different proportions, flow rates and analysis times were assayed for the separation of the
146 derivatized carbohydrates for each evaluated column, obtaining the following optimum
147 gradient conditions:
148 *BEH amide stationary phase:* acetonitrile (A) and water (B) eluted using the following
149 gradient: 0 min, 15 % B; 4 min, 15 % B; 14 min, 40 % B; 19 min, 40 % B.
150 *C_{18} column*: Water (A) and acetonitrile (B) eluted using the following gradient: 0 min, 5
151 % B; 30 min, 40 % B; 31 min, 60 % B; 36 min, 60 % B.
152 *Porous graphitic carbon (PGC) column*: Water (A) and acetonitrile (B) eluted using the
153 following gradient: 0 min, 60 % B; 20 min, 90 % B; 21 min, 90 % B.
154 For all of them 10 minutes was used as equilibration time, a flow rate of 0.4 mL min^{-1}
155 was selected and the injection volume was 5 µL.
156 Different chromatographic parameters were considered for optimization of 1DLC
157 methods, namely, retention time (t_R), peak width at half height (w_h), symmetry factor (S),
158 as the ratio of the front half to back half widths at 10% of the peak height, and resolution
159 (R_S), calculated according to

160 $$R_S = 2\frac{(t_{R2} - t_{R1})}{(w_{b1} + w_{b2})} \qquad (1)$$

161 where sub-indexes 1 and 2 refer to two consecutive eluting carbohydrates, and w_b is the
162 peak width at baseline.
163

164 **2.4. LC × LC analysis of carbohydrates**

165 *2.4.1. Instrumentation*

166 Comprehensive two-dimensional liquid chromatography instrumentation consisted on a
167 first dimension (^{1}D) composed of an Agilent 1200 series liquid chromatograph (Agilent
168 Technologies, Waldbronn, Germany) equipped with an autosampler and a diode array
169 detector which was connected at the exit of the second dimension. The second dimension
170 (^{2}D) was carried out using an additional LC pump (Agilent 1290 Infinity). An
171 electronically-controlled two-position ten-port switching valve (Rheodyne, Rohnert Park,
172 CA, USA) was used as modulator to connect both dimensions. Two identical sampling
173 loops (50 or 80 µL) or two C_{18} trapping columns (10×3 mm, 2.6 µm, Accucore, Thermo
174 Scientific, Waltham, MA, USA) were connected to the switching valve to collect the
175 fractions from the ^{1}D and to inject them into the ^{2}D. Separations were recorded at 304 nm
176 at the maximum available sampling rate (20 Hz). For the active modulation configuration,
177 an additional make-up flow was provided by a third LC pump (Agilent 1200 series)
178 connected through a T-piece between the outlet of ^{1}D and the switching valve. LC image
179 software (Zoex Corp., Houston, TX, USA) was used to plot the results as 2D and 3D
180 images.
181

182 *2.4.2. LC × LC separation conditions*

183 A HILIC separation in the ^{1}D was coupled to a reversed phase separation in the ^{2}D. After
184 optimization (see Sections 3.1 and 3.2), the following final conditions were employed:
185 *^{1}D separation:* A BEH amide column (150×2.1 mm; 3.5 µm particle size, Waters,
186 Hertfordshire, UK) was employed at a flow rate of 0.05 mL min^{-1}. Under optimum
187 conditions, H_2O (A) and ACN (B) were used as mobiles phases, eluted using the
188 following gradient: 0 min, 90% B, 60 min 50 % B; 70 min, 50% B; 71 min, 90% B; 85
189 min 90% B.

190 ^{2}D *separation:* two columns with different length (partially-porous C_{18} Ascentis Express
191 30 or 50×4.6 mm, 2.7 µm, Supelco, Bellefonte, CA, USA) were tested. Gradient, flow
192 rate, temperature, type of acid and solvents used were optimized separately. The optimum
193 conditions were achieved using the column of 50 mm length and 2.7 µm particle size with
194 H_2O (solvent A) and ACN (solvent B) as mobile phases. The optimum gradient was as
195 follow: 0 min, 10% B; 0.75 min, 40% B; 0.76 min, 90% B; 0.9 min, 10% B; and re-
196 equilibrium at starting conditions until 1 min. The flow rate was set at 2.5 mL min^{-1}.
197 Once these parameters were optimized, the coupling between both dimensions was
198 achieved employing 1 min repetitive ^{2}D separations; thus, the modulation time was 1 min.
199 The third pump used to achieve the active modulation was set at 500 µL min^{-1}, delivering
200 solvent with the same composition than the initial ^{2}D gradient conditions (10% B).
201

202 **2.5. Calculations**

203 To study the performance of the developed methods, the attainable peak capacity and
204 orthogonality were calculated. Individual peak capacities in each dimension were
205 calculated according to eq. 2 [13] as follows:

206 $$n_c = 1 + \frac{t_g}{(\frac{1}{n})\sum_1^n w} \qquad (2)$$

207 Where, n_c represents the peak capacity, n is the number of compounds, t_g is the gradient
208 time and, w is the peak width. With the individual peak capacity values of each dimension,
209 the total theoretical peak capacity of the two-dimensional method was calculated as,

210 $$^{2D}n_c = {}^{1}n_c \times {}^{2}n_c \qquad (3)$$

211 This value is clearly overestimated since it does not consider the effect of undersampling
212 of the ^{1}D and the peak broadening effect occurred during modulation. To solve this
213 problem, the effective peak capacity was calculated according to eq. 4 [14]:

214 $$^{2D}n'_c = \frac{^1n_c \times {}^2n_c}{\sqrt{1+3.35\times\left(\frac{^2t_c\,{}^1n_c}{^1t_G}\right)^2}} \qquad (4)$$

215

216 In this case, $^{2D}n'_c$ is the effective 2D peak capacity, ^2t_c is the second dimension cycle time

217 and ^1t_G is the first dimension gradient time. Lastly, the corrected peak capacity was

218 defined as:

219 $$^{2D}n_{c,corrected} = {}^{2D}n'_c \times A_0 \qquad (5)$$

220 Where A_0 is the orthogonality value calculated in agreement with the asterisk equations

221 method [15].

222

223 **3. Results and discussion**

224 **3.1. 1DLC analysis of di- and trisaccharides**

225 Preliminary experiments in 1DLC were carried out to select the most appropriate

226 stationary phase combination for further LC×LC separation of ABEE oligosaccharide

227 mixtures. Several di- and trisaccharide standards with different monomeric units and

228 glycosidic linkages were selected for the optimization of the separation in each stationary

229 phase. Three columns based on different HPLC operation mechanisms were assayed. In

230 all cases, chromatographic conditions were optimized in terms of the shortest t_R, the best

231 peak symmetry, the highest R_s and the lowest w_h, using acetonitrile and water as mobile

232 phases.

233 As it can be observed in Table 1, on BEH amide stationary phase, ABEE disaccharides

234 eluted at shorter retention times than trisaccharides, with an excellent resolution between

235 them. For instance, a resolution of R_s= 2.6 between the last disaccharide (1,6-

236 galactobiose) and the first trisaccharide to elute (laminaritriose) was achieved. Similar

237 results had been previously observed by Brokl et al. [16] and Hernández-Hernández et al.

238 [17] for non-derivatized carbohydrates, who found that this column was useful for the
239 separation of oligosaccharides with different degree of polymerization. However, a poor
240 resolution (R_s<0.48) among the different disaccharides was obtained, except for kojibiose
241 and 1,6-galactobiose and for maltose and 1,4-galactobiose. On the contrary, trisaccharides
242 were, in general, appropriately resolved (R_S >0.90), except for galactotriose and 3'-
243 galactosyl-lactose and for isomaltotriose and kojitriose. For a given glycosidic linkage,
244 di- and trisaccharides composed by glucosyl units eluted before those containing
245 galactosyl units (e.g., laminaribiose and 1,3-galactobiose; maltose and 1,4-galactobiose;
246 isomaltose and 1,6-galactobiose; maltotriose and 4'-galactosyl-lactose). For each
247 polymerization degree, in general, glucosyl-di- and trisaccharides with 1→3 glycosidic
248 linkages eluted first, while the most retained compounds were those with 1→6 linkages.
249 A similar behavior was observed for galactosyl-di- and trisaccharides, although isomers
250 with 1→4 linkages were the first to elute.
251 In general, good peak widths (w_h: 0.16-0.21 min) and symmetry values (0.79-1.55) were
252 observed for trisaccharides that eluted under the optimized conditions in BEH amide
253 stationary phase. Poorer results were obtained for disaccharides (w_h: 0.25-0.37 min and
254 symmetry: 1.02-1.95).
255 Regarding C_{18} stationary phase, the separation of ABBE oligosaccharides was not size-
256 dependent (Table 1), as it had been previously observed for oligosaccharides released
257 from glycosphingolipids [12]. Trisaccharides eluted in the range 10.70 min (6'-
258 galactosyl-lactose) – 14.72 min (maltotriose), while disaccharides eluted between 13.63
259 min (kojibiose) and 15.17 min (maltose). Contrary to that observed in the BEH amide
260 column, some trisaccharides eluted before disaccharides. The elution order was neither
261 associated to the composition of monomeric units (Glc and Gal) nor to the glycosidic
262 linkages. This column provided good w_h values (0.11-0.19 min) for all the carbohydrates.

263 In general, resolution values were acceptable for some carbohydrates although several
264 coelutions were observed for both di- and trisaccharides (e.g., laminaribiose and
265 laminaritriose). Peaks also showed a satisfactory symmetry (0.8-0.9), except for 6'-
266 galactosyl-lactose (0.38).

267 Graphitized carbon emerged as stationary phases in LC as an alternative to RP columns
268 for the analysis of polar compounds [18] and it has been successfully used for the
269 separation of neutral oligosaccharides, *N*-linked-oligosaccharides or chito-
270 oligosaccharides, providing good resolution for non-derivatized carbohydrates [19]. In
271 general, ABEE carbohydrates showed low retention times under optimal
272 chromatographic conditions in PGC column (Table 1). Most disaccharides eluted earlier
273 than trisaccharides; only maltose (5.77 min) exhibited a relatively high retention in this
274 stationary phase, eluting at the end of the chromatogram, surpassed only by maltotriose
275 (7.75 min). Broad peaks (from 0.20 to 0.41 min) with poor symmetry (0.25-0.53) were
276 obtained. Regarding R_s, only galactotriose, maltose and maltotriose were well resolved
277 (R_s,>0.99).

278 Retention times of ABEE di- and trisaccharides eluting on the three evaluated columns
279 were reconstructed in two dimensions (see Figure S1). As can be observed, the
280 combination of C_{18} column with both, BEH amide and PGC columns provided the best
281 separation of target carbohydrates. In both combinations, coelutions occurred in one
282 dimension could theoretically be resolved in the second dimension. However, considering
283 the peak width and symmetry values commented before, the use of the BEH amide
284 column in the ^{1}D and a C_{18} column in the ^{2}D was considered, in theory, the most promising
285 alternative for LC×LC separation of oligosaccharide mixtures.

286

287 **3.2. Optimization of the LC×LC separation of oligosaccharides**

288 To find appropriate chromatographic conditions compatible with LC×LC for the
289 separation of carbohydrates is a great challenge, mainly considering the broad distribution
290 of the compounds in the ^{1}D available separation space sought, as well as the limited
291 separation time afforded in the ^{2}D. As a result, different pre-requisites were imposed to
292 the separation conditions for optimization; in the ^{1}D, the attainment of good separations
293 with a maximum flow rate of 0.05 mL min^{-1} was targeted, whereas proper individual
294 separations of less than 1 min were aimed in the ^{2}D.
295 Starting from the mobile phases already selected in conventional LC (acetonitrile and
296 water), the gradient profile was adapted to the flow rate reduction and subsequently
297 optimized. Figure S2 shows a comparison between the different chromatograms obtained
298 during the optimization study of the ^{1}D separation using commercial disaccharide and
299 trisaccharide standards with different linkages and monomeric units. The best separation
300 of target carbohydrates was achieved varying acetonitrile from 90% to 50% in 60 min.
301 As it is known, under HILIC conditions, the sample solvent has a strong influence on the
302 attainable separation, which is even greater in the case of narrow bore columns as the one
303 used in the present work. A high water content in the sample may break the interaction
304 between analytes and the aqueous layer surrounding the stationary phase particles
305 hampering the separation due to lack of retention. To avoid this problem, the derivatized
306 samples were diluted with acetonitrile before injection.
307 On the other hand, two ^{2}D C_{18} columns with diverse length (30 and 50 mm) were tested.
308 Acetonitrile and water were also chosen as mobile phases under RP conditions. For each
309 column, different flow rates (1, 2 and 2.5 mL min^{-1}) and gradients were studied with the
310 aim to obtain a good separation within the previously allotted time (1 min). Optimum
311 conditions involved the use of 2.5 mL min^{-1} as flow rate using the following gradient: 0
312 min, 10% B; 0.75 min, 40% B; 0.76 min, 90% B; 0.9 min, 10% B; and re-equilibrium at

313 starting conditions until 1 min. Chromatographic profiles of a disaccharide mixture
314 obtained at different flow rates under the selected optimum gradient conditions for ^{2}D are
315 shown in Figure S3. Under those conditions, both tested columns provided very similar
316 performance (Figure S3D,E). Considering that the separation was finished in less than 1
317 min in both cases, the 50 mm length column was selected for further LC×LC optimization
318 due to its longer separation space available.
319 Once the individual conditions for each dimension compatible with LC×LC were
320 determined, the overall optimization of the two-dimensional separation was carried out.
321 The coupling between the two dimensions was established by using two 50 μL identical
322 sampling loops installed in the switching valve. This internal volume was the smallest
323 needed to collect each complete fraction eluting from the ^{1}D during the modulation
324 period. Under those conditions, the analysis of a di- and trisaccharide mixture was carried
325 out. Results obtained are shown in Figure 1A. Although some separation was observed,
326 peaks were grouped in two areas of the 2D plot corresponding to the beginning and half
327 of each ^{2}D analysis, suggesting that no proper retention/elution was obtained. The lack of
328 retention (peaks doubled) was most probably related to solvent strength incompatibility
329 between dimensions. In fact, the fraction solvent was by far stronger for the ^{2}D than the
330 initial gradient conditions. This problem has been widely reported in HILIC×RP
331 couplings [20]. In other applications, an increase of the fraction volume was shown to be
332 positive towards the elimination of this issue; the use of sampling loops with internal
333 volume larger than strictly needed to accommodate the ^{1}D fraction was demonstrated to
334 be useful to produce a dilution effect with ^{2}D compatible solvent [21]. Consequently, this
335 approach could be effective in reducing the overall fraction solvent strength and, thus,
336 improving the attainable separation. To test this potential solution, the same separation

337 conditions were applied increasing the sampling loops internal volume to 80 µL. As it
338 can be observed in Figure 1B, no noticeable improvement was produced.
339 The next step carried out to overcome this problem was the use of trapping columns in
340 the interface instead of sampling loops, looking for a focusing modulation able to increase
341 analyte retention. C_{18} trapping columns were employed using the same 2D analytical
342 conditions (Figure 1C). The selection of the stationary material was made in order to
343 match the selectivity used in the ^{2}D. The two tested trapping columns possessed a void
344 volume of 50 µL, thus, capable of collecting the whole fraction coming from the ^{1}D and
345 were eluted in forward elution mode. However, under the tested conditions, the problem
346 persisted and no complete retention was obtained using this focusing modulation
347 approach.
348 For this reason, the use of active modulation was investigated in this application. The
349 ability of active modulation to resolve solvent strength mismatch problems between
350 dimensions has been previously demonstrated [20-22]. The same trapping columns
351 previously installed were maintained but an additional make-up flow was introduced at
352 the exit of the ^{1}D and mixed with the fraction eluting from the ^{1}D column and directed to
353 the modulator. The make-up flow composition was selected to match the initial conditions
354 found in the ^{2}D gradient (90:10 water/acetonitrile), whereas the flow rate was set
355 according to our previous experience as 10-times the ^{1}D flow rate, i.e., 0.5 mL min^{-1} [20].
356 The rest of separation conditions were maintained. As can be seen in Figure 1D, the use
357 of active modulation resolved the retention issue observed in the other set-ups. In this
358 case, the separation in the ^{2}D was improved, and no doubled peaks were observed. Thus,
359 in spite of a more complicated set-up, including an additional pump for the make-up flow,
360 this strategy clearly permitted to produce a concentration step in the trapping columns
361 thanks to the dilution on a fully compatible solvent composition. Once the fraction

362 collection was finished, the ^{2}D gradient started and the compounds were eluted from the
363 trapping column accordingly. These separation conditions were, therefore, selected for
364 further analysis.
365

366 **3.3. Analysis of oligosaccharides by LC×LC.**

367 The optimized separation method was applied to the analysis of different di- and
368 trisaccharide mixtures with different glycosidic linkages (1→2, 1→3, 1→4 and 1→6
369 bonds) and monomeric units (Glc and Gal). Figure 2 shows the LC×LC contour plots of
370 the mixtures of glucosyl-disaccharides (panel A, kojibiose, laminaribiose, maltose,
371 cellobiose and gentiobiose), glucosyl-trisaccharides (panel B, kojitriose, laminaritriose,
372 maltotriose and isomaltotriose), galactosyl-disaccharides (panel C, 1,3-galactobiose, 1,4-
373 galactobiose, lactose and 1,6-galactobiose) and galactosyl-trisaccharides (panel D,
374 galactotriose, 3'-galactosyl lactose, 4'-galactosyl lactose and 6'-galactosyl lactose).
375 These mixtures were chromatographically resolved in the two-dimensions while some of
376 these compounds coeluted in monodimensional LC. This is the case, for instance, of
377 glucosyl-disaccharides (Figure 2A): kojibiose (with α1→2 linkage) was separated from
378 isomaltose (with α1→6 linkage) and cellobiose (with β1→4 linkage) from laminaribiose
379 (with β1→3 linkage) in the ^{2}D, while maltose (with α1→4 linkage) was separated from
380 cellobiose in ^{1}D. A similar pattern was also observed for their corresponding glucosyl-
381 trisaccharides (having the same glycosidic linkages and monomeric composition; Figure
382 2B). The formation of group-type patterns on the two-dimensional plane has been
383 previously observed for other type of compounds in LC×LC [10]. It has been seen that
384 regular variation of the compound structures could lead to ordering in a chromatogram.
385 Then, this method can be useful for the identification of unknown carbohydrates with a
386 certain degree of structural similarities present in complex samples.

387 Regarding galactosyl-di- and trisaccharides (Figure 2C and 2D, respectively), a good
388 separation was observed for each mixture; however, a group-type pattern could not be
389 established due to the different linkages and monomeric units constituting these
390 carbohydrates.
391 Then, to evaluate the advantages of the new LC×LC method for the separation of di- and
392 trisaccharides with different glycosidic linkages and monomeric units, three commercial
393 prebiotic mixtures of GOS and one commercial preparation of GEOS were analyzed. The
394 2D plots obtained for these samples are shown in Figure 3. Identification of the different
395 di- and trisaccharides was considered tentative and it was carried out based on the
396 comparison of retention times in ^{1}D and ^{2}D of the target carbohydrates with those of the
397 corresponding standards, when available, and/or data from literature regarding GOS
398 composition [17, 23]. Carbohydrates were properly resolved using the developed LC×LC
399 method despite the usual differences in concentration levels of target carbohydrates
400 present in real samples. This was the case, for example, for the structurally different
401 galactosyl-lactoses (4', 3' and 6') present in GOS1 sample at dissimilar concentrations
402 and which were efficiently resolved (Figure 3A). Furthermore, the optimized LC×LC
403 method provided the benefit of resolving isomeric oligosaccharides respect to 1DLC in
404 all assessed samples. For instance, 4'-galactosyl-lactose was efficiently resolved in GOS1
405 and GOS3 samples by LC×LC separation from an unknown trisaccharide which coeluted
406 by 1DLC (Figures 3A and 3C). This was also the case for the separation of several β-
407 galactosyl-galactoses from β-galactosyl-glucoses observed in GOS2 (Figure 3B) and
408 GOS3 samples (Figure 3C), as well as among the complex mixture of glucobioses present
409 in the GEOS sample (Figure 3D).
410 To establish a comparison of the method performance for the different samples analyzed,
411 peak capacities and orthogonality values were estimated in each case (Table 2). As it can

412 be observed, high effective peak capacity values, up to 1425 when undersampling from
413 the ^{1}D was already considered, were obtained for all the studied samples. These values
414 demonstrate theoretically the high separation power obtained using the optimized
415 methodology. However, more realistic peak capacity figures can be offered derived from
416 the calculation of the orthogonality degree. To do that, the orthogonality degree of each
417 separation was also estimated. In this case, relatively good values around 40% were
418 obtained for all the samples except GOS3. In general, good use of the available 2D space
419 was obtained, considering that the separated peaks were not grouped in the diagonal, as
420 it often happens with not entirely non-correlated separation mechanisms. However, the
421 ^{2}D separation space was not completely used, as peaks tended to elute around a 20 s
422 window in spite of the longer gradient employed. This fact is the main responsible for not
423 attaining even higher A_0 values, and clearly illustrates some of the challenges behind
424 carbohydrates analysis. In any case, when the effective peak capacity values were
425 corrected by orthogonality following eq. (5), good values were obtained for GOS1, GOS2
426 and GEOS of up to 531, which are comparable to other corrected peak capacities in other
427 successful applications [20].
428

429 **4. Conclusions**

430 A new HILIC×RP method has been developed for the separation of di- and trisaccharides
431 with different glycosidic linkages and monomeric unit. After preliminary analyses by
432 1DLC, the column combination involving the use of BEH amide column in the ^{1}D and
433 C_{18} in the ^{2}D was selected for the separation of these components by LC×LC. Active
434 modulation was shown to be useful to solve the solvent mismatch problems encountered
435 during the transfer to the ^{2}D. Thanks to the optimized methodology, it was possible to
436 analyze, for the first time, commercial prebiotic oligosaccharide mixtures of different

437 nature (GOS and GEOS) involving the presence of diverse glycosidic linkages that were
438 not separated by conventional LC. Our data demonstrate the utility of on-line LC×LC to
439 analyze complex oligosaccharide mixtures of low degree of polymerization, and open the
440 door to further applications in the field of carbohydrates analysis.

441

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447

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515 **FIGURE LEGENDS.**

516 **Figure 1**. 2D HILIC×RP separation of a di- and trisaccharide mixture using: A, non-
517 focusing modulation with 50 µL sampling loops; B, non-focusing modulation with 80 µL
518 sampling loops; C, focusing modulation using two trapping columns (C_{18}, 10×3 mm, 2.6
519 µm); D, focusing modulation with trapping columns and active modulation. For detailed
520 experimental conditions, see text.

521

522 **Figure 2.** 2D plots (304 nm) obtained under optimum HILIC×RP separation conditions
523 for different di- (A and C) and trisaccharide (B and D) mixtures. For detailed analytical
524 conditions, see text.

525

526 **Figure 3.** 2D plots (304 nm) obtained under optimum HILIC×RP separation conditions
527 for different commercial prebiotic oligosaccharide products. For detailed analytical
528 conditions, see text.

529 530 531

Table 1. Retention time (t_R, min), peak width (w_h), resolution (R_s) and symmetry of standard carbohydrates analyzed using a BEH amide, a C_{18} and a PGC column.

Column	Compound	t_R	w_h	S	R_s
BEH amide	Laminaribiose	14.00	0.36	1.16	0.04
	Cellobiose	14.03	0.34	1.20	0.22
	Maltose	14.16	0.37	1.48	1.04
	1,4-Galactobiose	14.80	0.35	1.95	0.08
	Lactose	14.84	0.27	1.18	0.48
	Isomaltose	15.09	0.32	1.41	0.01
	1,3-Galactobiose	15.09	0.34	1.73	0.16
	Kojibiose	15.17	0.25	1.02	1.39
	1,6-Galactobiose	15.77	0.25	1.08	2.60
	Laminaritriose	16.79	0.21	1.47	1.31
	Maltotriose	17.26	0.21	0.91	1.32
	4'- Galactosyl lactose	17.71	0.19	1.55	0.94
	Galactotriose	18.02	0.19	0.98	0.67
	3'-Galactosyl lactose	18.24	0.19	1.27	0.90
	Isomaltotriose	18.53	0.19	1.21	0.28
	Kojitriose	18.62	0.17	0.79	1.38
	6'-Galactosyl lactose	19.01	0.16	0.81	
C_{18}	6'-Galactosyl lactose	10.70	0.19	0.38	5.41
	Kojitriose	12.03	0.10	0.84	3.32
	Galactotriose	12.63	0.11	0.85	2.25
	4'-Galactosyl lactose	13.06	0.11	0.88	1.37
	Isomaltotriose	13.32	0.11	0.86	1.59
	Kojibiose	13.63	0.11	0.86	0.44
	1,4-Galactobiose	13.71	0.11	0.86	0.11
	3'-Galactosyl lactose	13.73	0.11	0.84	1.38
	1,3-Galactobiose	14.00	0.11	0.86	0.21
	Lactose	14.04	0.12	0.86	0.62
	Isomaltose	14.15	0.11	0.91	1.11
	Laminaritriose	14.36	0.12	0.83	0.08
	Laminaribiose	14.38	0.12	0.82	0.69
	Cellobiose	14.51	0.11	0.86	1.09
	Maltotriose	14.72	0.11	0.86	0.56
	1,6-Galactobiose	14.83	0.12	0.87	1.71
	Maltose	15.17	0.11	0.87	
PGC	Cellobiose	3.23	0.22	0.41	0.75
	1,4-Galactobiose	3.50	0.20	0.38	0.36
	Isomaltose	3.66	0.31	0.51	0.20
	Lactose	3.76	0.28	0.28	0.05
	Laminaribiose	3.78	0.33	0.43	0.14
	1,6-Galactobiose	3.87	0.35	0.53	0.08

1,3-Galactobiose	3.91	0.34	0.25	0.24
Kojibiose	4.04	0.27	0.31	0.87
4’-Galactosyl lactose	4.42	0.25	0.38	0.35
Laminaritriose	4.59	0.29	0.34	0.27
Isomaltotriose	4.72	0.31	0.37	0.56
Kojitriose	5.02	0.31	0.32	0.18
3’-Galactosyl lactose	5.11	0.31	0.34	0.18
6’-Galactosyl lactose	5.21	0.33	0.32	0.05
Galactotriose	5.23	0.26	0.39	0.99
Maltose	5.77	0.38	0.37	2.96
Maltotriose	7.75	0.41	0.36	

532

533 **Table 2.** Peak capacity and orthogonality values obtained for the 4 commercial prebiotic

534 oligosaccharide products analyzed using the optimum HILIC×RP conditions determined.

	GOS1	GOS2	GOS3	GEOS
^{1}D peak capacity, $^{1}n_c$	42	38	41	37
^{2}D peak capacity, $^{2}n_c$	62	54	48	64
Theoretical peak capacity, $^{2D}n_c$	2604	2052	1968	2368
Effective peak capacity, $^{2D}n'_c$	1425	1176	1084	1370
Corrected peak capacity, $^{2D}n_{c,corr}$	457	454	103	531
Orthogonality, A_0	32%	39%	10%	39%

535 A_O, orthogonality; $^{2D}n_c = {}^{1}n_c \times {}^{2}n_c$; $^{2D}n'_c$: calculated according to [13]; $^{2D}n_{c\ corr.}$: $^{2D}n'_c \times A_O$;

536