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On-line two-dimensional liquid chromatography hyphenated to mass spectrometry and ion mobility-mass spectrometry for the separation of carbohydrates from lignocellulosic biomass

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Abstract

Lignocellulosic biomass is a promising resource of newable energy. Its transformation into ethanol requires efficient pretreatment leading to complex liquid mixtures made of hundreds of oxygenated analytes. A large part of the released compounds belong to the carbohydrate family. To overcome the complexity of such samples, a comprehensive on-line two-dimensional reversed-phase liquid chromatography hyphenated to high-resolution mass spectrometry (RPLC×RPLC-HRMS) was dedicated to the separation of carbohydrates and more specifically for oligomers coming from pretreated lignocellulosic biomass. The first part of this study consisted in the optimization of such hyphenation (*i.e.* selection of stationary phases, mobile phases, sampling time, etc.). Then, the analytical method was applied to an industrial aqueous biomass product coming from the sulfuric acid-based pretreatment of a wheat straw. Around 70 well-resolved chromatographic peaks corresponding to oligomers were obtained. Occupation of the separation space between each chromatographic dimension was estimated to 75%. In the last part of this study, the interest of ion mobility-mass spectrometry in addition to RPLC×RPLC was discussed. Some examples highlighted the additional separation that can bring ion mobility into RPLC×RPLC-IMS-HRMS method. This four-dimensional hyphenation described each analyte with two retention times, the collisional cross section and the molecular formula allowing to reach a level of detail never seen for biomass sample compositions.

Keywords

Biomass; two-dimensional liquid chromatography; high-resolution mass spectrometry; ion mobility spectrometry; RPLC×RPLC-IMS-HRMS

33 1 Introduction

34 In the context of a depletion of fossil fuel as well as a global planet warming, renewable energies have
35 gained a growth of interest [1,2]. Among the renewable technologies investigated, conversion of
36 lignocellulosic biomass, also called the second generation of biomass, is a promising resource for the
37 production of biofuels and valuable bio-based chemicals either via the biochemical or thermochemical
38 conversion routes [3–5]. Lignocellulosic biomass is the most abundant material of plant cell walls and
39 consists mainly of three polymeric components: lignin, hemicelluloses and cellulose. The proportions
40 and compositions of these later could vary according to the plant species (*i.e.* hardwoods, agricultural
41 residues, etc.) [6]. Concerning the biochemical conversion of biomass into ethanol, transformation of
42 the feedstocks requires first a pretreatment prior to enzymatic hydrolysis and fermentation to
43 deconstruct the cell wall architecture of biomass and enhance the cellulose accessibility [7]. Aqueous
44 matrices made of several hundreds of oxygenated compounds result from the pretreatment. In order
45 to increase the understanding of the pretreatment mechanisms and thus to improve the conversion
46 process, the chemical characterization of the products is needed. Previous work demonstrated thanks
47 to successive liquid-liquid extractions that highly water-soluble compounds (*i.e.* carbohydrates)
48 represented up to 89% of the liquid biomass products [8]. Generally, carbohydrates from plant
49 materials or food are usually analyzed with HPAEC [9,10], hydrophilic interaction chromatography
50 (HILIC) [11–14] or RPLC with porous graphitic carbon stationary phase (PGC) [15], hyphenated most of
51 the time with MS. GC can be as well be employed however it requires a prior derivatization step
52 [16,17].

53 In order to achieve the separation of carbohydrates in such complex biomass samples, comprehensive
54 on-line two-dimensional liquid chromatography (LC×LC) hyphenated to HRMS may be a suitable
55 solution. The success of a two-dimensional method depends on a large number of parameters
56 including the complementarity/orthogonality of the two dimensions. Indeed, to maximize the
57 coverage of the retention space, it is essential to couple two dimensions of separation offering
58 different selectivities. HILIC mode can be a dimension of choice for the analysis of carbohydrates
59 [18,19] and the complementarity of HILIC and RPLC has been widely described as complementary to
60 RPLC in terms of retention [20]. The hyphenation of HILIC and RPLC therefore appears to be an
61 alternative to be considered for the two-dimensional analysis of carbohydrates mixture to achieve a
62 high degree of orthogonality. However, other constraints must be considered. Acetonitrile is used in
63 HILIC as the weak solvent while it becomes the strong solvent in RPLC. The coupling of these two
64 dimensions therefore leads to significant injection effects in the second dimension which result in band
65 broadening and peak distortion, severely degrading the total peak capacity despite an often unequaled
66 degree of orthogonality between the two dimensions [21]. Various technological solutions are
67 proposed in the literature to counter these phenomena (reducing the volume injected in the second
68 dimension, the use of active solvent modulation with or without a trapping column, or the
69 implementation of "total breakthrough" conditions) [22]. These solutions have shown their
70 effectiveness, they nevertheless complicate the implementation of the hyphenation.

71 An alternative to HILIC is the use of a porous graphitic carbon (PGC) column in the first dimension
72 coupled to grafted silica based column in the second dimension. The interest of this configuration for
73 the analysis of a complex mixture of carbohydrates in biomass samples can be justified for various
74 reasons: (1) it allows full compatibility between the two dimensions, the weak solvent remaining water
75 in both cases, which simplifies the hyphenation of the two dimensions, (2) the nature of the weak

76 solvent also remains much more compatible with the biomass samples which are insoluble in
77 acetonitrile (predominant solvent in HILIC), (3) the PGC column has been reported for the separation
78 of carbohydrates. This stationary phase proposed a significant retention for polar compounds
79 associated to a high selectivity for very close structure like isomers. This selectivity can play a key role
80 for the separation of carbohydrates contained in very complex biomass samples. For example, many
81 carbohydrates differ only from one methyl group [15,23,24]. More recently, ion mobility spectrometry
82 (IMS)) has gained in interest since its hyphenation with mass spectrometry (IMS-MS) as well as the
83 development of more powerful devices such as the trapped IMS (TIMS). The separation is based on
84 the size, shape and charge of compounds. From the measured mobilities, the collisional cross sections
85 (CCS) can be calculated and used as a supplementary descriptor for compound identifications in
86 addition to m/z . Hofmann *et al.* demonstrated using synthetic carbohydrates that IMS-MS succeeded
87 in the separation of isomers [25] as well as other teams [26–28]. Additionally, IMS-MS can be easily
88 coupled with LC as the time scales of each dimension are ideal for such hyphenation. This kind of three-
89 dimensional method was already performed for glycans [29], plant metabolites [30,31], lipids [32] or
90 biological sample analysis [33]. To the best of our knowledge, LC×LC-IMS-MS hyphenation has been
91 reported in only two publications [34,35]. They demonstrated that this kind of hyphenation offered
92 unequalled resolution, nevertheless data processing remained an issue for the visualization and
93 interpretation of data [34].

94 In the present study, first, the aim was to develop a three-dimensional LC×LC-HRMS for the untargeted
95 analysis of carbohydrates from biomass samples. We proposed to optimize and evaluate the interest
96 of a two dimensional separation consisted in the hyphenation of PGC and silica-based columns.
97 Optimization of LC×LC-HRMS included the selection of stationary and mobile phases and the
98 optimization of the interface between each dimension. The optimized method was applied to an
99 industrial biomass sample. Finally, the relevance of ion mobility spectrometry implied in the four-
100 dimensional RPLC×RPLC-IMS-HRMS method was discussed by presenting some examples to highlight
101 the input of IMS.

102 **2 Experimental section**

103 *2.1 Samples*

104 The biomass sample investigated in this work was the water-soluble fraction obtained after a sulfuric
105 acid-based pretreatment of wheat straw. A pH of 2.1 was measured with a combined pH electrode
106 (Fisher Scientific, France). The sample was provided by IFP Energies nouvelles (France). A filtration was
107 performed prior to analysis using Macherey-Nagel PTFE membrane filters (pore size: 0.20 μm ,
108 diameter: 13 mm, Germany).

109 *2.2 Chemicals*

110 Methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and formic acid (FA) were MS grade purchased
111 from VWR (France). Deionized water was produced with a Milli-Q water purifier (Millipore SAS,
112 France).

113 *2.3 Apparatus*

114 One and two-dimensional liquid chromatographies were performed using two Agilent 1290 UHPLC
115 systems (Agilent, USA) consisting in a high-pressure binary pump, an autosampler with a flow-through
116 needle of 20 μ L injection sample loop, a temperature-controlled column compartment and a
117 photodiode array (PDA) UV detector for each system equipped with 1 and 0.6 μ L flow-cells for the first
118 (D1) and the second dimension (D2) respectively. The interface connecting the two dimensions
119 included a 2-position/4-port duo valve with two sampling loops of either 20 or 40 μ L. Delay volumes
120 were estimated to 160 and 140 μ L for D1 and D2 respectively.

121 LC and LC \times LC were hyphenated to trapped ion mobility-quadrupole-time-of-flight mass spectrometer
122 (timsTOF, Bruker Daltonics, Germany) via an electrospray source. timsTOF included a dual TIMS
123 analyzer, a quadrupole, a collision cell and a temperature-controlled TOF analyzer.

124 2.4 RPLC-HRMS

125 Injection volume was 1 μ L. Separation was performed on a Kinetex C18 column (100 \times 3 mm, particle
126 size 2.6 μ m, Phenomenex, France) at 30°C. The mobile phases consisted in (A) 0.01% (v/v) of FA in
127 water and (B) 0.01% of FA in MeOH. A linear gradient at 0.6 mL/min was used as follows: 0.0–4.0 min,
128 1% B; 4.0–30.0 min, from 1 to 99% B; 30.0–35.0 min, 99% B; 35.0–36.0 min, from 99 to 1% B; 36.0–
129 40.0 min, 1% B.

130 Endplate offset, capillary voltage, nebulizer pressure, dry gas flow rate and temperature were set to
131 500 V, 3000 V, 3 bar, 8.5 L/min and 250°C respectively. Ions were transferred with an electric field
132 gradient. A stepping for collision cell RF and transfer time to the flight tube was applied (400-600 Vpp,
133 60-80 μ s respectively). The acquisition frequency was 5 Hz.

134 2.5 RPLC \times RPLC-HRMS and RPLC \times RPLC-IMS-HRMS

135 The development of the first dimension (D1) was conducted on a Hypercarb column (100 \times 2.1 mm, 5
136 μ m, ThermoFisher Scientific, USA). The solvent flow rate was 0.3 mL/min with a temperature of 60°C.
137 For the second dimension (D2), six 50 \times 2.1 mm columns were compared: Zorbax Bonus RP (1.8 μ m,
138 Agilent), Zorbax Eclipse Plus C18 (1.8 μ m, Agilent), Kinetex PFP (2.6 μ m, Phenomenex), Kinetex Phenyl-
139 Hexyl (2.6 μ m, Phenomenex), Acquity BEH Shield RP18 (1.7 μ m, Waters) and Acquity CSH Phenyl-Hexyl
140 (1.7 μ m, Waters). First experiments on these columns were performed using a gradient with (A) 0.01%
141 (v/v) of FA in water and (B) 0.01% of FA in MeOH at 0.3 mL/min at 30°C. A linear gradient was used as
142 follows: 0.0–2.0 min, 0% B; 2.0–16.0 min, from 0 to 99% B; 16.0–18.0 min, 99% B; 18.0-18.1, from 99
143 to 0% B; 18.1–21.0 min, 0% B. Nebulizer pressure, dry gas flow rate and temperature were set to 2.6
144 bar, 7 L/min and 215°C respectively. Other settings for HRMS were similar as in section 2.4.

145 Final chromatographic conditions of RPLC \times RPLC are summarized in Table 3. The solvent flow rate was
146 split with a 1:2 ratio for the hyphenation with HRMS. MS acquisition frequency was adjusted to 20 Hz.
147 Nebulizer pressure, dry gas flow rate and temperature were set to 4 bar, 11 L/min and 250°C
148 respectively. Other settings for HRMS were similar as in section 2.4.

149 For IMS settings, 1/ K_0 range varied from 0.45 to 1.80 V.s/cm² and the ramp time was set to 100 ms.

150 3 Results and discussion

151 3.1 RPLC-HRMS for the selection of carbohydrates of interest

152 One-dimensional reversed-phase liquid chromatography (RPLC) is one of the most employed
153 techniques when dealing with non-targeted analysis of biomass samples. In this study, the biomass
154 sample was injected on a C18 column hyphenated to HRMS (see Experimental section for the
155 conditions). Figure 1 shows the total ion chromatograms (TIC) obtained in positive and negative modes
156 electrospray ((+)ESI and (-)ESI) as well as the UV chromatogram at 210 nm. Although a large number
157 of compounds were retained and eluted during the gradient step (*i.e.* retention time between 5 and
158 25 min), intense peaks were detected with mass spectrometry during the isocratic step (retention time
159 < 5 min). Despite the recording of the UV chromatogram at a relative low wavelength, the intensity of
160 UV signal between 0 and 5 min was quite weak. In a previous work, the same sample was characterized
161 and it was demonstrated thanks to the mass accuracy of HRMS and fragmentation experiments, that
162 the highly polar analytes were related to carbohydrate family [8]. This family of compounds was
163 defined by a high number of hydrogen atoms/number of carbon atoms (H/C) between 1.3 and 2.3, and
164 a high number of oxygen atoms/number of carbon atoms (O/C) between 0.7 and 1.2.

165 Moreover, other compounds having stronger retention corresponded mainly to phenolic compounds,
166 furans and lignin-carbohydrate complexes. As an example, the most intense peak eluted at 12.7 min
167 was tentatively assessed to a feruloyl xyloside (C₁₅H₁₈O₈)[8].

168 Compounds eluted after 5 min, like phenolic compounds, have already been studied in many works,
169 therefore a focus was done here on the first part non elucidated of the chromatograms. Eight ions
170 among the most intense ones detected with (-)ESI belonging to the investigated retention time window
171 are presented in Table 1. It clearly appeared that carbohydrates in the biomass sample were not
172 oligomers made of simple sugars (glucose, xylose, etc.). For example, cellobiose is a disaccharide of
173 glucose obtained by the hydrolysis of cellulose, with the formula C₁₂H₂₂O₁₁ and a double bond
174 equivalent (DBE) of 2. In Table 1, two ions have twelve carbon atoms, however their DBE were 3 and
175 4. These higher DBE values might be linked to additional chemical groups such as carboxylic acid groups
176 (*i.e.* glucuronic acid) or/and unsaturated bonds coming from dehydration reactions occurring during
177 the biomass pretreatment step.

178 Although HRMS could identify the analytes, the lack of resolution of RPLC led to peak co-elutions and
179 thus may conduct to matrix effects in MS ionization as well as complex data interpretation of mass
180 spectra. Therefore, the objective of this study was to develop a method to achieve the separation of
181 these compounds co-eluted in RPLC.

182 3.2 On-line two-dimensional liquid chromatography RPLC×RPLC-HRMS

183 In order to achieve the separation of carbohydrates in our biomass sample, a comprehensive on-line
184 two-dimensional liquid chromatography consisted in the hyphenation of PGC and silica-based
185 columns, coupled to HRMS, was developed.

186 3.2.1 Optimization of the first dimension (D1)

187 The lack of extensive knowledge on biomass samples and more specifically on sugar oligomers from
188 hydrolyzed biomass prevented the method optimization by means of model molecules. Thus, the
189 optimization of the separation was directly conducted by using the chromatograms of biomass
190 samples. The Hypercarb column showed a promising separation of carbohydrates as illustrated in
191 Figure 2 representing the base peak chromatograms (BPC) obtained from analysis in (-)ESI mode. The

192 eight labeled peaks seemed to be eluted according to the number of oxygen atoms and more likely to
193 the number of hydroxyl groups [36].

194 However, under the previous experimental conditions using an elution gradient ranging from 1 to 99%
195 of methanol with 0.01% (v/v) of formic acid (FA), two main issues were highlighted.

196 The first issue was the elution of sulfuric acid, used during the pretreatment of the biomass,
197 throughout the chromatogram as demonstrated in Figure S1. Indeed, using 0.01% of FA, the intensity
198 of sulfuric acid in (-)ESI mode was higher than the intensities of most peaks in Figure 2. Therefore, a
199 strong competition between analytes and sulfuric acid may take place during the ionization process in
200 the mass spectrometry ionization source, which can potentially lead to the ionization suppression of
201 the compounds of interest. Thus, the chromatographic conditions were modified by increasing the
202 amount of FA in the mobile phases (water and methanol). As illustrated in Figure S1, by increasing the
203 percentage of formic acid from 0.01 to 0.5%, the elution of sulfuric acid moved to the beginning of the
204 run while only a small decrease in retention was observed for the carbohydrates. Moreover, some
205 inversions in the elution order of the eight carbohydrates from Table 1 were observed (Figure S2). In
206 the light of the impact of the percentage of FA in mobile phases, 0.5% of FA was selected. Although a
207 high amount of acid in the mobile phases could be problematic for the hyphenation with mass
208 spectrometry, in this study, the Hypercarb column was selected as the first dimension of the 2D
209 system, thus FA will be diluted in the high flow rate of the mobile phase of the second dimension
210 before entering into the ionization source.

211 The second issue was the non-elution of a part of the sample. When developing a chromatographic
212 method directly using an industrial-like sample, it is required to pay special attention to the elution of
213 all analytes. In order to elute the entire sample from the column, isopropanol (IPA) was required as a
214 replacement for methanol (Figure S3). The elution order according to the chemical family was as
215 follows: sugars < phenolic compounds < lignin-carbohydrate complexes. The strong retention of lignin-
216 carbohydrate complexes on PGC may be linked to the addition of retention mechanisms of
217 carbohydrates and phenolic compounds. Although IPA allowed the elution of the entire sample, it also
218 decreased the retention of the carbohydrates of interest. In order to improve the separation of
219 carbohydrates, the mobile phase composition during the isocratic step was changed to 100% water
220 instead of 99%. As illustrated in Figure 3, the modification of the isocratic step allowed to increase the
221 retention of carbohydrates which were previously co-eluted with sulfuric acid. Some co-elutions
222 remained, however by adjusting the IPA gradient, separation between carbohydrates should be
223 improved. In order to select the correct gradient, composition at the elution (C_e) of compound [1] was
224 calculated as follows:

$$225 \quad C_e = C_i + \frac{C_f - C_i}{t_G} \times (t_R - t_0 - t_D - t_{iso}) \quad (\text{Eq. 1})$$

226 where C_i and C_f are the initial and final gradient compositions respectively, t_G the gradient time, t_R the
227 retention time, t_0 the column dead time, t_D the dwell time and t_{iso} the time of the isocratic step with
228 100% water.

229 t_0 was calculated according to Eq. 2

$$230 \quad t_0 = \frac{V_0}{F} \quad (\text{Eq. 2})$$

231 where F is the mobile phase flow rate and V_0 the column dead volume estimated by Eq. 3

$$232 \quad V_0 = \frac{\pi \times d^2 \times L}{4} \times \varepsilon \quad (\text{Eq. 3})$$

233 with d the column diameter, L the column length and ε the column porosity estimated to 0.7.

234 The composition at elution was 10% of IPA for the carbohydrate [1] eluted at 10.8 min. Based on this
235 measurement, the gradient of the first dimension consisted in first an isocratic step with 100% of
236 water, followed by a gradient from 0 to 10% of IPA with a slow slope (0.083% of IPA/min), then a faster
237 second gradient from 10 to 99% of IPA (2.225% of IPA/min). Finally, to ensure the complete elution of
238 all analytes, an isocratic step with 99% of IPA was added at the end. The mobile phases (water and IPA)
239 contained 0.5% of FA.

240 3.2.2 *Choice of the stationary phase in the second dimension (D2)*

241 Six silica-based columns were considered for the second dimension. BPC between m/z 50-1000
242 obtained from the analysis of the biomass sample with the six columns are presented in Figure S4.
243 Direct comparison of the chromatograms in terms of retention window is possible because the same
244 conditions and column geometry (length and internal diameter) were used. Two main criteria were
245 taken into account to select the second dimension stationary phase. The peak shape was the first one.
246 The Zorbax Bonus RP column showed strong peak distortion and thus was no longer considered. The
247 second criterion was the size of the elution window for the carbohydrates of interest (highlighted with
248 black boxes in Figure S4). Although the Acquity CSH Phenyl-Hexyl column met both criteria, its
249 stationary phase has surface charges that induced interactions with sulfuric acid. Indeed, sulfuric acid
250 was eluted in the middle of the gradient (between 5.5 and 9.2 min). Thus, the Zorbax Eclipse Plus C18
251 column was selected.

252 3.2.3 *Designing of the RPLCxRPLC method*

253 Once the nature of the stationary and mobile phases have been selected in the two separation
254 dimensions, the next step was the designing of the RPLCxRPLC system, *i.e.* mainly the choice of the
255 internal diameters, lengths and particle sizes of the columns as well as the loop volume and sampling
256 time. The designing of the on-line RPLCxRPLC analysis is essential in order to maximize the peak
257 capacity in a given analysis time. In the literature, several approaches were proposed models have
258 been developed to optimize the several parameters [37–41]. However, today no commercial interface
259 is available to achieve this development. Nevertheless, a fairly pragmatic approach, using the
260 conclusions of the various studies described in the literature, was used to define satisfactory
261 conditions. Thus, the following principles have been followed to carry out the dimensioning of the
262 analysis:

263 (1) The flow rate of the first-dimension analysis should allow a sampling time between the first and
264 second dimensions that is compatible with the second-dimension analysis time. It is very common to
265 use a flow rate far-below the column optimum.

266 (2) It is necessary that each first-dimensional peak is sampled at least 3 to 4 times towards the second
267 dimension, otherwise undersampling will lead to a loss in peak capacity [42].

268 (3) A special attention must be paid to the volume injected in the second dimension. Injection effects
269 may be encountered when the first-dimensional solvent, which becomes the second-dimensional

270 injection solvent, has a strong eluent strength relative to the initial second-dimensional elution
271 conditions.

272 (4) Short analysis time is required for the second dimension separation. The best compromise has to
273 be found to maximize the sampling rate while maintaining a sufficient second dimensional analysis
274 time. A too long analysis time in the second dimension necessarily reduces the sampling time while a
275 too short analysis time limits the second dimensional peak capacity. It is very common to use a flow
276 rate that is beyond the optimum column flow rate.

277 3.2.3.1 Dimensioning of D1

278 Taking into account the principles listed above, a column with an internal diameter of 1 mm with 5 μ m
279 particles and working at a temperature of 60°C was chosen for the first dimension, which allowed a
280 relatively slow flow rate of mobile phase of 0.01 mL/min. A length of 100 mm was selected as a good
281 compromise between first-dimensional peak capacity and analysis time.

282 3.2.3.2 Interface between D1 and D2: determination of the sampling rate

283 As stated above, the objective was to sample sufficiently the first-dimensional outflow, controlling the
284 effects of second-dimensional injection and finding the right compromise with the second-dimensional
285 analysis time.

286 Model compounds were used in order to evaluate the effects of the volume and solvent injection on
287 the shape of the second dimensional peaks. To do so, mixtures of vanillin and salicin were prepared in
288 25, 50 and 90% of IPA. Figure S5 represents the EIC of model solutions implying 50% of IPA for injection
289 volumes of 1, 2, 5, 8, 10 and 20 μ L, corresponding to sampling times of 6, 12, 30, 48, 60 and 120 seconds
290 respectively in RPLCxRPLC with a first-dimensional flow rate of 0.01 mL/min. For vanillin, a peak
291 distortion was observed from an injection volume of 8 μ L while breakthrough phenomenon (peak
292 splitting) appeared for salicin with an injection volume of 5 μ L. Thus, distortion and peak splitting
293 appeared to be highly analyte-dependent. Similar experiments were conducted with standards
294 prepared in 25 and 90% of IPA. The results are summarized in Table 2: the red boxes indicate a
295 breakthrough phenomenon and the green boxes correspond to a correct peak shape or with a slight
296 distortion. As already noted, salicin was more prone to strong distortion than vanillin. For example,
297 breakthrough already appeared with an injection volume of 2 μ L with 90% of IPA while peak splitting
298 for vanillin was observed when the injection volume was four times higher. The chromatograms in
299 Figure 3 showed that the last carbohydrate of interest was eluted with about 10% of IPA. Thus, by
300 limiting the injection volume, no phenomenon of breakthrough should be observed.

301 In order to alleviate the injection effects, several second dimension gradients were compared, starting
302 from 0, 20 and 40% of MeOH in the mobile phase to 100% (Figure S6). The idea was to increase the
303 solvent eluent strength of D2 to be closer to the composition of the injection and thus limit the
304 breakthrough. However, the opposite trend was surprisingly observed for both standards. Thus, 100%
305 of water was selected for the initial composition of the 2D gradient.

306 Until now, for the tests presented above, injection was performed via the autosampler to mimic what
307 might happen in the second dimension of separation. However, in a two-dimensional analysis, the
308 fractions were collected in sampling loops. In order to avoid any sample loss, these loops have to be

309 filled to a maximum of 80% by the flow coming from the first dimension[37]. The remaining volume of
310 the loop was filled with the mobile phase of the second dimension at the starting conditions of the
311 gradient (*i.e.* water in this study). To better understand the role played by water in the phenomena
312 undergone during injection, injections of 10 μL via the autosampler or via 20 μL sampling loops were
313 compared. Therefore the sampling loops were only 50% filled, the remainder being water. These
314 experiments were conducted with three IPA injection compositions: 25, 50 and 90% (Figure 4). With
315 25% of IPA, the peak widths were similar for both types of injection. However, from 50% of IPA in the
316 injection solvent, the peaks obtained using sampling loops was significantly thinner than when using
317 the autosampler. The presence of water within the loop might allow diffusion of the sample into water.
318 Weakening the eluent strength of D1 could help to promote the retention on the column head of D2
319 (focusing effect) and thus improve the peak shapes. Finally, 40 μL sampling loops were also evaluated.
320 However, the increase in the volume of the loops did not accentuate this phenomenon due to a slow
321 diffusion kinetics of the sample in water within the loop. Conversely, the increase in loop volume led
322 to an increase of the injection volume, which resulted in a widening of the peak. (Figure S7).

323 The study on the injection effects provided a number of elements to select the best conditions. Thus,
324 an injection volume between 5 and 10 μL appeared to be a wise choice, taking into account that the
325 carbohydrates of interest were eluted with a maximum IPA content of 10% and that the presence of
326 water in the sampling loops played a positive role on the peak shape.

327 Therefore, 20 μL sampling loops were used and 40% filled with a volume of 8 μL from D1 corresponding
328 to a sampling time of 48 s. It must also be checked that this sampling rate allowed 3 to 4 modulations
329 per first-dimensional peak. Figure S8 illustrates a part of the BPC obtained from the analysis of the
330 biomass sample under the optimized first-dimensional conditions. The widths of the chromatographic
331 peaks varied from 2.5 to 3.5 min depending on the abundance of the compounds, which means 3 to 5
332 modulations per peak.

333 3.2.3.3 Dimensioning of D2

334 In the second dimension, for very fast analysis, a column with an internal diameter of 2.1 mm was
335 selected with a length of 5 cm and a particle size of 1.8 μm ; a temperature of 50°C and a flow rate of
336 2 mL/min were applied. This was the maximum flow rate allowed to avoid reaching the pressure limits
337 of the apparatus. The chosen column temperature also allowed for rapid analysis while limiting the
338 pressure generated. Moreover, acetonitrile was preferred to methanol in order to limit the pressure.

339 The sampling time settled the duration of D2 analysis at 48 s. Considering the equilibration time as
340 four times the dead volume and a dwell time, and taking into account the column geometry, 18 s were
341 required for equilibration which led to a D2 gradient time of 30 s.

342 The EIC of vanillin obtained under the optimized second dimensional conditions is shown in Figure S9.
343 The peak width measured at half height in (-) ESI was 0.023 min, which was equivalent to a peak width
344 of about 2.3 s measured at 13.5% height. To correctly describe second dimensional peaks, the TOF
345 acquisition frequency was set at 20 Hz (other HRMS conditions are detailed in the Experimental
346 section).

347 3.2.4 Application of the optimized RPLCxRPLC-HRMS to an aqueous biomass product

348 Optimized conditions of the 2D method are summarized in Table 3. The D1 solvent flow rate was
349 increased during the isocratic step with 99% of IPA as no carbohydrates of interest eluted during this
350 step.

351 The RPLC×RPLC-HRMS method was applied to the investigated biomass sample. The 2D chromatogram
352 obtained in (-)ESI is shown in Figure 5. The dotted lines represent the elution window for the
353 carbohydrates of interest. Peak splitting due to injection effects was not observed in the elution
354 window and did not appear until 220 min. Each peak in the retention window was labeled (Figure S10)
355 and the most intense ion under each chromatographic peak was identified (Table S1). A total of 69
356 chromatographic peaks were detected in the elution window of interest.

357 In order to estimate the occupation of the separation space (γ), the ratio of the effective and
358 theoretical retention areas was calculated according to a methodology proposed in the literature [43].
359 Figure S11 illustrates the methodology. The occupation of the separation space for the hyphenation of
360 non-silica and silica-based columns was measured to 75% which reflected a high orthogonality
361 between both dimensions.

362 3.3 Comparison of RPLC×RPLC-HRMS and RPLC-HRMS approaches

363 The addition of a separation dimension allowed to increase the number of detected and resolved
364 chromatographic peaks. Although all compounds could be distinguished within a single
365 chromatographic dimension using HRMS, the large number of co-elutions, even for one given m/z
366 ratio, prevented to well determine the retention times of analytes. EIC of six ions from Table 1 obtained
367 with RPLC-HRMS method were plotted in Figure 6. For example, the ion m/z 381 had an EIC with many
368 peaks without baseline return between 1.0 and 2.5 min in RPLC-HRMS analysis. Usually, ions with the
369 same retention time may be considered to come from the same ionized compound under several
370 forms (adducts, ion-molecule adducts, fragments, etc.). In the case of RPLC-HRMS analysis, the too
371 large number of co-elutions during the isocratic step made it difficult to process MS data using this
372 approach. For example, at 1.9 min, it seemed that the top of a chromatographic peak correspond to
373 the m/z 513, 381 and 321 EIC. A hypothesis is that m/z 513 fragmented in-source to form m/z 381 and
374 321. By subjecting m/z 513 to MS-MS experiments, m/z 381 and 321 fragments were observed, but
375 the possibility of a second compound also forming these ions could not be excluded.

376 In RPLC×RPLC-HRMS analysis, the EIC were represented by maps with the retention time of D1 on the
377 y-axis and the retention time of D2 on the x-axis (Figure 7). The use of two retention times as filters in
378 addition to m/z ratios measured in HRMS, allowed a more specific characterization of compounds and
379 made easier the identification and differentiation of a pseudo-molecular ion *versus* in-source
380 fragments. For example, the EIC of m/z 513 did not have a common chromatographic peak with the
381 EIC of m/z 381 and 321, indicating that m/z 513 ion was not fragmented into m/z 381 and 321 in
382 RPLC×RPLC-HRMS. At the opposite, m/z 381 and 321 were detected at the same retention times
383 indicated by black boxes in Figure 7. These two ions most probably correspond to a single compound,
384 m/z 321 coming from the fragmentation of m/z 381 (corresponding to a loss of 60 u, classically
385 observed for carbohydrate compounds).

386 In addition, a smaller number of chromatographic peaks for m/z 381, as well as for m/z 321, were
387 obtained in RPLC×RPLC-HRMS compared to RPLC-HRMS. This observation suggested that the flow rate

388 and mobile phase composition associated with the ionization conditions used in RPLC×RPLC-HRMS
389 analysis tended to form less fragments than in RPLC-HRMS analysis.

390 Finally, the reduction of the number of co-elutions thanks to the two separation dimensions upstream
391 of HRMS allowed to obtain cleaner and specific mass spectra that were easier to interpret. As an
392 example, Figure 8 presents the mass spectra of the compound m/z 471 highlighted by an asterisk on
393 the EIC with both methods (Figure 6 and Figure 7). Two main ions identified as $[M-H]^-$ and $[2M-H]^-$
394 forms were observed with RPLC×RPLC-HRMS, while a multitude of ions were observed with RPLC-
395 HRMS due to co-elutions with other compounds. The annotation of such a mass spectrum is therefore
396 much more complex and subjected to misidentification compared to the one obtained with
397 RPLC×RPLC-HRMS.

398 3.4 Ion mobility spectrometry as an additional dimension to the RPLC×RPLC-IMS-HRMS hyphenation

399 For targeted analysis, the timsTOF is capable of achieving a resolution of 200 with specific optimization.
400 At the opposite, during this work, untargeted analysis was performed. In order to have access to the
401 mobility of all the carbohydrate of interest, a wide range of $1/K_0$ ($V.s/cm^2$) was selected with a ramp
402 time of 100 ms, thus reducing the resolution. An average resolution of 50 was measured based on the
403 mobilograms of carbohydrates from Table 1.

404 Classically, IMS-HRMS data are visualized on a map representing $1/K_0$ according to m/z ratio. Figure
405 S12 illustrates this map for the RPLC×RPLC elution window of interest (between 0 and 205 min in D1).
406 Two areas were observed: the first one for $[M-H]^-$ ions and a second one that might be related to ion-
407 molecule adducts. If an ion $[2M-H]^-$ formed in the ion source is fragmented in $[M-H]^-$ after the ion
408 mobility cell (during ion transfer between IMS and TOF), the extracted ion mobilogram (EIM) of $[M-H]^-$
409 will therefore present two peaks: the first one for the $[M-H]^-$ formed in the ion source and the other
410 one produced after the IMS due to the fragmentation of the ion-molecule adduct. An example was
411 presented in Figure S13 for m/z 381. The first EIM was obtained by extracting m/z 381, while the
412 second corresponded to the ion-molecule adduct $[2M-H]^-$ (m/z 763). EIM of m/z 763 gave a $1/K_0$ of
413 $1.243 V.s/cm^2$ for the most intense peak, which corresponded to a CSS of 253.4 \AA^2 ; whereas m/z 381
414 ion had a CCS of either 181.7 or 185.1 \AA^2 depending on the retention time as shown previously. This
415 example showed that IMS data processing may be more complex than the interpretation of RPLC×RPLC
416 data.

417 Considering all the ions, the occupation of the separation space between IMS and HRMS could be
418 estimated to 70%. However, if only $[M-H]^-$ ions were considered, the orthogonality between IMS and
419 HRMS led to a much lower occupation of the separation space evaluated to 20%.

420 IMS offers an intermediate time scale between LC (or LC×LC) and TOF analyzer leading to an ideal
421 sampling between each dimension. Moreover, the addition of IMS to the RPLC×RPLC-HRMS
422 hyphenation does not increase the analysis time. In RPLC×RPLC-IMS-HRMS analysis, each compound
423 was defined by two retention times, one $1/K_0$ value that could be converted into CCS value and one
424 mass spectrum necessary to determine the molecular formula. Furthermore, UV spectra as well as MS-
425 MS spectra could also be collected. All these descriptors can be used simultaneously to support
426 identification. However, due to the lack of commercial software to represent and process these four-
427 dimensional data, data analysis was performed manually. In order to highlight the input of IMS
428 combined to the RPLC×RPLC separation, the Figure 9 represented $1/K_0$ as a function of the D2 retention

429 time during one modulation for m/z 321. RPLC×RPLC allowed to distinguish two non-resolved
430 chromatographic peaks. The combination of the two chromatographic dimensions and the IMS
431 improved the resolution between these two peaks. The most compact compound eluted first in D2
432 and had a $1/K_0$ of 0.876 V.s/cm² corresponding to a CCS of 181.7 Å²; while the second peak had a $1/K_0$
433 of 0.892 V.s/cm² or a CCS of 185.1 Å². The three-dimensional separation made it possible to distinguish
434 the MS spectra of these two isomers.

435 Although figure 9 presented only one example, other examples also demonstrated that the
436 hyphenation of the four separation dimensions allowed to increase the resolution of the method and
437 thus decrease the number of co-eluted compounds. The unique RPLC×RPLC-IMS-HRMS hyphenation
438 will make it possible to reach a level of detail never seen for biomass sample compositions.

439 **4 Conclusion**

440 The aim of this work was to develop suitable two-dimensional conditions for the separation of
441 carbohydrates coming from the biochemical conversion of lignocellulosic biomass and that are not
442 retained in conventional RPLC analysis. Firstly, the nature of carbohydrates of interest was determined
443 thanks to a RPLC-HRMS analysis. Although these later co-eluted in the first part of the run during the
444 isocratic step with a high water content, the high-resolution of mass spectrometry allowed to separate
445 and identify the carbohydrates. However, when a large number of compounds are co-eluted, mass
446 spectrometry may suffer from matrix effects due to ion competition during the ionization and
447 desolvation processes. Moreover, poor quality of mass spectrum leads to a complex assignment. The
448 optimized two-dimensional method consisted in the hyphenation of a PGC and a silica-based columns.
449 Its application to a biomass sample allowed to distinguish *ca.* 70 chromatographic peaks with an
450 occupation of the separation space of 75%.

451 Finally, RPLC×RPLC-IMS-HRMS hyphenation was performed for the first time on biomass sample. A
452 major difficulty of this kind of hyphenation is the representation and processing of four-dimensional
453 data. Moreover, although IMS data interpretation must be done carefully, we demonstrated that such
454 hyphenation improved the separation of compounds still co-eluted in RPLC×RPLC. RPLC×RPLC-IMS-
455 HRMS method is highly specific by describing each analyte with two retention times, one mobility that
456 can be converted in CCS and one mass spectrum allowing to determine the molecular formula. All
457 these descriptors can be used simultaneously to support an identification and thus increase the level
458 of confidence.

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- 608

609 **Figure captions:**

610 Figure 1: TIC in (+)ESI, (-)ESI and UV chromatogram at 210 nm

611 Figure 2: BPC between m/z 100-1000 in (-)ESI mode of the biomass sample using Hypercarb (100×2.1
612 mm) column. Refer to Table 1 for labelled peaks

613 Figure 3: BPC between m/z 50-1000 in (-)ESI of the biomass sample with Hypercarb column, IPA as
614 organic solvent phase from 0 to 99% (gradient from 4 to 30 min)

615 Figure 4: EIC of vanillin in (-)ESI with three D1 solvent compositions (25, 50, 90% IPA) injected either
616 via the autosampler (black) or 20 μ L sampling loops (red)

617 Figure 5: Contour plot of on-line RPLC×RPLC-HRMS for the separation of the biomass sample in (-)ESI.
618 Dotted lines represent the elution window of carbohydrates of interest.

619 Figure 6: EIC (± 0.005) of six ions listed in Table 1 obtained from the RPLC-HRMS analysis of the biomass
620 sample

621 Figure 7: EIC (± 0.005) of six ions listed in Table 1 obtained from the RPLC×RPLC-HRMS method

622 Figure 8: Mass spectra obtained by (A) RPLC-HRMS at 0.85 min and (B) RPLC×RPLC-HRMS at 133 min
623 in D1 and 8 s in D2

624 Figure 9: $1/K_0$ as a function of D2 retention time (D1: 126.4 min) for m/z 321 and mass spectra of the
625 two peaks