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1 A rational strategy based on experimental designs to optimize parameters of a
2 liquid chromatography-mass spectrometry analysis of complex matrices

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10

11 **Abstract**

12 The ability of a method based on hyphenation of liquid chromatography and mass spectrometry to
13 successfully separate, ionize and detect compounds in complex matrices is determined by a high
14 number of factors controlled by the experimenter. Key steps to manage such hyphenation are
15 focused on desolvation and ionization processes. In this study, a design of experiments approach was
16 used to optimize decisive parameters (nebulizing, drying and sweep gas flow rates, ion transfer
17 capillary voltage and temperature) for electrospray ionization and atmospheric pressure chemical
18 ionization sources both in positive and negative modes. Central composite designs of 131
19 experiments were built in such a way as to cover rationally a sufficiently wide range of operating
20 conditions and make possible the establishment of models. Each run was repeated three times to
21 insure stable conditions of ionization and thus a satisfactory repeatability. Extracted ion
22 chromatograms of twelve model compounds were integrated and entered as responses for
23 experiment designs. Quadratic models for each standard allowed to take into account interactions
24 between factors. Then responses were simultaneously maximized to achieve optimized factors. To
25 illustrate the efficiency of this methodology, optimal conditions were applied to a lignocellulosic
26 biomass fast pyrolysis oil. High sensitivity was obtained in LC/MS, especially for negative-ion mode
27 electrospray which enabled identification of 5500 molecular formulae whereas direct introduction
28 allowed to attribute twice less. In short, this study proposed a rational methodology to optimize
29 ionization efficiency of a LC/MS analysis of complex mixtures.

30 *Keywords:*

31 *LC-UV/MS; High resolution mass spectrometry; Design of experiments approach; Ionization efficiency;*
32 *Lignocellulosic biomass*

33

34 **1. Introduction**

35 Liquid chromatography (LC) hyphenated with mass spectrometry (MS) began to be studied much
36 later than gas chromatography/mass spectrometry (GC/MS) because of the technical difficulties
37 imposed by such hyphenation (desolvation in particular). It is only since thirty years that LC/MS has a
38 real rise thanks to the development of ionization sources at atmospheric pressure. Despite the strong
39 interest of the scientific community specially in metabolomics research, LC/MS is not systematically
40 used as a routine technique [1]. Preliminary tests and an important step of optimization must be
41 done when an analysis is set up. The main causes are the incompatibility of some chromatographic
42 methods to mass spectrometry (solvent flow rate, additive, etc.) and the large number of parameters
43 to manage in order to control the ionization efficiency inside the atmospheric pressure ionization
44 source (API). Indeed, the signal obtained depends on several factors such as the flow rates of
45 nebulising and drying gases, the sprayer position, the temperatures of the API and transfer capillary,
46 capillary voltage, etc. Optimizing each of these parameters can be tedious and most of the time it is
47 based on the experience that can have a regular user of a LC/MS system. Moreover, the procedure of
48 varying one variable at a time does not guarantee the achievement of optimal conditions.
49 Conversely, although barely used in the literature, the chemometric approach is based on a rational
50 experimental model, which allows the simultaneous variation of all the experimental parameters.
51 The use of an experimental plan also makes it possible to determine the number of necessary
52 experiments and avoids the redundancies of information. It can also take into account the possible
53 interactions between the different parameters. In our knowledge, only few publications used a
54 design of experiments approach to optimize the ion source-mass spectrometry parameters mainly
55 for pharmaceuticals issues [2–4]. Main of them focus on improving signal of target analytes for
56 pharmaceuticals issues. Perrenoud *et al.* compared the sensitivity of a UHPSFC-ESI/MS/MS versus a
57 UHPLC-ESI/MS/MS analysis for pharmaceutical compounds using a face-centered central composite
58 design to optimize capillary voltage, desolvation temperature and drying gas flow rate [3].

59 In addition, LC/MS has become more and more interesting to analyze complex matrices containing
60 for example heavy or thermosensitive products that cannot be analyzed by GC/MS. This is
61 particularly true for oxygenated compounds coming from the conversion of lignocellulosic biomass
62 [5–7]. In the present challenging energy transition context, biomass seems as a promising source to
63 produce renewable energy, biofuels and chemical intermediates by mean of several transformation
64 pathways [8,9]. As regards thermochemical pathway, lignocellulosic materials, mainly composed of
65 lignin, cellulose and hemicellulose, are liquefied by fast pyrolysis at 400-450°C to produce bio-oils
66 which might be further upgrated to biofuels [10,11]. The resulting liquid matrices are highly complex
67 since they contain a wide diversity of oxygenated compounds (carboxylic acids, ketones, aldehydes,
68 phenols, furans, carbohydrates, etc.). The large range of polarities and molecular weights but also
69 heat sensitivity behavior of some compounds explain why chemical characterization of such liquids is
70 still a challenge today [12]. Without prior derivatization step, GC and comprehensive two-
71 dimensional gas chromatography (GC×GC) could hardness detect compounds with a molecular
72 weight higher than 200 g/mol [13–15]. However liquid chromatography (LC and LC×LC) appears to be
73 a suitable technique thanks to its capacity to elute compounds with high polarity such as
74 carbohydrates and/or high molecular weight [16,17]. More recently Fourier-transform ion cyclotron
75 resonance mass spectrometry (FT-ICR/MS) has gained an increasing attention [18–23]. High-
76 resolution mass spectrometry (HRMS) is a powerful technique for the analysis of many kinds of
77 mixtures and provides an important list of chemical molecular formulae allowing a deeper

78 understanding of the composition of bio-oils. Moreover, the use of several atmospheric pressure
79 ionization sources is essential to detect a large range of molecules [24]. Some works have also
80 demonstrated that additives like ammonium chloride or sodium hydroxide could enhance ionization
81 efficiency of hemicellulosic and lignin derivative compounds [18,25,26]. However, one issue
82 associated with complex mixture analysis based on mass spectrometry is signal suppression due to
83 competition between analytes during the ionization process, especially with ESI. The hyphenation
84 with liquid chromatography should theoretically reduce this phenomenon but also allow to
85 differentiate isomers. Although a quite high number of papers using HRMS with sample direct
86 introduction have been published on biomass samples [18,21,23–25], a much smaller number of
87 studies was dedicated to the hyphenation of LC with HRMS [5,7,27].

88 In the present study, the aim was to develop an effective LC-UV/MS method to reach optimal signal.
89 We have used a response surface methodology with central composite designs (CCD) in order to
90 increase the ionization efficiency of model compounds related to bio-oils. LC/MS experiments were
91 carried out using a LC/FT-ICR/MS hyphenated system. One experiment design were done for each
92 ionization mode: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)
93 both in positive and negative modes. Ahead the experiment designs, preliminary tests were made to
94 fix some parameters like additives, split ratio and sprayer position). The objective was to develop a
95 rational optimization of LC/MS method and thus to have a better control of the ionization efficiency.
96 Our four methods were then applied to a lignocellulosic biomass pyrolysis oil.

97 ## 2. Experimental

98 ### *2.1. Chemicals*

99 Methanol and formic acid were MS grade, purchased from VWR (Fontenay sous Bois, France).
100 Deionized water was produced by a Milli-Q water purifier (Millipore SAS, Molsheim, France).
101 Ammonium hydroxide, ammonium formate, 1-(4-hydroxy-3-methoxyphenyl)ethan-1-one
102 (acetovanillone), phenylethanone (acetophenone), 1-(4-ethylphenyl)ethanone (4-
103 ethylacetophenone), 1,3-cyclopentanedione, 2-phenylbenzo[h]chromen-4-one (α-naphthoflavone),
104 2,5-hexanedione, 3-methyl-2-cyclohexen-1-one, 3-methyl-2-cyclopenten-1-one, 4-
105 methylcyclohexanone, benzene-1-,2-diol (catechol), 2-methoxyphenol (guaiacol), 1,3-dimethoxy-2-
106 hydroxybenzene (syringol), (3-phenoxyphenyl)methanol, 4-(hydroxymethyl)-2-methoxyphenol
107 (vanillyl alcohol), butane-1,2,3,4-tetrol (erythritol), but-2-ene-1,4-dioic acid (fumaric acid),
108 octodecanoic acid (stearic acid), benzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-
109 methylbenzoic acid (p-toluic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 4-hydroxy-3,5-
110 dimethoxybenzoic acid (syringic acid), benzaldehyde, 4-hydroxybenzaldehyde and 4-hydroxy-3-
111 methoxybenzaldehyde (vanillin) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier,
112 France).

113 The fast pyrolysis bio-oil used in this study was produced from softwood sawdust and provided by IFP
114 Energies nouvelles (Solaize, France).

115 ### *2.2. Sample preparation*

116 Model mixtures used for CCD were prepared in methanol at a concentration of 1 mM for each
117 compound. One mixture contained acetovanillone, acetophenone, 4-ethylacetophenone, 1,3-

118 cyclopentanedione, α-naphthoflavone, catechol, guaiacol, syringol, (3-phenoxyphenyl)methanol,
119 vanillyl alcohol and erythritol. The second was composed of fumaric acid, stearic acid, benzoic acid,
120 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, p-toluic acid, o-toluic acid, vanillic acid, syringic acid,
121 benzaldehyde, 4-hydroxybenzaldehyde and vanillin (Table 1). The fast pyrolysis bio-oil was 10 times
122 diluted in methanol before injection.

123 *2.3. Instrumentation*

124 All experiments were performed using an Agilent 1290 UHPLC system (Palo Alto, CA) consisting of a
125 binary pump, an autosampler, a temperature-controlled column compartment and a photo diode
126 array (PDA) detector (volume UV cell 1 µL). The extra-column variance was measured at 9 μL^2. The
127 chromatographic system was hyphenated to a Thermo Scientific LTQ-FT-ICR (Bremen, Germany)
128 composed of a linear ion trap and an ion cyclotron resonance cell in a 7 Tesla superconducting
129 magnet. Ionization was carried out with the ESI and APCI sources both working in positive or negative
130 modes.

131 *2.4. High performance liquid chromatography/high-resolution mass spectrometry*

132 For LC/MS analysis, 1 µL of each sample was injected. Separation was achieved on a Kinetex C18
133 column (100 × 3 mm, particle size 2.6 µm, Phenomenex, Le Pecq, France) at 30°C. The mobile phase
134 solvents used were a 0.01% (v/v) formic acid in water (A) and a 0.01% (v/v) formic acid in methanol
135 (B) at 600 µL/min. A linear gradient was used as follows: 0.0 - 4.0 minutes, 1%B; 4.0 - 30.0 minutes,
136 from 1% B to 99% B; 30.0 - 35.0 minutes, 99% B; 35.0 - 36.0 minutes, from 99% B to 1% B; 36.0-40.0
137 minutes, 1% B. UV signal was recorded from 210 to 400 nm.

138 To avoid excessive pressure on the UV cell and to be in optimal conditions for desolvation in ESI, LC
139 flow was splited between UV detector and ESI source (Figure 1). Flow rates of nebulising, drying and
140 sweep gases as well as transfer capillary temperature and voltage were optimized using a design of
141 experiments approach (DoE). The spray voltage was -3.5 kV in negative mode and 4 kV in positive
142 one. For APCI source, flow rates of nebulising and drying gases, as well as vaporizer temperature,
143 transfer capillary temperature and voltage were optimized with a DoE. Discharge current was fixed at
144 20 µA in positive and negative modes. No sweep gas was used. For both API sources, a delta of 25 V
145 between tube lens voltage and transfer capillary voltage was kept constant. The sprayer position was
146 also optimized.

147 In order to have at least 10 points for each chromatogram peak, a resolving power of 12500 at *m/z*
148 400 was used for the FT-ICR spectrometer. The mass range was set to *m/z* 92-1000 with 2 µscans
149 averaging. External calibration was accomplished with CalMix+ and CalMix- (Thermo Scientific) in
150 positive and negative mode respectively.

151 For Flow Injection Analysis (FIA), LC conditions were unchanged. MS conditions with APCI source
152 were as follow: nebulising gas flow rate 40 AU, drying gas flow rate 20 AU, sweep gas flow rate 0 AU,
153 capillary voltage -50V, capillary temperature 250°C, tube lens voltage -75V. MS conditions with ESI
154 source were : nebulising gas flow rate 30 AU, drying gas flow rate 25 AU, sweep gas flow rate 0 AU,
155 capillary voltage -30V, capillary temperature 350°C, tube lens voltage -55V.

156

157 *2.5. Direct introduction (–)ESI/FT-ICR/MS*

158 Sample was diluted in methanol (1:50; v/v) prior to the introduction by infusion (5 µL/min). The
159 number of µscans was set at 8 and 50 scans were accumulated with a resolution of 100000. Flow rate
160 of nebulising gas was set to 5 AU. No drying and sweep gases were used. The spray voltage was -2.8
161 kV. Transfer capillary temperature, transfer capillary voltage and tube lens voltage were respectively
162 set to 275 °C, -35 V and -110 V.

163 *2.6. Software and MS data processing*

164 Extracted ion chromatograms (EICs) were traced and integrated with Xcalibur 2.1 software (Thermo
165 Scientific).

166 Construction of CCD, quadratic modellings of model molecules and optimization of MS parameters
167 were done on Design Expert 10 software (Stat-Ease).

168 For molecular formulae calculation, homemade software was used. The following parameters were
169 applied: elemental composition $^{12}C_{1-100}$, $^{1}H_{1-100}$, $^{16}O_{1-100}$ and mass accuracy ≤ 2 ppm.

170

171 **3. Results and discussion**

172 *3.1. Methodology for optimizing desolvation and ionization parameters*

173 The objective of this work was to develop a rational strategy to optimize parameters of a LC-UV/MS
174 analysis of complex matrices coming from biomass such as bio-oils. When liquid chromatography is
175 hyphenated to mass spectrometry, desolvation and ionization of molecules are critical steps. Indeed,
176 when LC is used without MS, there is no restriction about additive/salt concentrations neither about
177 the LC flow rate which is directly related to chromatographic column's dimensions. To make LC/MS
178 hyphenation possible, many parameters have to be adjusted: LC flow rate, split ratio between LC and
179 MS system, nebulising, drying and sweep gas flow rates, transfer capillary temperature and voltage,
180 tube lens voltage, vaporizer temperature for APCI, spray voltage or discharge current, API position
181 are the main conditions to be investigated. To increase ionization efficiency, a make-up such as
182 ammonium hydroxide in negative mode could also be added at the outlet of the LC system. In light of
183 the large number of factors, to succeed this hyphenation and reach the real optimum, we propose a
184 DoE methodology using central composite designs. Thereafter, a quadratic model was built, taking
185 into account second order interactions.

186 This development was carried out on model compounds which are representative of bio-oils
187 according to previous work [28]. Two mixtures were used (Table 1):

188 - one for negative-ion ESI and APCI modes made of carboxylic acids and aldehydes
189 - one for positive-ion ESI and APCI modes made of ketones and alcohols.

190 The main advantage to use model compounds was to cleverly optimize each ionization mode
191 depending on the ability of compounds to accept or lose proton. Indeed, for example there is no
192 point to improve the ionization of carboxylic acids in positive mode while they are well ionized in
193 negative one thanks to the labile proton on the COOH function.

194 To build the four CCD, five significant parameters were chosen for each ionization mode in order to
195 limit the number of experiments: flow rates of nebulising, drying and sweep gases, transfer capillary
196 temperature and voltage (see part 3.2.). Tube lens voltage was associated with transfer capillary
197 voltage by keeping a difference of 25 V between them in order to prevent ion source fragmentation.
198 Other parameters which need manual adjustments were not included in the DoE and thus were
199 optimized and fixed in advance: make-up and split of LC mobile phase at the entrance of mass
200 spectrometer as well as the API source position. Impact of additives also called make-ups was also
201 studied (see 3.1.). Chromatographic conditions were chosen to be compatible with mass
202 spectrometry: for example concentration of formic acid was as low as possible but sufficient to keep
203 analytes in their protonated form. Figure 1 illustrates the parameters which were either optimized in
204 advance or with a DoE for ESI mode. To avoid any discharge and/or damage, authors decided to keep
205 spray voltage at -3.5 kV and 4.0 kV for negative and positive ESI modes respectively. Moreover,
206 according to previous publication about effects of several parameters linked to ion generation region
207 on signal intensity using a similar ion source (electrospray ionization MAX2 source, Thermo
208 Scientific); spray voltage was not found as a significant parameters at the opposite of the nebulising
209 gas, capillary voltage and tube lens voltage. [29].

210 *3.2. Adaptation of the LC downstream flow for MS compatibility*

211 To optimize a LC/MS interface, it is primordial to well understand the process of ionization in order to
212 focus on key parameters and adjust the flow rate and/or the composition of mobile phases to drive
213 them compatible with ion source specificities.

214 In the case of ESI, the compounds diluted in the mobile phase are introduced by a capillary in an area
215 at atmospheric pressure where an electric field of a few kV is applied. This electric field causes an
216 accumulation of charges on the surface of the liquid causing the formation of a cone at the end of
217 the capillary (Taylor cone). When coulombic repulsions exceed the surface tension of the liquid,
218 charged droplets are then emitted from the tip of the cone. At this stage, several theories concerning
219 the ionization process compete. The best known one is the residual charge theory introduced in 1968
220 by Dole *et al.* [30]. According to them, a succession of fissions of the droplets alternating with the
221 evaporation of the solvent cause the release of ions in the gas phase. Conversely, according to other
222 theories, like the model of ion evaporation (Iribarne and Thomson), the ions would be ejected from
223 the droplets or even ejected directly from the Taylor cone [31]. In fact, the models may be
224 complementary, and ionic evaporation may occur in the later stages of the residual charge theory
225 [32,33]. It should be noted that the formation of ions in the gas phase often ends not in the
226 atmospheric pressure area but at the interface of the latter and the next area at reduced pressure.

227 Unlike ESI for which ions are pre-formed in solution, APCI requires that the molecules go into the gas
228 phase prior to ionization. For this reason, the ionization source is heated between 300 and 500°C
229 (vaporizer temperature) depending on the flow rate and the composition of the liquid. The formed
230 vapor then passes through a stream of electrons emitted by a corona discharge which creates plasma
231 around the needle.

232 *3.2.1. Flow splitting*
233 LC flow rate was 600 µL/min, which was too high for ESI. According to manufacturer, ESI could
234 manage a flow of 1 mL/min, but optimal operation is obtained between 100 and 400 µL/min. To
235 reduce the flow rate, a T-split was installed after the chromatographic column to prevent UV cell

236 damages (Figure 1). Split ratio was calculated by divided flow rate at the outlet of the UV detector by
237 the LC flow rate. The main drawback to split the mobile phase is solute dispersion, especially when a
238 low split ratio has to be considered. Regarding theoretical considerations, total solute dispersion
239 (σ^2_{total}) can be assessed by peak variance coming from dispersion inside the column (σ^2_{col}) and extra-
240 column dispersion (σ^2_{ext}) (Eq 1). Extra-column dispersion results from injection, tubing, detector and
241 split ratio (Eq 2) [34].

242 (1) $\sigma^2_{total} = \sigma^2_{col} + \sigma^2_{ext}$

243 (2) $\sigma^2_{ext} = \sigma^2_{injection} + \sigma^2_{tubing} + \sigma^2_{detector} + \sigma^2_{split}$

244 To limit the loss of theoretical plates (N_{col}), extra-column dispersion must be as low as possible. The
245 ratio between σ^2_{col} and σ^2_{ext} (β^2) represents the percentage of remaining plates. Here, we were
246 interested in the ratio between σ^2_{col} and σ^2_{split}. For a compound with a retention factor at elution (k_e)
247 of 3, σ^2_{col} is equal to 187 µL² according to Eq 3.

248 (3) $\sigma^2_{col} = \frac{V_O^2(1+k_e)^2}{N_{col}}$

249 With V_0 the column dead volume and N_{col} estimated by dividing the column length (L_{col}) by 3 times
250 the pore size (d_p).

251 To calculate the total dispersion, Dorsey-Foley equation was used (Eq.4).

252 (4) $\sigma^2_{total} = \frac{(\mathrm{As}+1.25)\, F^2 W_{0.1}^2}{41.7}$

253 With F the mobile phase flow rate, As the peak asymmetry and $W_{0.1}$ the peak width both at 10% of
254 the peak height respectively.

255 By removing the chromatographic column, σ^2_{total} corresponds to σ^2_{ext}. In order to measure only the
256 dispersion due to flow splitter, T-split was first removed to calculate the dispersion from injection,
257 tubing and detection. Then this measured dispersion was subtracted to σ^2_{total} to obtain only the split
258 dispersion. Several split ratios were tested by changing the LC flow rate and keeping constant the
259 flow coming into the mass spectrometer. By doing this, we could consider that dispersion of mass
260 spectrometer was constant. The variation of tubing's dispersion related to LC flow modification was
261 considered negligible before split dispersion (from 100 to 600 µL/min). Figure 2 shows the
262 percentage of remaining plates according to split ratios. With a ratio of 0.20 (1:5), around 60% of
263 plates were lost only because of the T-split which may lead to mix resolved peaks. To keep at least
264 70% of theoretical plates, a split ratio of minimum 0.45 (1:2.2) was needed which corresponds to a
265 flow of 270 µL/min directed into the mass spectrometer. Thus, this ratio was used later on.

266 For APCI, no flow splitter was needed. Thanks to the tubular oven, APCI can handle higher flow rate
267 than ESI. Moreover, APCI is a mass dependent ionization technique, which means a T-split would
268 decrease the MS signal. At the opposite, ESI is concentration dependent, thus with adapted
269 desolvation conditions, a flow splitter does not impact the MS signal [35].

270 *3.2.2.Sprayer position*

271 To optimize the source position of APCI and ESI, 4 model molecules were injected in FIA (Flow
272 Injection Analysis) at a concentration of 1 mM. On the API used, four positions were possible from A

273 which is the closest position to the transfer ion capillary, to D the furthest one. Each EIC was
274 integrated and measured areas as a function of the source position are plotted in Figure 3.

275 For APCI, the source position did not have a real impact on the areas of EIC and thus on the ionization
276 efficiency of model compounds. For ESI, areas of compounds could be 5 times lower for position A
277 compared to position D. So for all other experiments, API was fixed in D position. These observations
278 were consistent with the ionization process of each API source. Indeed, in APCI source, the whole
279 mobile phase is vaporized and the ionization occurs in the gas phase by the use of a corona
280 discharge. At the opposite in ESI source, ionization takes place in liquid phase with the formation of a
281 spray due to high voltage applied between the ESI needle and the ion sweep cone. Thus the position
282 of ESI sprayer have a direct impact on the electric field (E) which becomes more intense by bringing
283 the sprayer closer (Eq.5).

284 (5) $E = \frac{V}{A \cdot r \cdot ln\left(\frac{4d}{r}\right)}$

285 With V the applied voltage, A an empirical constant estimated by Smith to be equal to 0.667, r the
286 capillary emitter radius (cm) and d the distance between the ESI sprayer and the ground plane (cm)
287 [36,37].

288 It is important to note that position source optimum depends on many other parameters such as
289 nebulising gas flow rate or transfer ion capillary voltage. These parameters were optimized later with
290 a DoE.

291 *3.2.3. Influence of additives*

292 It is well known that mobile phase composition plays an important role during desolvation and
293 ionization steps [38–42]. In ESI, the solvent used must have a sufficient dielectric constant to form
294 charged droplets as well as a low surface tension. Moreover, additives should be used with a
295 concentration as low as possible. Indeed, ESI is a competitive ionization process. When several
296 compounds are present in an electrospray droplet, they compete for a limited number of charged
297 sites at the droplet surface, which can lead to ion suppression. In this study, the mobile phase
298 contained 0.01% (v/v) of formic acid in order to keep carboxylic acids under their protonated forms
299 during chromatographic separation. The measured pH of aqueous solvent was 3.2. For ESI positive-
300 ion mode, formic acid was a good source of protons; however for negative-ion mode, a pH around 8
301 would be preferred to help anion formation in the liquid phase. To evaluate the effect on the mass
302 signal of formic acid added in the mobile phase as well as two other additives, 4 model compounds
303 (vanillic acid, syringic acid, vanillin and syringaldehyde) were injected in negative ESI using formic
304 acid, ammonium hydroxide and ammonium formate at concentrations varying from 0.1 µM to 50
305 mM.

306 Unexpectedly, formic acid did not deteriorate ionization efficiency at moderate concentrations
307 (Figure 4). Loss of signal started around 1 mM. Between 0.1 µM and 1 mM, ionization of carboxylic
308 acids was not really impacted by formic acid, whereas aldehyde responses were exalted. This
309 phenomenon is called “wrong-way-round ionization” [41,43]. ESI process is complex and many
310 characteristics of mobile phase composition influence ionization process such as volatility, surface
311 tension, conductivity, ionic strength, pH, gas-phase ion-molecule reactions, etc. Indeed, even if an
312 analyte is ionized in liquid phase, ionization of neutral analytes present in the gas phase could also

313 occur by proton transfer reactions involving charged eluent species from solvents and/or additives.
314 Such exaltation of aldehydes responses may be explained by the fact that ionization took place by
315 gas-phase proton transfer or it may happen at the liquid-gas interface droplet. It is known that redox
316 reactions occurring at ESI sprayer, *i.e.* reduction for negative-ion mode, create a significant difference
317 of pH between the solution and the droplets [42]. Addition of protons from acids could facilitate
318 chemical reduction and so decrease the pH at the surface of droplets [44]. Thus even if solutes were
319 not ionized inside the mobile phase, modification of pH droplets could make ionization in liquid
320 phase possible. However, this theory was not in agreement with the fact that carboxylic acids had an
321 almost constant response although aldehydes shown an increasing of their responses, except if
322 carboxylic acids were already well ionized without formic acid because of their low pKa.

323 Similarly to formic acid behavior, ammonium hydroxide and ammonium formate enhanced aldehyde
324 responses, especially vanillin signal. These additives increased the pH, thus ionization could occur
325 directly inside mobile phase. The main differences between ammonium hydroxide and formate were
326 an enhancement less important for ammonium formate than ammonium hydroxide and an
327 important ion suppression at high concentration of ammonium formate which did not occur for
328 ammonium hydroxide. This last phenomenon could be due to the anionic part of ammonium formate
329 $HCOO^-$ which is bigger than OH^- for ammonium hydroxide and so had a better affinity for droplet
330 surface leading to a competition with solutes at high concentrations.

331 During this study, 0.01% (v/v) of formic acid was added to mobile phase, which corresponds to 2.7
332 mM. These conditions were not unfavorable as predicted; thereby no other additive was used at the
333 outlet of LC system.

334 *3.3. Optimization of ESI and APCI desolvation conditions by mean of central composite design*

335 CCD was chosen in this study in order to take into account second order interactions. For each
336 ionization mode, a DoE was established including the 5 key parameters. For ESI, nebulising, drying
337 and sweep gas flow rates, transfer ion capillary temperature and voltage were studied. For APCI,
338 preliminary experiments showed that sweep gas flow rate did not influence ionization, thus it was
339 excluded of the design and vaporizer temperature was added to the study. The investigated ranges
340 for each variable are summarized in Table 2. These ranges had to be restricted to make the later
341 second order modelling possible and in the same time sufficient to consider a relevant signal.

342 Each CCD was composed of

343 - a factorial design with 2^k experiments (k is the number of factors, here 5), each factorial
344 point was repeated 3 times
345 - 2×k axial points also repeated 3 times
346 - 5 central points.

347 In this work, points were repeated whatever the conditions were, not only on central point as it
348 usually done in literature. In this way repeatability of runs was estimated and unstable ionization
349 conditions were pointed out, especially spray that may be not stable in the ESI source. In total, 131
350 injections of our model mixture were done for the four ionization modes. EIC were integrated for the
351 12 model compounds. Measured areas were used as responses of CCD and were exploited by
352 creating one quadratic model per molecule. Models were constructed with 80% of experiments, the

353 remaining 20% part was used as validating points. Last step was optimization of model compound
354 responses simultaneously with desirability function in order to obtain optimized conditions.
355 Optimization of negative APCI is developed in this paper as example.

356 To correlate responses with factors, a second order model with interaction was fitted to the
357 experimental data. The general form of second order function is:

358 $$(6)\quad y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j>i}^{k} \beta_{ij} x_i x_j$$

359 Where y is the response, β_0 the constant, β_i the first order coefficients, β_{ii} the quadratic coefficients
360 and β_{ij} the interactive coefficients.

361 A model involving 5 factors requires calculation of 21 coefficients. First, to simplify the model, only
362 significant quadratic and interactive coefficients with a p-value lower than 0.1 were kept. Analysis of
363 variance (ANOVA) for response surface reduced quadratic model of vanillic acid is shown in Table 3.
364 The model F-value was equal to 58.42 indicating that the model is significant ($P<0.05$) and there was
365 only 0.01% chance that the F-value this could occur due to noise. P-value less than 0.05 indicates that
366 model terms are significant (with $\alpha = 0.05$). In this case A, B, D, E, AB, AC, AE, BC, BD, CD, CE, A^2, C^2
367 and D^2 were significant model terms. First order coefficient C, corresponding to capillary
368 temperature, was not significant; however quadratic coefficient C^2 and several interactive
369 coefficients with C were significant.

370 R-squared value was 0.9148 representing a good agreement between experimental results and the
371 model predictions. Moreover R-squared increased every time when an independent variable was
372 added to the model, thus regression model containing more variables than another one is expected
373 to provide a better fit (main risk being model overfitting in this case). On the contrary, adjusted R-
374 squared increased only if a new term improved the model fit otherwise it decreased. Predicted R-
375 squared was used to determine how well model makes predictions. To calculate this value, a data
376 point from the dataset was removed, then the regression equation was calculated, the last step was
377 to evaluate how well the model predicts the missing point. This procedure was done for all data
378 points. In this case, predicted R-squared was 0.8786 and it was in good agreement with the adjusted
379 R-squared of 0.8992.

380 Once the model was built, several model evaluation plots could be traced. Figure 5 shows the
381 experimental data *versus* predicted value plot for the vanillic acid model. It can be observed that
382 points are around the line, which means model fit to experimental data. Moreover, validation points,
383 notched points on the graph, were quite well predicted.

384 Interactions between factors could be represented with response surface. As an example, Figure 6
385 illustrates the response surface of ion transfer capillary temperature and ion transfer capillary
386 voltage for vanillic acid. For low capillary voltage, the optimum capillary temperature was higher than
387 350°C while for high capillary voltage, a capillary temperature lower than 350°C was preferred.

388 When all the models for compounds were built, a desirability function was used to maximize at the
389 same time all the responses for predicted optimal conditions (Table 4). Figure 7 illustrates
390 perturbations at optimized conditions for the desirability and for two model compounds (vanillic acid
391 and 4-hydroxybenzaldehyde). Optimal conditions will be applied for complex and unknown samples
392 containing similar analytes than chosen model compounds in this study. Thus a compromise was

393 made in order to obtain a sufficient signal for all model compounds without reaching the maximum.
394 For example, the value of C factor, ion transfer capillary temperature, was optimized at 376°C, but it
395 would be better for vanillic acid to use a lower temperature. As regards 4-hydroxybenzaldehyde, a
396 slightly higher temperature would give a better response. Another remark concerns the stability of
397 optimal conditions. The perturbation of desirability shows a plate, except for B factor (drying gas).
398 This means that for example, if needed, smaller vaporizer temperature (E factor) could be used
399 without having important consequence on signal. Globally, higher optimal nebulising and drying gas
400 flow rates were obtained in positive modes than in negative ones. To facilitate desolvation, high
401 temperatures were preferred in negative modes.

402 *3.4. Application to a lignocellulosic biomass pyrolysis oil*

403 Optimized ionization conditions were applied to a lignocellulosic biomass pyrolysis oil. LC-UV/HRMS
404 analysis resulted from five complementary detections: UV, ESI and APCI in positive and negative
405 modes. Moreover, all UV spectra were recorded from 210 to 400 nm. Molecular formulae were
406 identified based only on carbon, hydrogen and oxygen elemental composition with an accuracy of 2
407 ppm (see part 2.5).

408 UV chromatogram at 254 nm and base peak chromatogram (BPC) of a lignocellulosic biomass
409 pyrolysis oil are shown in Figure 8 as examples. Optimized ESI negative-ion mode showed a high
410 sensitivity, especially between 10 and 30 minutes.

411 By integrating all BPC data coming from ESI negative-ion mode, two main zones are clearly
412 distinguished on the mass spectrum (Figure 9). The first one includes *m/z* ratios varying between 100
413 and 450 whereas the second area ranges from 450 to 900 *m/z* ratios. When the pyrolytic oil was
414 infused into the FT-ICR mass spectrometer without any chromatographic separation dimension, the
415 second zone was not as well detected because of ion suppression.

416 When dealing with complex samples producing a large number of data to cope with, van Krevelen
417 diagram is a good way to represent MS data. The highest number of molecular formulae was
418 achieved with ESI negative-ion mode (*ca.* 5500 formulae). At the opposite only 500 formulae were
419 identified with APCI negative-ion mode. Since they contain significant contents of sugars and other
420 heat sensitive compounds, lignocellulosic bio-oils are known to be thermally unstable. So during MS
421 analysis, compounds burned and formed a black substrate deposited on the tube oven of the APCI
422 source because of the too high temperature (450°C). Experiments at 350°C did not improved the
423 signal. ESI is a softer ionization source than APCI, thus it is more suitable for this kind of sample.
424 However, APCI do not formed adducts during ionization process which can simplify data
425 interpretation. In comparison to negative ionization mode, only 900 molecular formulae could be
426 calculated with positive ionization mode (APCI and ESI). A van Krevelen diagram including all
427 ionization modes was built (Figure 10). Compounds eluted with less than 20% of MeOH were
428 represented in red, in blue those eluted between 20 and 80% of MeOH and in green with more than
429 80%. Red points correspond mainly to sugaric compounds coming from the degradation of cellulose
430 and hemicellulose. These carbohydrates are very polar compounds and thus were non-retained on
431 apolar stationary phase. Compounds showing a higher affinity with the stationary phase (blue points)
432 correspond to pyrolytic lignin (phenolic compounds). Finally, green points were eluted with more
433 than 80% of MeOH, they might correspond to lipids (fatty and resin acids).

434 It has to be noted that only 2500 molecular formulae could be attributed by direct introduction in
435 negative ESI against 5500 with LC/MS due to matrix effects leading to ion suppression. Figure 9
436 already proved that heavy compounds (*m/z* > 500) were not well ionized with direct introduction. To
437 target molecule class which suffer from ion suppression in infusion, the heteroatom class distribution
438 for LC/MS and direct introduction, with oxygen families ranging from O_0 to O_{25} is presented in Figure
439 11. It appears that molecules having low number of oxygen atoms (< 4) were not detected with
440 direct introduction. Thus prior separation with liquid chromatography greatly enhances the detection
441 of heavy and moderately polar molecules.

442 In addition to limit ion suppression, liquid chromatography hyphenated to mass spectrometry
443 allowed to distinguish isomers which is not possible with direct introduction. An example is
444 presented in Figure 12. EIC of *m/z* 259.0975 ± 0.0005 in negative ESI corresponding to $C_{15}H_{16}O_4$
445 shows four intense peaks and other smaller ones at different retention times indicating the presence
446 of several isomers.

447 **4. Conclusion**

448 Despite LC-UV/HRMS analysis has shown a great interest for analysis of complex matrices since
449 several years, no rational optimization methodology was proposed in order to obtain a sensitive but
450 also controlled analytical method. In this work, a design of experiment was used to improve the
451 desolvation and ionization steps of a system involving a reversed phase column hyphenated to a FT-
452 ICR high resolution mass spectrometer. The effects of five experimental key parameters were studied
453 using ESI and APCI sources in negative and positive-ion modes. In our study, more than 130
454 experiments were performed for each ionization mode, using a synthetic solution containing 12
455 oxygenated model compounds. This chemometric approach allowed to take into account all possible
456 second order interactions, which will not be possible with the classic optimization 'one variable at a
457 time'. Quadratic models were built for each model compound, then their responses were maximized
458 at the same time to conclude about optimal conditions.

459 Moreover, some parameters (split ratio, sprayer position and additives) were investigated prior to
460 carry out the design of experiments. As regards ESI analyses, a T-split was added in order to decrease
461 the mobile phase flow rate at the entrance of the mass spectrometer. However dispersion induced
462 by such system must be considered to keep enough theoretical plates and narrow peaks and achieve
463 a satisfactory chromatographic separation. Signal detected in ESI was highly dependent on sprayer
464 position, contrary to APCI. In our conditions (LC mobile phase containing 0.01% of formic acid), no
465 supplementary additive was needed at the outlet of the chromatographic column.

466 Optimized conditions were applied to a lignocellulosic biomass fast pyrolysis oil to illustrate the
467 efficiency of such a methodology. LC-FT-ICR/MS analysis in ESI negative-ion mode provides a detailed
468 characterization of our complex bio-oil, delivering more than 5500 molecular formulae. Hyphenation
469 coupling a chromatographic separation to high-resolution mass spectrometry was demonstrated as
470 on one hand, LC first dimension enables to alleviate ion matrix effects and differentiate isomers
471 having the same molecular formulae but different retention times, and on the other hand, precise
472 and reliable molecular formulae are proposed for a large number of oxygenated compounds.

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Figure captions

Color should be used for Figure 1 , Figure 3 , Figure 4, Figure 5, Figure 8, Figure 10 and Figure 11.

Table 1: Model mixtures for negative-ion (left) and positive-ion (right) modes

Figure 1: Key parameters for ESI detection optimized with DoE (red boxed text) and before DoE (purple boxed text)

Figure 2 : Percentage of remaining plates according to the split ratio

Figure 3: Areas of EIC for 4 model compounds according to the ESI and APCI position (negative-ion mode). [LC and MS conditions : see Experimental part]

Figure 4: Effect of formic acid, ammonium hydroxide and ammonium formate on negative-ion ESI mode responses of 4 model compounds. The vertical axis represents the percentage ratio of peak area in the presence of additive to peak area in the absence of additive

Table 2: Central composite designs for ESI and APCI both in positive and negative modes

Table 3: ANOVA for response surface reduced quadratic model of vanillic acid in negative APCI

Figure 5: Experimental data versus predicted value plot for the vanillic acid model

Figure 6: Response surface plot of ion transfer capillary temperature and ion transfer capillary voltage. Nebulising gas flow rate, drying gas flow rate and vaporizer temperature were fixed at respectively 30 AU, 20 AU and 350°C in this representation.

Figure 7: Perturbation graphs at optimized conditions for the desirability (left), vanillic acid (middle) and 4-hydroxybenaldehyde (right)

Table 4: Optimized conditions resulting from model predictions for ESI and APCI sources

Figure 8: UV chromatogram at 254 nm (red) and BPC of negative ESI (blue) of a lignocellulosic biomass pyrolysis oil

Figure 9: ESI Negative-ion mode mass spectra of a lignocellulosic biomass pyrolysis oil obtained with prior LC separation (top) and direct introduction (bottom)

650 **Figure 10**: Van Krevelen diagram of a lignocellulosic biomass pyrolysis oil.
651 **Figure 11**: Comparison of heteroatom class distributions between LC-(-)ESI/FT-ICR/MS and (-)ESI/FT-
652 ICR/MS
653 **Figure 12**: Extraction ion chromatogram of a lignocellulosic biomass pyrolysis oil for of *m/z* 259.0975
654 +/- 0.0005