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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 upgraded 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 hardly 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 ( $\alpha$ -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,  $\alpha$ -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  $\mu$ L). The extra-column variance was measured at 9  $\mu$ L<sup>2</sup>. 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  $\mu$ L of each sample was injected. Separation was achieved on a Kinetex C18  
133 column (100  $\times$  3 mm, particle size 2.6  $\mu$ m, 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  $\mu$ L/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 splitted 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  $\mu$ A 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  $\mu$ scans  
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  $\mu$ L/min). The  
159        number of  $\mu$ scans 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  $^{\circ}$ 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  $^{12}\text{C}_{1-100}$ ,  $^1\text{H}_{1-100}$ ,  $^{16}\text{O}_{1-100}$  and mass accuracy  $\leq 2$  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  $\mu\text{L}/\text{min}$ , 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  $\mu\text{L}/\text{min}$ . 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 ( $\sigma^2_{total}$ ) can be assessed by peak variance coming from dispersion inside the column ( $\sigma^2_{col}$ ) and extra-  
240 column dispersion ( $\sigma^2_{ext}$ ) (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  $\sigma^2_{col}$  and  $\sigma^2_{ext}$  ( $\beta^2$ ) represents the percentage of remaining plates. Here, we were  
246 interested in the ratio between  $\sigma^2_{col}$  and  $\sigma^2_{split}$ . For a compound with a retention factor at elution ( $k_e$ )  
247 of 3,  $\sigma^2_{col}$  is equal to  $187 \mu L^2$  according to Eq 3.

248 (3)  $\sigma^2_{col} = \frac{V_0^2(1+k_e)^2}{N_{col}}$

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

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,  $\sigma^2_{total}$  corresponds to  $\sigma^2_{ext}$ . 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  $\sigma^2_{total}$  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  $\mu L/min$ ). 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  $\mu L/min$  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).

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

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  $\mu$ M 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  $\mu$ M 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  $\text{HCOO}^-$  which is bigger than  $\text{OH}^-$  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 -  $2 \times k$  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:

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

359 Where  $y$  is the response,  $\beta_0$  the constant,  $\beta_i$  the first order coefficients,  $\beta_{ii}$  the quadratic coefficients  
360 and  $\beta_{ij}$  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 sugarc 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  $m/z 259.0975 \pm 0.0005$  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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#### 626 **Figure captions**

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

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

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

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

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

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

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

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

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

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

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

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

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

648 **Figure 9:** ESI Negative-ion mode mass spectra of a lignocellulosic biomass pyrolysis oil obtained with  
649 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