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On-line 2D-RPLC x RPLC – HRMS to assess wastewater treatment in a pharmaceutical plant

Fleur Marie Saint Germain^{1,2}, Karine Faure², Estelle Saunier¹, Jean-Michel Lerestif¹, Sabine Heinisch^{2*}

¹ORIL Industrie, 13 rue Auguste Desgenetais, 76210 Bolbec, France

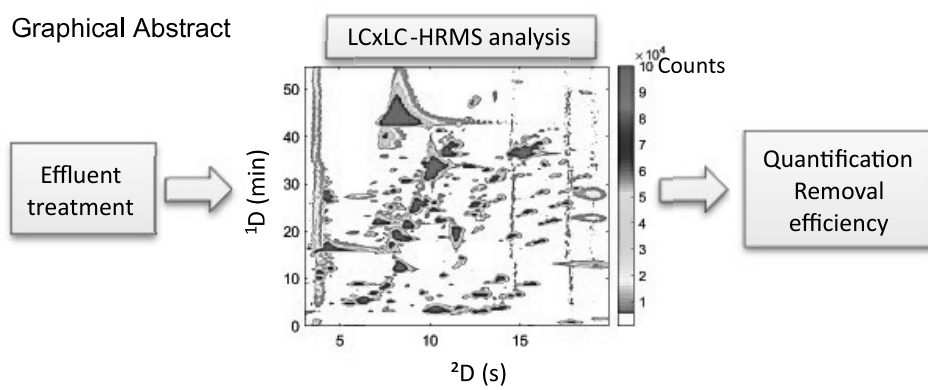
²Université de Lyon, Institut des Sciences Analytiques, CNRS UMR 5280, 5 rue de la Doua, 69100 VILLEURBANNE, France

*corresponding author: Sabine Heinisch; sabine.heinisch@univ-lyon1.fr

Abstract

Pharmaceutical effluents are complex media containing hundreds of compounds including active ingredients, intermediate products and unknown impurities. Bringing an industrial wastewater treatment plant (WWTP) into compliance with European directives requires a thorough analysis of the effluent. In this study, we demonstrate how online comprehensive two-dimensional liquid chromatography (on-line LC × LC) hyphenated to high resolution mass spectrometry (HRMS) can be a powerful analytical methodology to monitoring the outlet water, by analysing the content of known molecules while characterising unknown compounds. Reversed phase liquid chromatography (RPLC) was used in both dimensions, with a penta-fluoro-phenyl silica-based column at neutral pH in the first dimension (¹D) and a C18 column at acidic pH in the second one (²D). The conditions were optimized for a total analysis time of 60 min. The variability of both retention times and peak areas was evaluated. The average standard deviation on retention times was found to be less than 0.1 s in ²D. The relative standard deviation on peak area was about 7 % for run-to-run analysis. This analytical approach, applied to the pharmaceutical effluents before (inlet) and after (outlet) wastewater treatment permitted to detect 240 compounds. These included 27 priority pharmaceutical products, 8 of which were of very high priority and their concentrations could be compared to target values. The comparison of 2D-LC and 1D-LC approaches clearly highlights the power of on-line RPLC x RPLC technique, which allows both targeted quantitative analysis and non-targeted qualitative analysis of pharmaceutical effluents.

Graphical Abstract



1 On-line 2D-RPLC x RPLC – HRMS to assess wastewater treatment in a pharmaceutical plant

2 Fleur Marie Saint Germain^{1,2}, Karine Faure², Estelle Saunier¹, Jean-Michel Lerestif¹, Sabine
3 Heinish^{2*}

4 ¹ORIL Industrie, 13 rue Auguste Desgenetais, 76210 Bolbec, France

5 ²Université de Lyon, Institut des Sciences Analytiques, CNRS UMR 5280, 5 rue de la Doua,
6 69100 VILLEURBANNE, France

7 *corresponding author: Sabine Heinish; sabine.heinish@univ-lyon1.fr

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13 two-dimensional liquid chromatography (on-line LC × LC) hyphenated to high resolution mass
14 spectrometry (HRMS) can be a powerful analytical methodology to monitoring the outlet
15 water, by analysing the content of known molecules while characterising unknown
16 compounds. Reversed phase liquid chromatography (RPLC) was used in both dimensions, with
17 a penta-fluoro-phenyl silica-based column at neutral pH in the first dimension (¹D) and a C18
18 column at acidic pH in the second one (²D). The conditions were optimized for a total analysis
19 time of 60 min. The variability of both retention times and peak areas was evaluated. The
20 average standard deviation on retention times was found to be less than 0.1 s in ²D. The
21 relative standard deviation on peak area was about 7 % for run-to-run analysis. This analytical
22 approach, applied to the pharmaceutical effluents before (inlet) and after (outlet) wastewater
23 treatment permitted to detect 240 compounds. These included 27 priority pharmaceutical
24 products, 8 of which were of very high priority and their concentrations could be compared
25 to target values. The comparison of 2D-LC and 1D-LC approaches clearly highlights the power
26 of on-line RPLC x RPLC technique, which allows both targeted quantitative analysis and non-
27 targeted qualitative analysis of pharmaceutical effluents.

28

29 **Keywords:**

30 Comprehensive 2D-LC, pharmaceuticals, industrial wastewater analysis, treatment plant
31 efficiency.

32 **1. Introduction**

33 The pharmaceutical industry is a strictly regulated industrial sector. As a result of
34 environmental awareness, much more attention is now being paid to wastewater
35 management, which requires a specific internal policy to meet European directives [1,2]. The
36 content of wastewater effluent from industrial plants depends on the manufactured products
37 and may vary from batch to batch. Therefore, industrial operators promote on-site treatment
38 plants to better adapt the treatment to the physico-chemical properties of their products. The
39 process effluents usually consist of leftover chemical media and their rinsing water. They
40 therefore contain hundreds of molecules including known active pharmaceutical ingredients
41 (API), known intermediate products (IP), unknown impurities and degradation or
42 transformation products. The degradation strategy used in wastewater treatment plant
43 (WWTP) usually involves the application of multiple treatments to water, physical, chemical
44 and/or biological in order to reduce the number of organics they contain [3]. The effluent can
45 then be either reused in the industrial cycle or safely released [4]. De facto, the outlet water
46 should contain far fewer, if any, API or IP. However, it may contain new unknowns, such as
47 degradation products.

48 In order to comply with European recommendations [5] but also to maintain its technological
49 lead, a pharmaceutical manufacturer needs to continuously improve every step of its on-site
50 wastewater treatment infrastructure. To this end, analytical sciences must provide fast and
51 reliable results, by monitoring existing infrastructures or evaluating new treatment processes.
52 Focusing on a list of targets, usually consisting of API, IP and suspected impurities, the
53 industrial objectives are then multiple:

- 54 1) In order to tune the treatment, it is crucial to unambiguously identify the molecules
55 entering the treatment step.
- 56 2) The treatment step efficiency has to be evaluated in a timely manner, especially if
57 upgrades are performed. For that purpose, removal efficiency of targeted products
58 must be assessed.

59 3) In order to control the effluent and its fate, quantification of the outgoing molecules
60 is essential. If regulated molecules are of concern, this can trigger an alert, while
61 information on unregulated molecules can help industrial operators to prepare for
62 future regulatory changes.

63 4) Finally, with a view to continuous improvement, it is interesting to obtain informative
64 data on unknown molecules in outlet water, these molecules being either compounds
65 that have passed directly through the treatment or those produced by the treatment
66 itself (emerging substances).

67 The current analytical strategy for effluent monitoring is based on a set of specific LC-MS
68 methods. The need to use multiple methods increases the time required to rule on the
69 compliance of a given effluent and ultimately places the responsibility on the experience of
70 the analyst.. LC-triple quadrupole with selected reaction monitoring (SRM) is currently the key
71 technique for quantification analysis [6,7]. On the other hand, high resolution mass
72 spectrometry (HRMS) with time of flight (ToF) analyzers can help to distinguish the numerous
73 closely related compounds generated during pharmaceutical production. TOF analyzers
74 operate in full scan mode, and thus detect a very large number of compounds, enabling
75 retrospective interpretation of the data if new targets are suspected. Furthermore, HRMS can
76 help the screening of unknown transformation products that may derive from the water
77 treatment itself [8]. Beyond their primary identification purpose, recent QToF analyzers with
78 improved sensitivity were found to be well suited for quantification [9].

79 Increasing both the selectivity and the peak capacity is an absolute requirement to avoid
80 isobar co-elution and matrix effects in complex samples such as industrial wastewater. The
81 potential of comprehensive multidimensional separation techniques hyphenated to HRMS
82 was therefore explored for wastewater analysis. These techniques involved the combination
83 of either two GC dimensions (GC x GC) [10,11], liquid chromatography with ion mobility (LC x
84 IM) [12], or two LC dimensions (LC x LC), which was applied to the analysis of urban
85 wastewater [13] or industrial wastewater [14]. GC x GC is limited by the need for derivatization
86 in the case of non-volatile compounds such as most pharmaceuticals. IM is potentially
87 attractive for the separation of isobars but its high correlation with mass spectrometry
88 reduces its interest. Unlike the preceding combinations, the high potential of LC x LC for
89 pharmaceuticals has often been reported [15–18]. Furthermore, LC x LC hyphenated to MS

90 equipped with electrospray ionization (LC x LC – ESI-MS) was found to be much more efficient
91 than 1D-LC – ESI-MS to reduce ion suppression in wastewater analysis [19].

92 In light of the above, LC x LC - HRMS is expected to take up the challenge of a comprehensive
93 characterization of wastewater from the pharmaceutical industry. In the present work, we
94 propose a comprehensive analytical strategy for unequivocal detection and quantification of
95 targeted pharmaceutical compounds in complex wastewater samples. This strategy is based
96 on an optimized RPLC x RPLC – HRMS technique associated to a decision tree for data
97 processing. To illustrate our overall strategy, a list of 27 targeted compounds under
98 continuous monitoring, subsequently referred to as "priority products", has been established.
99 Of these, 8 were of very high priority because of their proven toxicity above known
100 concentrations. An in-depth analysis of these compounds was carried out in the inlet and
101 outlet effluents of the pharmaceutical WWTP. The comparison of RPLC x RPLC-HRMS and
102 RPLC-HRMS analyses shows that the two-dimensional method clearly outperforms the one-
103 dimensional method for the characterization of pharmaceutical effluents. Initial attempts to
104 identify unknowns are also discussed.

106 **2. Material and methods**

107 **2.1. Chemical and sample preparation**

108 Acetonitrile (ACN), and methanol (MeOH) were LC-MS grade from Sigma-Aldrich (Steinheim,
109 Germany). Water was obtained from an Elga Purelab Classic UV purification system (Veolia
110 water STI, Le Plessis Robinson, France). Formic acid and ammonium acetate were obtained
111 from Sigma-Aldrich (Steinheim, Germany). All eluents were prepared in dedicated glassware
112 to avoid contamination.

113 Process effluent samples as well as the 27 priority standard products (12 intermediate
114 products and 15 active pharmaceutical ingredients) were provided by ORIL Industrie (Servier
115 laboratories, Bolbec, France). However, for confidentiality reasons, their molecular structures
116 are not shown. Molecular weights were between 100 and 800 Da.

117 Sample A contained 0.5 mg/L of each 27 priority standard products in water-MeOH (90:10,
118 v/v). Samples B and C contained the 8 high priority products in water-MeOH (90:10, v/v) with

119 various concentrations ranging from 1 to 1000 µg/L. Samples A, B and C were used for a 3-
120 point calibration.

121 Real samples were obtained from the inlet (sample I) and the outlet (sample O) effluents of a
122 wastewater process treatment. The treatment was biological. Filtered aliquots were stored at
123 -25 °C. After thawing and homogenization, reference molecule P24 was added at 0.1 mg/L and
124 the samples were filtered on 0.2 µm filter.

2.2. Instruments

125 The LCxLC-HRMS system consists in an Infinity 1290 2D-LC system hyphenated to an Agilent
126 6560 Ion-Mobility Quadrupole Time-of-Flight LC/MS equipped with a JetStream electrospray
127 ionization (ESI) source, both from Agilent Technologies (Waldbronn, Germany). The 2D-LC
128 instrument includes two high-pressure binary solvent delivery pumps, an autosampler with a
129 flow-through needle of 20 µL and an extension loop of 80 µL, two column ovens with a
130 maximum temperature of 100 °C. A 2-position/4-port duo-valve was used as interface
131 between the two dimensions, and was equipped with two 20 µL sampling loops. The dwell
132 volumes were 140 µL and 80 µL for the first and the second dimensions, respectively, while
133 the measured extra-column volumes were 22 µL and 8.5 µL for the first and the second
134 dimensions. The extra-column variance estimations were 12 µL² and 4.5 µL², respectively.
135 Flow splitting was applied, after the ¹D column to reduce the injection volume in ²D and after
136 the ²D column to reduce the flow-rate entering MS.

137
138 1D-LC experiments were carried out on the first dimension of the Infinity 1290 2D-LC system,
139 with a diode array detector (1290 DAD FS), equipped with a 0.6 µL flow-cell, set at 254 nm
140 with an acquisition rate of 40 Hz. Experiments in 1D-LC were performed without flow-splitting.

141 Instrument controlling and data acquisition were performed using OpenLab software for
142 LCxLC and MassHunter software for MS, both from Agilent Technologies (Waldbronn,
143 Germany). 2D-data were exported to house-made Matlab program (R2019a version) designed
144 to construct 2D-contour plots.

2.3. Chromatographic and MS conditions

145 Two columns were used in LC x LC, both from Waters Corporation (Milford, MA, USA): Acquity
146 CSH PFP (50 x 2.1 mm, 1.7 µm) in ¹D and an Acquity CSH 18 (30 x 2.1 mm, 1.7 µm) in ²D. The
147 final optimised LC x LC conditions are listed in Table 1. LC-UV experiments for injection
148

149 optimization were carried out in the same conditions as in ¹D, except that the flow-rate was
150 increased to 0.5 mL/min, while maintaining the same normalized gradient slope (i.e. 1 %). 1D-
151 LC-QToF experiments were performed with an Acquity CSH-C18 (150 x 2.1 mm, 1.7µm)
152 column at 0.7 mL/min (maximum pressure). All other conditions were the same as in ²D
153 (normalized gradient slope maintained at 0.7 %).

154 The ESI source settings were as follows: drying gas temperature, 300 °C; drying gas flow rate,
155 11 L/min; nebulizer pressure, 40 psig; sheath gas temperature, 350 °C; sheath gas flow rate,
156 11 L/min; fragmentor 185 V; capillary voltage, 3500 V; positive mode. Mass spectra data were
157 collected in full scan mode with a mass range of 100-1700 m/z (with 18920 FWHM resolution
158 at $m/z = 322$) and an acquisition rate of 14 spectra/s. No collision energy was employed during
159 the MS data collection. Accurate mass of the precursor ion $[M+H]^+$ was recorded with a mass
160 window of ± 20 ppm in LC x LC and of ± 40 ppm in 1D-LC.

161 2.4. Calculations

162 The gradient conditions were optimized according to the Linear Solvent Strength theory [20].
163 The following equations were used.

164 For a given dimension, the normalized gradient slope s was defined as:

$$165 \quad s = \frac{C_f - C_i}{t_g} \times t_0 \quad (1)$$

166 C_i and C_f are the initial and final eluent compositions, t_g the gradient time and t_0 the column
167 dead time.

168 The composition at elution, C_e , was given by:

$$169 \quad C_e = C_i + \frac{C_f - C_i}{t_g} \times (t_R - t_0 - t_D) \quad (2)$$

170 With t_R the compound retention time and t_D the instrument dwell time.

171 The peak capacity, n_C was given by:

$$172 \quad n_C = 1 + \frac{t_n - t_1}{1.7 \times w_{0.5}} \quad (3)$$

173 With t_n and t_1 the retention times of the most and least retained compound, and $w_{0.5}$ the peak
174 width measured at half-peak height.

175 A 2D-chromatogram is constituted of a succession of ²D fraction separations. The ¹D retention
176 time, ¹t_R of a given solute was assigned to the peak apex of its most intense peak while the ²D
177 absolute retention time, ²t_R was calculated from both ¹t_R and the sampling time, t_s via the
178 modulo operation:

$$^{2}t_R = \text{mod}(^{1}t_R, ^{1}t_s) \quad (4)$$

180 Due to a slight time difference between the first valve switch and the start of MS acquisition,
181 a relative retention time was considered in ²D (²t'_R). It was calculated from the retention time
182 of a non-retained reference product (²t_{reference}), added in known concentration to all analyzed
183 samples:

$$^{2}t'_R = ^{2}t_R - ^{2}t_{reference} \quad (5)$$

185 The compound P24 was chosen as the reference product.

187 3. Results and discussion

188 3.1. Selection of chromatographic conditions

189 A large set of comprehensive LC x LC conditions was recently screened with a large variety of
190 pharmaceuticals. 190 different LC x LC combinations were compared considering their peak
191 capacity and their degree of orthogonality [17]. It was concluded that the best results should
192 be obtained with highly efficient reversed phase columns in both dimensions operating at
193 neutral pH in ¹D and acidic pH in ²D with different organic modifiers in the two dimensions.
194 The combination of a PFP and a C18 stationary phases was found to be the most relevant for
195 those compounds. An earlier study also reported that combining a C18 and a PFP stationary
196 phase could be attractive for pharmaceuticals in sewage water [13]. As a result, RPLC x RPLC
197 conditions with ammonium acetate 5 mM in ¹D and formic acid 0.1 % in ²D were considered
198 in the present study. An Acquity CSH PFP column was used at 30 °C in ¹D with methanol as
199 organic modifier. In order to benefit from fast efficient separations in ²D, a short Acquity CSH
200 C18 column was used at 80 °C with acetonitrile as organic modifier. Gradient conditions were
201 optimized in both dimensions so that the analysis time did not exceed one hour. That was
202 achieved according to an optimization strategy, based on a pareto-optimality approach which
203 was developed in our laboratory. This one aims at maximizing the peak capacity while

204 minimizing the dilution factor [21,22] for a given analysis time (here 60 min). The optimized
205 parameters were the flow-rates, the gradient time in ²D, the column dimensions, the sampling
206 rate and the split ratio between the first dimension and the valve. Resulting optimized
207 conditions are listed in Table 1.

208 Some compounds were expected to be present at very low concentrations in both the inlet
209 and outlet water. It was therefore important to set the highest possible injection volume in ¹D
210 to increase the sensitivity (decrease the dilution factor) without compromising the separation
211 quality due to excessive band broadening. The effluent samples being in pure water, on-
212 column focusing was expected to be important and in favor of large injected volumes in ¹D.
213 Increasing volumes (from 20 to 100 μ L) of the inlet sample (sample I) were injected in the ¹D
214 conditions (UV detection at 254 nm) in order to assess the maximum volume that could be
215 injected. For each injected volume, the peak capacity (Eq. 3) was calculated for three isolated
216 peaks well distributed in the first third of the separation. Fig. 1a shows the chromatograms
217 obtained by injecting 20 μ L (red) and 80 μ L (black), which represents 19 % and 77 % of the
218 column dead volume, respectively. The three considered peaks are indicated by arrows. As
219 can be observed the peak intensity increases for the three peaks. As expected, it is multiplied
220 by a factor of about four for the two most retained peaks. However, it is multiplied by a factor
221 of three only for the least retained one. The variation of the calculated peak capacity with the
222 injected volume is shown in Fig. 1b for the three peaks. While the least retained peak #1 ($C_e =$
223 8 %, Eq.2) clearly exhibits continuous decrease in peak capacity (down to less than 60% of the
224 initial value with 100 μ L injected), more retained peaks #2 ($C_e = 19$ %, Eq.2) and #3 ($C_e = 39$ %, Eq.2) globally maintain their initial peak capacity with up to 80 μ L injected (loss of 13 % and 8 % with 80 μ L injected). With the objective of losing less than 30% of the peak capacity, an injected volume of 80 μ L was considered as a good trade-off between peak intensity and peak capacity.

229 Online RPLC x RPLC - HRMS separation was carried out using a synthetic mixture containing all
230 targeted analytes that could be released in the effluents, considered as priority products and
231 denoted here Sample A. The full spectrum of each analyte made it possible to select the most
232 abundant ion for identification and quantification process. That corresponded to the
233 molecular ion for 25 out of 27 priority products (see Table 2). Two compounds (P11 and P15)
234 were suspected to be fragmented in the ionization source, leading to the use of the mass-to-

235 charge ratio (m/z) of their major fragment. The extracted ion chromatogram (EIC) of each of
236 the 27 priority products (listed by increasing monoisotopic mass in Table 2) was processed
237 with a maximum mass deviation of 20 ppm. The sum of EICs is represented by a 2D contour
238 plot as shown in Fig. 2, where the Y-axis represents the first dimension separation and the X-
239 axis the second one. The color-scale (Z-axis) represents the peak intensity as MS counts. It has
240 to be noted that very intense peaks may appear larger depending on the chosen Z-scale focus.
241 Retention data are expected to be diagonalized in RPLC x RPLC [17]. In Fig. 2, a fairly large
242 repartition of the peaks across the separation space and around a diagonal can be observed.
243 A retention surface coverage of 0.71 was evaluated using a method previously described [23],
244 which is based on the ratio of the confidence area in which solutes are evenly distributed
245 throughout the separation area. This value of 0.71 is of the same order of magnitude as that
246 previously determined with a similar 2D-configuration applied to drug samples [17], thus
247 confirming its relevance for the present study. It is interesting to note in Fig. 2 that many
248 compounds that were not separated in 1D were separated in 2D and vice versa.

3.2. Creation of a reference table in RPLC x RPLC - HRMS for the priority products

251 The signal-to-noise ratio (S/N) must be above 3 to detect a compound. In LC x LC, the most
252 intense peak among those obtained from the 2D -separations of successive fractions, was
253 considered for this purpose. The noise level was evaluated at 200 counts (white zones in Fig.
254 2). As can be observed in Fig. 2, the number of relevant spots exceeds the number of priority
255 products, suggesting the presence of isobars (circled in black in Fig. 2). They may result either
256 from another product present in the sample or from the product itself. Out of the 27 EICs, 22
257 resulted in only one spot, so that 22 priority products could be unambiguously identified and
258 related to three features, their two retention times (in first and second dimensions) and their
259 m/z value. The standards of the other five priority products (P9, P14, P15, P21 and P24) for
260 which the EIC of sample A presented multiple spots were individually injected to definitely
261 ascertain their retention times (in both 1D and 2D). Their identification was also confirmed by
262 in-house spectrum database of pharmaceuticals and finally, the code name of each target
263 compound could be duly informed in Fig. 2.

264 During the course of method development, it appeared a slight time difference between the
1 first valve switch (start of the ²D analysis) and the start of MS acquisition, which could lead to
2 265 retention time errors in ²D. This time lag was in the range 1.8 s to 2.2 s and had, of course, no
3 266 impact on the determination of the retention time in ¹D. However, since the peaks widths in
4 267 ²D were of about 1 s only, an error of 2 s could lead to false identification. Consequently,
5 268 absolute retention times were considered in ¹D (¹t_R, peak apex of the most intense fraction)
6 269 while relative ones in ²D (²t'_R determined by Eq. 5). The compound P24, retained in ¹D but not
7 270 retained in ²D, was chosen as the reference compound. It was added in known concentration
8 271 (0.1 mg/L) to all samples to ensure its presence.
9 272

10 273 Table 2 lists the codes for the priority products, the retention times in both dimensions (¹t_R,
11 274 ²t'_R) obtained from the analysis shown in Fig. 2 and the mass data. For confidentiality reasons,
12 275 the m/z values cannot be given and only the considered extracted ions are reported. Such a
13 276 table was used to unambiguously identify the 27 priority products in any real samples analyzed
14 277 in a sequence.
15 278

16 279 **3.3. Establishing a mass window and a time window for the identification of priority 17 280 products**

18 281 As indicated above, the identification of priority products in a given sample is entirely based
19 282 on three data (m/z, ¹t_R, ²t'_R), as listed in Table 2. The reliability of these measurements is hence
20 283 of prime importance to ensure proper identification of the compounds. A variability study was
21 284 therefore carried out, to find out the repeatability of these measurements (run-to-run) as well
22 285 as their intermediate precision (day-to-day).
23 286

24 287 The variability of mass accuracy (expressed in ppm) was evaluated from the analysis of sample
25 288 A. The accurate masses of the 27 priority products were calculated from their chemical
26 289 formulas considering the isotopic mass distribution. For the 27 products, analyzed by the
27 290 proposed RPLC x RPLC – HRMS method, the mean mass error value was found to be 2.0 ppm
28 291 which is quite satisfactory according to both the instrument specifications (i.e. 1 ppm) and the
29 292 literature [24]. The maximum deviation was observed for the less retained compounds, P1
30 293 and P2, with a value of 4.7 and 8.6 ppm, respectively. However, we had to be aware of the
31 294 possible existence of matrix effects, interfering components or highly concentrated products,
32 all of which could shift the measured mass and lead to false negatives [25]. Mass extraction
33 windows of 10 and 20 ppm were therefore assessed. It was found, for some products such as

295 P19, which have a very good MS response and therefore a risk of detector saturation, that the
1 296 10 ppm window could be insufficient at high concentrations. In order to analyze highly
2 397 concentrated inlet samples, a mass extraction window of 20 ppm was definitely chosen.
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6 298 Both retention time repeatability (3 consecutive injections of sample A) and retention time
7 299 intermediate precision (3 inter-day injections of sample A) were assessed in both dimensions.
8
9 300 The resulting retention time standard deviations are shown in Fig. S1 for the 27 priority
10 301 products. For a given solute in ¹D, the considered retention time was that of the most intense
11 302 peak among those obtained from the two or three analyzed fractions. This peak could easily
12 303 be shifted by one sampling time (i.e. 0.4 min) between two analyses. Despite this, the standard
13 304 deviation of three consecutive measurements was always lower than 0.25 min (Fig. S1a) with
14 305 an average value over all compounds of 0.15 min. In light of these results and taking into
15 306 account the possible difference in time that could be up to one sampling time, a detection
16 307 window of ± 0.40 min was defined for run-to-run analyses in ¹D. As can be observed in Fig.
17 308 S1a, the retention times usually varied with a greater extent from day-to-day analyses, with
18 309 much larger standard deviations for five products. At a closer look, the related peaks
19 310 presented a large tailing, making probably the time at the peak apex more subject to
20 311 variations.
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34 312 In ²D (Fig. S1b), the retention time standard deviations were similar for run-to-run and for day-
35 313 to-day analyses with an average standard deviation of 0.07 s and 0.05 s respectively. For the
36 314 large majority of compounds, the standard deviations were lower than 0.05 s, which is
37 315 remarkable considering that the average peak width at half-peak height was ten times higher
38 316 (i.e. 0.5 s). This highlights the high degree of reliability of the second dimension separation.
39 317 For run-to-run analyses, the retention time window was set at twice the average standard
40 318 deviation (i.e. ± 0.15 s). The relative standard deviations (RSD) were informed in Table S1.
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46
47

48 319 Due to the large variability in ¹D retention times for some products from day to day (Fig. S1a),
49 320 it was decided to systematically include sample A in the analysis sequence in order to have a
50 321 more reliable comparison between the retention times of suspected compounds in real
51 322 samples with those of priority products unambiguously identified in sample A. In that respect,
52 323 a reference table similar to Table 2 (with updated retention times) was constructed for each
53 324 sequence. As said before, there was an identification ambiguity for 5 out of 27 compounds in
54 325 sample A. While not separated from their isobars in ¹D, they were well separated in ²D, making
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326 the corresponding retention times and time windows (listed in Table 2), the references for
327 definite identification.

328 **3.4. Quantitative analysis in RPLC x RPLC – HRMS**

329 Once a priority product has been unambiguously identified in the effluent, it should be
330 quantified to monitor its fate in the treatment plant.

331 **3.4.1. Reliability study**

332 LC x LC – HRMS is well designed for qualitative analysis as it often permits unambiguous
333 identification of suspected compounds. However, it is quite rare to use QToF analyzers for
334 quantitative analysis. Two main issues have indeed to be addressed in LC x LC - HRMS: (i) how
335 to determine the peak volume in LC x LC and (ii) the variability of MS-response.

336 In LC x LC, each peak in ¹D is displayed in several peaks in ²D. Summing the consecutive peak
337 areas was found to be the most reliable method in LC x LC – UV for quantitative analysis
338 [15,26,27]. This method was therefore used in the present study. The same approach as for
339 retention times was followed for the sum of peak areas in order to assess the variability of the
340 measures and hence the reliability of quantitative analysis in RPLC x RPLC - HRMS. This was
341 achieved with sample A from three non-consecutive runs in the same sequence (repeatability)
342 and over three days (intermediate precision). With an acquisition rate of 14 Hz, each peak
343 could be described by about 12 points. As illustrative example, the peaks obtained for three
344 consecutive fractions of P13 are shown in Fig. S2. Increasing the acquisition rate up to 20 Hz
345 improved the precision of peak area with 17 data points but decreased the number of
346 accumulated mass spectra per point and hence the sensitivity.

347 In terms of repeatability, the average relative standard deviation (RSD) on the sum of peak
348 areas was found to be 6.9 % with QToF detection while only 1.4 % with UV detection. Such
349 difference between these two detection modes was ascribed to both variations in ionization
350 efficiency between runs and/or insufficient description of MS-peaks as discussed above. In
351 terms of intermediate precision, the obtained results were unsatisfactory with an average RSD
352 value of 49 %, ranging from 17 % for P4 to an impressive 135 % for low-responding P11. This
353 is not surprising since ionization conditions are expected to significantly vary from day to day,
354 thereby requiring external calibration standards, which was not an easy task in view of the
355 large number of compounds that needed to be quantified. In the light of these results,

356 quantitative analysis was performed by injecting the calibration solutions and the samples to
357 be analyzed within the same sequence.

358 **3.4.2. General strategy**

359 Quantitative analysis of effluents was completed in about six hours with a RPLC x RPLC - HRMS
360 analysis sequence that included in order: a blank, sample A (highest concentrations), sample I
361 (inlet sample), sample B (intermediate concentrations), sample O (outlet sample) and sample
362 C (lowest concentrations). Among the 27 priority products to look for in effluent samples, 8
363 were considered as high priorities for quantitative analysis. Four of them were already
364 regulated (P6, P7, P9 and P16) while the other four were monitored to anticipate future
365 regulatory changes (P4, P10, P19 and P20). In sample C, the concentrations of the high priority
366 products were set at the maximum permitted value in the treatment plant outlet, except P10
367 and P19 for which the concentrations were set at a lower value. The maximum permitted
368 concentrations (listed in Table 3) were either those defined by regulatory authorities or our
369 own target concentrations designed to improve treatment efficiency.

370 Fig. 3 shows the decision flowchart to process the analytical data. It was designed firstly to
371 detect the presence or absence of priority products in the effluents and then to quantify them
372 if necessary. The first step (Fig. 3a) was intended to create the reference table (similar to Table
373 2) for the analysis sequence. ICH guidelines recommend signal-to-noise ratio values (S/N)
374 above 3 for detection and above 10 for quantification [28]. Given the concentrations in sample
375 A, all compounds were expected to be detected unless there was an instrumental problem. In
376 this case, the compound could no longer be considered in the current analysis.

377 In the second step (Fig. 3b), the processing data continued with samples I, B, O and C. Each
378 EIC was scrutinized. If S/N was higher than 3, a product was detected. If the retention data
379 matched those of the reference table, the priority product identity was confirmed.

380 The third and fourth steps (Figs. 3c and 3d) were related to the characterization of the outlet
381 sample, including the calculation of the removal efficiency for all detected priority products
382 and the quantitative analysis of the eight high priority products when they were detected in
383 sample O. The quantification was done with a 3-point calibration. It is important to underline
384 the assumption that matrix effects did not hinder this process.

385 The removal efficiency, RE , for a given product, can be defined as the amount of product
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2 386 treated compared to the starting amount:

$$387 \quad RE (\%) = \frac{m_{inlet} - m_{outlet}}{m_{inlet}} \times 100 \quad (6)$$

388 where m_{inlet} and m_{outlet} are the amounts of product in the inlet and outlet samples respectively.

389 RE was calculated either from the sum of peak areas if $S/N > 10$ for at least one of the two
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12 390 samples (inlet or outlet) or from the main peak intensity if $3 < S/N < 10$ for both samples. In
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14 391 this case, the noise was subtracted from the main peak intensity for a fairer calculation. In the
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16 392 case where the product was not detected in the outlet sample while detected with $S/N > 10$ in
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18 393 the inlet sample, the removal efficiency was considered to be 100 %.

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20
21 394 In case of a high priority product, its concentration in the outlet sample was compared to that
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23 395 in sample C in order to check the compliance of the treatment plant. Quantitative analysis was
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25 396 possible provided that a calibration curve could be constructed from the sum of peak areas,
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27 397 that means if S/N values were above 10 for the three calibration samples (A, B and C).

29 398 **3.4.3. Application to industrial process effluents**

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31 399 The general strategy described above was applied to process effluents from ORIL Industrie
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33 400 (Bolbec, France). They were collected before (sample I) and after (sample O) the WWTP. The
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35 401 samples were collected over two months. Monitoring the continuous process of an industrial
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37 402 WWTP is a difficult task, as the inlet effluent varies from one production cycle to another
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39 403 (usually over a period of a couple of months). The sampling process (inlet and outlet water)
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41 404 was performed on the same day while the residence time of the analyzed products in the
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43 405 WWTP was estimated to seven days. The collected outlet effluent was therefore not directly
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45 406 a consequence of the treatment of the collected inlet effluent, but of the inlet effluent
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47 407 entering the WWTP seven days before. Nevertheless, as most products are continuously
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49 408 produced, the two samples were expected to be representative of the issues the
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51 409 environmental engineers may encounter in monitoring the WWTP.

52
53 410 Figures 4a and 4b show the obtained 2D-contour plots (sum of 27 EICs) of the inlet and outlet
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55 411 samples respectively. It is obvious that the number of peaks detected in these 2D-plots far
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57 412 exceeds the number of priority products revealed in Fig. 2. This can be ascribed to the
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59 413 presence of numerous isobars in the effluents as exemplified at the bottom of the figure,

414 which displays the EICs (m/z of P15) for samples A, I and O. Two spots can be observed for
415 sample A (Fig. 4c). One corresponds to P15 (identified via individual injection) while the other
416 one (circled in black solid line) resulted from a fragmentation of P20 in the MS-source. These
417 two spots are also observed with samples I (Fig. 4d) and O (Fig. 4e), revealing the presence of
418 P15 and P20 in both effluents. The additional observed spots are likely to be due to reaction
419 impurities and/or product degradation. According to their retention times, some are common
420 to both inlet and outlet water (circled spots in the black dotted line) while others are specific
421 to the outlet effluent. These 2D-contour plots clearly point out the separation power of the
422 RPLC x RPLC - HRMS analysis, which effectively separated the isobars of P15 when they would
423 not have been separated in either 1D-RPLC methods. Furthermore, the reference retention
424 times in both dimensions were decisive for the unambiguous identification of P15 in both
425 effluents without any bias due to the presence of isobars.

426 Based on the decision-making flowchart shown in Fig. 3 with a limit of detection set at 600
427 MS-counts, 21 out of 27 priority products were detected in the inlet sample (Fig. 4a). The
428 number of products and their concentrations depends on the production scheme of the
429 pharmaceutical plant and hence may greatly vary from one production to the next. For
430 example, P19 was highly concentrated in sample I (Fig. 4a). In outlet sample (Fig. 4b), 20 out
431 of 21 products were detected in much smaller amounts as reflected by the corresponding spot
432 intensities. P11 and P22, detected in sample O, were not detected in sample I, possibly due to
433 the production timeline or to the residence time of the products in the treatment plant of
434 several days as discussed above.

435 In Fig. 5, the removal efficiency of the 20 priority products that were detected both in inlet
436 and outlet effluents, is plotted against the ratio of the signal of the most intense peak (inlet or
437 outlet sample) to the noise (S/N). The data dot is blue or orange depending on whether the
438 inlet or outlet sample provides the most intense signal. The orange dots were obviously not
439 expected and can in no way reflect removal efficiency. They can be explained by the fact, as
440 mentioned above, that although both samples were collected at the same time, there was
441 necessarily a time lag between a possible change in production and the end of the treatment.
442 Intermediate precision on removal efficiency was assessed from three analysis sequence
443 performed on three different days. Resulting relative standard deviations were found to be in
444 the range 0.2 to 6 %, which underlines the good reliability of the method. For 12 out of 20

445 products, the treatment process reduced the amount present in the effluent by 80 %. For 3
1 446 products (P13, P21 and P24), the removal efficiency was found to be lower than 50 %.

447 Quantitative analysis of high priority products in sample O was intended to reassure or alert
448 to their concentration level, and thus draw attention to a possible problem in the operation
449 of the WWTP. Table 3 summarizes the obtained quantitative results. The determination
450 coefficients (R^2) were all above 0.99 (with calibration lines forced to 0) despite the very low
451 concentrations in sample C, thereby providing a fair estimation of the concentrations in the
452 sample O, with an error estimated at about 7.0 %. For one product (P16), the concentration
453 was well above the maximum concentration (56 vs 1 $\mu\text{g/L}$), showing a lack of efficiency of the
454 WWTP for this product. In contrast, the concentrations of other high priority products were in
455 line with expectations.

3.5. Advantages of RPLC x RPLC-HRMS analysis over RPLC-HRMS analysis

456 Compared to one-dimensional separations, on-line LC x LC separations are often perceived as
457 being more difficult to implement, due to complex optimization and complex data processing.
458 We therefore attempted to establish an objective comparison between these two techniques,
459 both coupled with high-resolution mass spectrometry. A RPLC gradient method was
460 developed with the same gradient time (60 min), injection volumes as in ¹D and stationary
461 phase (Acquity CSH C18) as in ²D. Finally, in order to obtain the same MS response, we used
462 the same mobile phase (water-acetonitrile-formic acid) and the same column temperature
463 (80 °C) as in ²D. A 15 cm column was selected so that the peak capacity per time unit was
464 maximized in such RPLC-conditions [22,29]. Samples I and O are two examples of very complex
465 media in which a tremendous number of molecules are present, including pharmaceutical
466 products (reactants, active ingredients, intermediate products, impurities) but also products
467 derived from the WWTP process (enzyme substrate, degradation products). The same
468 analytical strategy as described above (Fig. 3) was applied in RPLC-HRMS to the inlet and outlet
469 effluents.

470
471 Base peak chromatograms (BPC) of the outlet sample in RPLC x RPLC and RPLC are shown in
472 Figs. 6a and 6b respectively. The main points of comparison for these two methods are given
473 in Table 4. The peak capacities were calculated according to Eq. 3. In RPLC x RPLC, the product
474 of peak capacities was corrected by taking into account both the under-sampling [30] and the
475 occupation of the retention space as delimited by the red and black dotted lines (86 % space

476 occupation) [23]. The calculated peak capacity was found to be 900 in RPLC x RPLC while 430
1 477 in RPLC. The higher separation power in RPLC x RPLC allowed the detection of 240 peaks while
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3
4 478 only 130 could be detected in RPLC.
5

6 479 The great advantage of RPLC x RPLC - HRMS is highlighted in Fig.6 by the mass spectra of the
7
8 480 two peaks shown on the chromatograms (labels 1 and 2). The first peak corresponds to P19
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10 481 while the second peak is an unknown compound. For both peaks, the mass spectra are much
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12 482 more complex in RPLC, revealing the presence of various masses in addition to the main one.
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14 483 In addition, the signal of the most intense mass for peak 2 is about six times higher in RPLC x
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16 484 RPLC.
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19 485 Peak identification was sometimes biased in RPLC for inlet and outlet samples as illustrated in
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21 486 Fig. S3a, showing overlaid EICs of P10 for samples A, C and I. As can be observed, both the
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23 487 time at the peak apex and the peak shape changed for this poorly retained compound
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25 488 depending on the sample. One explanation could be a change in the surface state of the
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27 489 stationary phase by the matrix when a large amount of sample I is injected (here 80 μ L). This
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29 490 hypothesis was confirmed by injecting smaller volumes of sample I (Fig. S4). In this case, the
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31 491 peaks became similar to those obtained by injecting samples A or sample C (both without
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33 492 matrix). In contrast, no retention shift was observed in RPLC x RPLC for sample I compared to
34
35 493 samples A and C (Fig. S3b), as the upstream separation greatly simplifies the sample injected
36
37 494 in the second dimension. Due to such a retention shift, identification of the corresponding
38
39 495 peaks in RPLC was made impossible unless a sufficiently large retention window was used (i.e.
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41 496 as large as several peak widths in this case). This did not suit our purpose.
42

43 497 A quantitative analysis problem, resulting from the presence of numerous isobars in the
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45 498 effluents, was also specifically highlighted in RPLC-HRMS. In addition to a higher effective peak
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47 499 capacity, the second dimension provides another retention mechanism, which increases the
48
49 500 chances of separating new isobars. Whereas all standard products could be unambiguously
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51 501 identified by their time windows in RPLC as in RPLC x RPLC, we often observed far fewer peaks
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53 502 in the EICs of inlet and outlet samples in RPLC than in RPLC x RPLC. As a result, in some cases,
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55 503 isobars were found to co-elute with the standard product in RPLC, thus distorting the
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57 504 quantitative results. An example is given in Figure S5 where many isobars of P1 were separated
58
59 505 in ¹D and not in ²D, suggesting that they were not separated in 1D-LC either, the conditions
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61 506 being the same as in ²D.
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507 Finally, by improving the separation of the priority products with the interfering matrix
1 508 components, RPLC x RPLC is expected to reduce matrix effects in the ionization source
2 509 compared to RPLC. In case of industrial wastewater coming from WWTP, it was reported that
3 510 matrix effects were more important for the inlet sample than for the outlet one and also in
4 511 the first part of the RPLC separation [31]. An in-depth comparison between RPLC and RPLC x
5 512 RPLC in terms of matrix effects was beyond the scope of this study. However, we compared
6 513 the mass error obtained for the priority products, which can increase with high concentrated
7 514 compounds present in the matrix [25]. In RPLC, the mass error attained up to 20 ppm with an
8 515 average value of 10 ppm (calculated for the detected priority products) while only 3.5 ppm in
9 516 RPLC x RPLC. This probably highlights more important matrix effects in RPLC and forced us to
10 517 consider a larger mass error in RPLC (i.e 40 ppm vs 20 ppm in RPLC x RPLC) for EICs. In addition
11 518 to an increase in the mass error, we observed an increase in noise in RPLC (300 vs 200 counts),
12 519 hence increasing the limits of both detection and quantification. This is probably the reason
13 520 why (i) less products were detected in sample O (i.e. 10 in RPLC vs 12 in RPLC x RPLC) and (ii)
14 521 their quantification was expected to be less accurate considering the R^2 value much lower
15 522 than 0.99 while higher in RPLC x RPLC.

31 523 All the above results allowed us to confirm that RPLC x RPLC far outperforms RPLC when it
32 524 comes to analysing such complex matrices as industrial effluents.

36 525 **3.6. Characterization of unknown compounds in process effluents**

37 526 The high potential of both RPLC x RPLC in term of separation power and HRMS in term of mass
38 527 accuracy permitted to gain valuable information on the various molecules that are either
39 528 transported through or produced during the WWTP process. While knowledge of the
40 529 molecules sent to the WWTP helps to reduce sources of pollution, knowledge of the molecules
41 530 released by the WWTP helps to monitor emerging contaminants, which can sometimes
42 531 represent a higher risk to the environment or health than their parent products [8].

43 532 The 2D-contour plots (BPC) obtained from the separations of the inlet and outlet samples are
44 533 shown in Figs. 7a and 7b respectively. In both separations, some spots seem to be highly
45 534 correlated, suggesting the presence of homologous series. The different correlation lines are
46 535 plotted on graphs that reconstruct the presence of these spots. Each line is labelled with a
47 536 number and a letter. The spots located on the same line differ from one spot to another by
48 537 the same mass value (44.026, 58.042 and 14.015 Da for lines 1, 2 and 3 respectively).

538 According to the literature, a mass of 44.026 Da might be related to ethoxylate [32], 58.042
1 Da to propylene oxide [32] and 14.015 Da to methylene. Surprisingly, similar differences in
2 539 Da to propylene oxide [32] and 14.015 Da to methylene. Surprisingly, similar differences in
3 mass were observed from one line to another as highlighted by coloured arrows in Fig. 7, with
4 540 mass were observed from one line to another as highlighted by coloured arrows in Fig. 7, with
5 colour depending on the mass value (14.015 Da in black, 0.036 Da in blue, 15.995 Da in purple).
6 541 colour depending on the mass value (14.015 Da in black, 0.036 Da in blue, 15.995 Da in purple).
7 542 As said above, 14.015 is most probably related to a methylene group, 0.036 could correspond
8 542 As said above, 14.015 is most probably related to a methylene group, 0.036 could correspond
9 to a difference in mass between O and [CH₃ + H], 15.995 is the monoisotopic mass of an oxygen
10 543 to a difference in mass between O and [CH₃ + H], 15.995 is the monoisotopic mass of an oxygen
11 544 atom. Finally, a last correlation can be noticed between lines 1a and 1c (in red). These are
12 544 atom. Finally, a last correlation can be noticed between lines 1a and 1c (in red). These are
13 545 compounds with the same m/z, probably isobars. Overall, these correlations and repetitive
14 545 compounds with the same m/z, probably isobars. Overall, these correlations and repetitive
15 546 mass differences most certainly reflect the presence of polymeric structures that have yet to
16 546 mass differences most certainly reflect the presence of polymeric structures that have yet to
17 547 be identified. Further studies are being carried out in our laboratory on this subject. However,
18 547 be identified. Further studies are being carried out in our laboratory on this subject. However,
19 548 it is interesting to note that the compounds in question are mostly different in the two
20 548 it is interesting to note that the compounds in question are mostly different in the two
21 549 samples.
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26 551 **4. Conclusions**

28 552 Effluent from wastewater treatment plants requires a specific internal policy to meet official
29 552 Effluent from wastewater treatment plants requires a specific internal policy to meet official
30 553 guidelines. The performance of RPLC × RPLC - HRMS was explored with respect to its potential
31 553 guidelines. The performance of RPLC × RPLC - HRMS was explored with respect to its potential
32 554 for the analysis of WWTP effluents. The analysis of the samples was carried out without
33 554 for the analysis of WWTP effluents. The analysis of the samples was carried out without
34 555 extraction and/or preconcentration, which limits the bias for quantification. HRMS allowed to
35 555 extraction and/or preconcentration, which limits the bias for quantification. HRMS allowed to
36 556 perform full-scan acquisition with detection limits in the ng/L range and high mass accuracy
37 556 perform full-scan acquisition with detection limits in the ng/L range and high mass accuracy
38 557 (mass errors lower than 5 ppm).
39

41 558 An excellent retention surface coverage was achieved for these samples with the PFP x C18
42 558 An excellent retention surface coverage was achieved for these samples with the PFP x C18
43 559 column combination, allowing, together with the MS data, the unambiguous identification of
44 559 column combination, allowing, together with the MS data, the unambiguous identification of
45 560 all suspected contaminants in the inlet and outlet samples of the treatment step.
46

47 561 In addition to the removal efficiency for the 27 priority products, the concentration in the
48 561 In addition to the removal efficiency for the 27 priority products, the concentration in the
49 562 outlet sample could be assessed for 8 high priority products and compared to target values.
50

52 563 The RPLC x RPLC method was compared to the RPLC method performed under optimized 1D-
53 563 The RPLC x RPLC method was compared to the RPLC method performed under optimized 1D-
54 564 LC conditions and was proved to give much more reliable results. The peak capacity was more
55 564 LC conditions and was proved to give much more reliable results. The peak capacity was more
56 565 than doubled between RPLC and RPLC x RPLC. Matrix effects, identification and quantification
57 565 than doubled between RPLC and RPLC x RPLC. Matrix effects, identification and quantification
58 566 problems were clearly highlighted in RPLC-HRMS unlike RPLC x RPLC.
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567 The proposed RPLC x RPLC - HRMS method should therefore make it possible to qualify both
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2 568 known and unknown products of a wastewater treatment plant and to accurately quantify the
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4 569 known compounds. Work is underway to complete the identification of unknown compounds
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6 570 whose retentions were found to be fully correlated in both dimensions.
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11 572 **References**

- 13 573 [1] Directive 2000/60/CE du parlement européen et du conseil du 23 octobre 2000
14 574 établissant un cadre pour une politique communautaire dans le domaine de l'eau n.d.:73.
15 575 [2] Directive 2010/75/UE du Parlement européen et du Conseil du 24 novembre 2010
16 576 relative aux émissions industrielles (prévention et réduction intégrées de la pollution)
17 577 n.d.:103.
18 578 [3] Mao G, Hu H, Liu X, Crittenden J, Huang N. A bibliometric analysis of industrial
19 579 wastewater treatments from 1998 to 2019. *Environmental Pollution* 2021;275:115785.
20 580 <https://doi.org/10.1016/j.envpol.2020.115785>.
21 581 [4] Guerra-Rodríguez S, Oulego P, Rodríguez E, Singh DN, Rodríguez-Chueca J. Towards the
22 582 Implementation of Circular Economy in the Wastewater Sector: Challenges and
23 583 Opportunities. *Water* 2020;12. <https://doi.org/10.3390/w12051431>.
24 584 [5] Consolidated Annual Activity Report 2019 (CAAR) - EEA annual report — European
25 585 Environment Agency n.d. [https://www.eea.europa.eu/publications/consolidated-](https://www.eea.europa.eu/publications/consolidated-annual-activity-report-2019)
26 586 [annual-activity-report-2019](https://www.eea.europa.eu/publications/consolidated-annual-activity-report-2019) (accessed May 6, 2021).
27 587 [6] Meng Y, Liu W, Liu X, Zhang J, Peng M, Zhang T. A review on analytical methods for
28 588 pharmaceutical and personal care products and their transformation products. *Journal*
29 589 *of Environmental Sciences* 2021;101:260–81. <https://doi.org/10.1016/j.jes.2020.08.025>.
30 590 [7] Chen X, Wu X, Luan T, Jiang R, Ouyang G. Sample preparation and instrumental methods
31 591 for illicit drugs in environmental and biological samples: A review. *Journal of*
32 592 *Chromatography A* 2021;1640:461961. <https://doi.org/10.1016/j.chroma.2021.461961>.
33 593 [8] Agüera A, Martínez Bueno MJ, Fernández-Alba AR. New trends in the analytical
34 594 determination of emerging contaminants and their transformation products in
35 595 environmental waters. *Environmental Science and Pollution Research* 2013;20:3496–
36 596 515. <https://doi.org/10.1007/s11356-013-1586-0>.
37 597 [9] Gómez M, Gómez-Ramos M, Gómez-Ramos M, Malato O, Mezcua M, Fernández-Alba A.
38 598 Rapid automated screening, identification and quantification of organic micro-
39 599 contaminants and their main transformation products in wastewater and river waters
40 600 using liquid chromatography-quadrupole-time-of-flight mass spectrometry with an
41 601 accurate-mass database. *Journal of Chromatography A* 2010;1217:7038–54.
42 602 <https://doi.org/10.1016/j.chroma.2010.08.070>.
43 603 [10] Blum KM, Gallampois C, Andersson PL, Renman G, Renman A, Haglund P. Comprehensive
44 604 assessment of organic contaminant removal from on-site sewage treatment facility
45 605 effluent by char-fortified filter beds. *Journal of Hazardous Materials* 2019;361:111–22.
46 606 <https://doi.org/10.1016/j.jhazmat.2018.08.009>.
47 607 [11] Lacina P, Mravcová L, Vávrová M. Application of comprehensive two-dimensional gas
48 608 chromatography with mass spectrometric detection for the analysis of selected drug
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 609 residues in wastewater and surface water. *Journal of Environmental Sciences*
1 610 2013;25:204–12. [https://doi.org/10.1016/S1001-0742\(12\)60006-0](https://doi.org/10.1016/S1001-0742(12)60006-0).
- 2 611 [12] Stephan S, Hippler J, Köhler T, Deeb AA, Schmidt TC, Schmitz OJ. Contaminant screening
3 612 of wastewater with HPLC-IM-qTOF-MS and LC+LC-IM-qTOF-MS using a CCS database.
4 613 *Analytical and Bioanalytical Chemistry* 2016;408:6545–55.
5 614 <https://doi.org/10.1007/s00216-016-9820-5>.
- 6 615 [13] Ouyang X, Leonards P, Legler J, van der Oost R, de Boer J, Lamoree M. Comprehensive
7 616 two-dimensional liquid chromatography coupled to high resolution time of flight mass
8 617 spectrometry for chemical characterization of sewage treatment plant effluents. *Journal*
9 618 *of Chromatography A* 2015;1380:139–45.
10 619 <https://doi.org/10.1016/j.chroma.2014.12.075>.
- 11 620 [14] Purschke K, Zoell C, Leonhardt J, Weber M, Schmidt TC. Identification of unknowns in
12 621 industrial wastewater using offline 2D chromatography and non-target screening.
13 622 *Science of The Total Environment* 2020;706:135835.
14 623 <https://doi.org/10.1016/j.scitotenv.2019.135835>.
- 15 624 [15] Iguiniz M, Corbel E, Roques N, Heinisch S. Quantitative aspects in on-line comprehensive
16 625 two-dimensional liquid chromatography for pharmaceutical applications. *Talanta*
17 626 2019;195:272–80. <https://doi.org/10.1016/j.talanta.2018.11.030>.
- 18 627 [16] Iguiniz M, Heinisch S. Two-dimensional liquid chromatography in pharmaceutical
19 628 analysis. Instrumental aspects, trends and applications. *Journal of Pharmaceutical and*
20 629 *Biomedical Analysis* 2017;145:482–503. <https://doi.org/10.1016/j.jpba.2017.07.009>.
- 21 630 [17] Iguiniz M, Rouvière F, Corbel E, Roques N, Heinisch S. Comprehensive two dimensional
22 631 liquid chromatography as analytical strategy for pharmaceutical analysis. *Journal of*
23 632 *Chromatography A* 2018;1536:195–204. <https://doi.org/10.1016/j.chroma.2017.08.070>.
- 24 633 [18] Brynish RD, Alex J, Subham D, Angel TA. Multiple Heart-Cutting Two-Dimensional Liquid
25 634 Chromatography: Recent Developments and Applications. *Current Analytical Chemistry*
26 635 2021;17:339–54.
- 27 636 [19] Groskreutz SR, Swenson MM, Secor LB, Stoll DR. Selective comprehensive
28 637 multidimensional separation for resolution enhancement in high performance liquid
29 638 chromatography. Part II: Applications. *Journal of Chromatography A* 2012;1228:41–50.
30 639 <https://doi.org/10.1016/j.chroma.2011.06.038>.
- 31 640 [20] Snyder LR, Dolan JW, Gant JR. Gradient elution in high-performance liquid
32 641 chromatography: I. Theoretical basis for reversed-phase systems. *Journal of*
33 642 *Chromatography A* 1979;165:3–30. [https://doi.org/10.1016/S0021-9673\(00\)85726-X](https://doi.org/10.1016/S0021-9673(00)85726-X).
- 34 643 [21] Sarrut M, D'Attoma A, Heinisch S. Optimization of conditions in on-line comprehensive
35 644 two-dimensional reversed phase liquid chromatography. Experimental comparison with
36 645 one-dimensional reversed phase liquid chromatography for the separation of peptides.
37 646 *Journal of Chromatography A* 2015;1421:48–59.
38 647 <https://doi.org/10.1016/j.chroma.2015.08.052>.
- 39 648 [22] Sarrut M, Rouvière F, Heinisch S. Theoretical and experimental comparison of one
40 649 dimensional versus on-line comprehensive two dimensional liquid chromatography for
41 650 optimized sub-hour separations of complex peptide samples. *Journal of Chromatography*
42 651 *A* 2017;1498:183–95. <https://doi.org/10.1016/j.chroma.2017.01.054>.
- 43 652 [23] D'Attoma A, Grivel C, Heinisch S. On-line comprehensive two-dimensional separations of
44 653 charged compounds using reversed-phase high performance liquid chromatography and
45 654 hydrophilic interaction chromatography. Part I: Orthogonality and practical peak capacity

- 655 considerations. *Journal of Chromatography A* 2012;1262:148–59.
656 <https://doi.org/10.1016/j.chroma.2012.09.028>.
- 657 [24] Brenton AG, Godfrey AR. Accurate mass measurement: Terminology and treatment of
658 data. *J Am Soc Mass Spectrom* 2010;21:1821–35.
659 <https://doi.org/10.1016/j.jasms.2010.06.006>.
- 660 [25] Calbiani F, Careri M, Elviri L, Mangia A, Zagnoni I. Matrix effects on accurate mass
661 measurements of low-molecular weight compounds using liquid chromatography-
662 electrospray-quadrupole time-of-flight mass spectrometry. *Journal of Mass
663 Spectrometry* 2006;41:289–94. <https://doi.org/10.1002/jms.984>.
- 664 [26] Pól J, Hohnová B, Jussila M, Hyötyläinen T. Comprehensive two-dimensional liquid
665 chromatography–time-of-flight mass spectrometry in the analysis of acidic compounds
666 in atmospheric aerosols. *Journal of Chromatography A* 2006;1130:64–71.
667 <https://doi.org/10.1016/j.chroma.2006.04.050>.
- 668 [27] Kivilompolo M, Hyötyläinen T. Comprehensive two-dimensional liquid chromatography
669 in analysis of Lamiaceae herbs: Characterisation and quantification of antioxidant
670 phenolic acids. *Journal of Chromatography A* 2007;1145:155–64.
671 <https://doi.org/10.1016/j.chroma.2007.01.090>.
- 672 [28] Tietje C, Brouder A, editors. International Conference On Harmonisation Of Technical
673 Requirements For Registration Of Pharmaceuticals For Human Use. Handbook of
674 Transnational Economic Governance Regimes, Brill | Nijhoff; 2010, p. 1041–53.
675 <https://doi.org/10.1163/ej.9789004163300.i-1081.897>.
- 676 [29] Chapel S, Rouvière F, Heinisch S. A Theoretical and Practical Approach to Manage High
677 Peak Capacity and Low Dilution in On-line Comprehensive Reversed-Phase LC ×
678 Reversed-Phase LC: A Comparison with 1D-Reversed-Phase LC. *Chromatography Online*
679 2020. <https://www.chromatographyonline.com/view/theoretical-and-practical-approach-manage-high-peak-capacity-and-low-dilution-line-comprehensive-rev>
680 (accessed July 19, 2021).
- 681 [30] Stoll DR, Carr PW. Two-Dimensional Liquid Chromatography: A State of the Art Tutorial.
682 *Anal Chem* 2017;89:519–31. <https://doi.org/10.1021/acs.analchem.6b03506>.
- 683 [31] Rossmann J, Gurke R, Renner LD, Oertel R, Kirch W. Evaluation of the matrix effect of
684 different sample matrices for 33 pharmaceuticals by post-column infusion. *Journal of
685 Chromatography B* 2015;1000:84–94. <https://doi.org/10.1016/j.jchromb.2015.06.019>.
- 686 [32] Loos M, Singer H. Nontargeted homologue series extraction from hyphenated high
687 resolution mass spectrometry data. *Journal of Cheminformatics* 2017;9:12.
688 <https://doi.org/10.1186/s13321-017-0197-z>.
- 689
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695 Figure captions

696 Figure 1: Effect of the injection volume on the RPLC separation of an inlet sample of WWTP;
697 (a) Overlaid chromatograms with injection volumes of 20 μ L (red) and 80 μ L (black). (b) Peak
698 capacity loss as a function of injection volume for three different peaks (shown in the top
699 figure). Peak width measured at half peak height. Flow rate: 0.5 mL/min; gradient time: 18.69
700 min. UV-detection at 254 nm. Other conditions given in Table 1 (¹D conditions).

701 Figure 2: On-line RPLC x RPLC separation of the 27 priority standard products (sum of EICs).
702 Products are numbered close to their corresponding spots (P_n). Isobars are circled in black
703 and numbered (ISO-P_n). Experimental conditions are given in Table 1.

704 Figure 3: Decision making flowchart for qualitative and quantitative analysis of a given
705 suspected priority standard product in treatment plant samples: (a) Creation of the reference
706 table (purple dotted frame); (b) Detection of priority products in samples B, C, I and O (green
707 dotted frame); (c) Removal efficiency for priority products according to Eq.6 (red dotted
708 frame) and (d) quantitative analysis for high priority products (blue dotted frame). "I", "O" and
709 "C" stand for samples I, O and C respectively. See text for explanations.

710 Figure 4: On-line RPLC x RPLC separations of (a) sample I (sum of 27 EICs), (b) sample O (sum
711 of 27 EICs), (c) sample A (EIC P15), (d) sample I (EIC P15) and sample O (EIC P15). Product
712 numbers are located close to the corresponding spots. The spot circled in solid black line
713 corresponds to a fragment of P20. The spots circled in dotted black line are common to
714 samples I and O. Experimental conditions are given in Table 1.

715 Figure 5: Removal efficiencies against the most intense S/N for the priority products detected
716 in both inlet and outlet samples. RPLC x RPLC – HRMS analyses shown in Figure 5. Blue and
717 orange data dots correspond to products that were more concentrated in the inlet and outlet
718 samples respectively. The vertical dotted lines indicate the limits of detection (LoD) and of
719 quantification (LoQ).

720 Figure 6: Analysis of sample O (Base Peak Chromatogram) in (a) on-line RPLC x RPLC – HRMS
721 (2D and 3D-plots) and (b) RPLC-HRMS. The given mass spectra correspond to peaks 1 and 2 as
722 indicated on the chromatograms. Black and red dotted lines delimit the available separation
723 space and the occupied separation space respectively. Experimental conditions are given in
724 the experimental section.

725 Figure 7: Unknown compound correlations in (a) the inlet sample and (b) the outlet sample
 726 after an on-line RPLC x RPLC – HRMS separation. The arrows highlight the differences in mass
 727 (values shown with the same colour on the figure) between two spots. Experimental
 728 conditions are given in the experimental section.

729

730 **Tables**

731 Table 1: Experimental conditions in RPLC x RPLC.

	First dimension (¹ D)	Second dimension (² D)
Injection volume	10 µL (samples A and B) and 80 µL (samples C, I and O)	15 µL
Column parameters	Acquity CSH PFP (50 x 2.1 mm, 1.7 µm)	Acquity CSH C18 (30 x 2.1 mm, 1.7 µm)
Temperature	30 °C	80 °C
Flow rate	0.15 mL/min	1.8 mL/min
Mobile phase A	¹ A: water-ammonium acetate (5 mM)	² A: water-formic acid (0.1 %)
Mobile phase B	¹ B: MeOH	² B: acetonitrile-formic acid (0.1 %)
Gradient conditions	1-90 % ¹ B in 61.41 min, 90-1 % ¹ B in 0.8 min, 1 % ¹ B from 62.2 to 66 min	1-86 % ² B in 0.24 min, 86-1 % ² B in 0.04 min, 1 % ² B from 0.28 to 0.4 min
Split ratio	1:3 interface:waste	1:2 MS:waste
Sampling time	0.4 min	

732

733 Table 2: Main characteristics, reference retention times and retention time windows for the
 734 27 priority products for the RPLC x RPLC separation shown in Fig. 2. ${}^2t'_R$ is the relative retention
 735 time in 2D (calculated according to Eqs. 4 and 5). High priority products are indicated by an
 736 asterisk (*). These retention times are used as reference for the identification of the 27
 737 products in any sample A. They are further updated for each analysis sequence. The conditions
 738 are given in Table 1.

Product number	Function (pKa)	Extracted ion	1t_R (min)	${}^2t'_R$ (s)	1t_R window (min)	${}^2t'_R$ window (s)	
					Run-to-run	Run-to-run	Day-to-day
P1	Basic	$[M_1+H]^+$	6.10	0.48	± 0.4	± 0.15	-
P2	Basic	$[M_2+H]^+$	8.50	0.24	± 0.4	± 0.15	-
P3	Acidic	$[M_3+H]^+$	3.76	4.02	± 0.4	± 0.15	-
P4*	Basic	$[M_4+H]^+$	14.57	4.74	± 0.4	± 0.15	-
P5	Basic	$[M_5+H]^+$	31.79	5.82	± 0.4	± 0.15	-
P6*	Neutral	$[M_6+H]^+$	15.05	9.60	± 0.4	± 0.15	-
P7*	Acidic/Basic	$[M_7+H]^+$	4.59	5.52	± 0.4	± 0.15	-
P8	Basic	$[M_8+H]^+$	35.87	10.50	± 0.4	± 0.15	-
P9*	Basic (9.0)	$[M_9+H]^+$	22.55	3.48	± 0.4	± 0.15	± 0.15
P10*	Basic (4.5/9.2)	$[M_{10}+H]^+$	22.15	3.60	± 0.4	± 0.15	-
P11	Basic	$[M_{11}-C_4H_{10}O_3P]^+$	30.59	6.06	± 0.4	± 0.15	-
P12	Basic	$[M_{12}+H]^+$	35.79	5.64	± 0.4	± 0.15	-
P13	Acidic/Basic (14.1/9.8)	$[M_{13}+H]^+$	37.81	6.78	± 0.4	± 0.15	-
P14	Basic (7.0)	$[M_{14}+H]^+$	39.78	4.98	± 0.4	± 0.15	± 0.15
P15	Basic	$[M_{15}-NH_2]^+$	31.79	6.06	± 0.4	± 0.15	± 0.15
P16*	Neutral	$[M_{16}+H]^+$	33.46	9.78	± 0.4	± 0.15	-
P17	Acidic/Basic (4.1/1.4)	$[M_{17}+H]^+$	19.09	11.82	± 0.4	± 0.15	-
P18	Acidic (8.9)	$[M_{18}+H]^+$	25.86	9.96	± 0.4	± 0.15	-
P19*	Basic (3.8/5.5)	$[M_{19}+H]^+$	21.03	8.10	± 0.4	± 0.15	-
P20*	Acidic/Basic (4.2/8.1)	$[M_{20}+H]^+$	32.22	7.62	± 0.4	± 0.15	-
P21	Basic (9.4)	$[M_{21}+H]^+$	42.60	6.48	± 0.4	± 0.15	± 0.15
P22	Acidic/Basic (14.3/7.5)	$[M_{22}+H]^+$	51.89	11.52	± 0.4	± 0.15	-
P23	Acidic (4.3)	$[M_{23}+H]^+$	33.91	12.90	± 0.4	± 0.15	-
P24 ⁽¹⁾	Basic	$[M_{24}+H]^+$	27.31	0.00	± 0.4	± 0.15	± 0.15
P25	Acidic (8.5)	$[M_{25}+H]^+$	35.41	7.26	± 0.4	± 0.15	-
P26	Acidic (9.5)	$[M_{26}+H]^+$	28.62	7.32	± 0.4	± 0.15	-
P27	Basic (6.6)	$[M_{27}+H]^+$	42.20	6.18	± 0.4	± 0.15	-

59 739 ⁽¹⁾Reference product

740 Table 3: Quantitative results for the high priority products in the outlet sample.

Pharmaceutical code	Allowable concentration (µg/L)	Calibration range (µg/L)	Slope (counts.min.ng ⁻¹)	Determination coefficient, R ²	Estimated concentration (µg/L)
P4	40	40 – 500	4298	0.990	< 40 ^b
P6	100	100 – 500	3047	0.999	6 ^a
P7	100	100 – 500	4083	0.990	31 ^a
P9	10	10 - 500	37505	0.995	< 10 ^b
P10	1000	500 - 1000	10090	1	818
P16	1	1 - 500	15764	1	56
P19	200	100 - 300	68961	0.991	114
P20	20	20 - 500	24241	0.990	11 ^a

741 a: extrapolated value

742 b: not detected in the outlet sample

743

744 Table 4 : Comparison of the results obtained for the analyses of the outlet sample in RPLC -

745 HRMS and RPLC x RPLC – HRMS (see Fig. 6)

	RPLC -HRMS	RPLC x RPLC -HRMS
Occupation of the retention space (γ)	-	86 %
Effective peak capacity	430	900
Number of peaks detected	130	240
Average mass error (27 priority products)	10.0 ppm	3.5 ppm
Analysis time	60 min	60 min

746

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

FIG1

[Click here to access/download;Figure;21-10-14_V2-Fig1-RPLCXRPLC\(WWTP\).pdf](#)

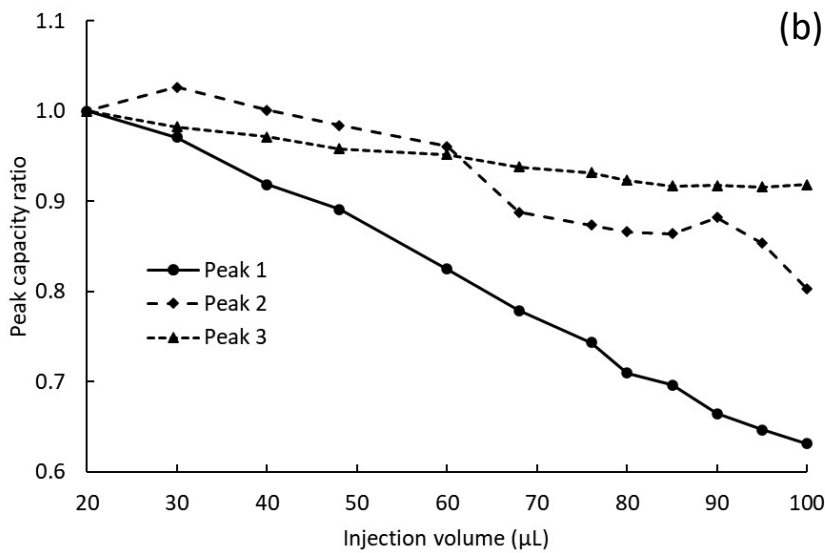
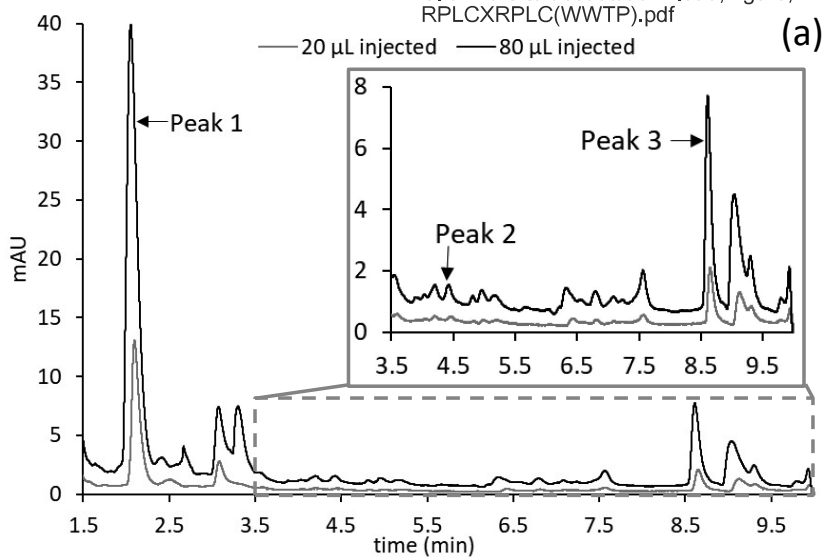
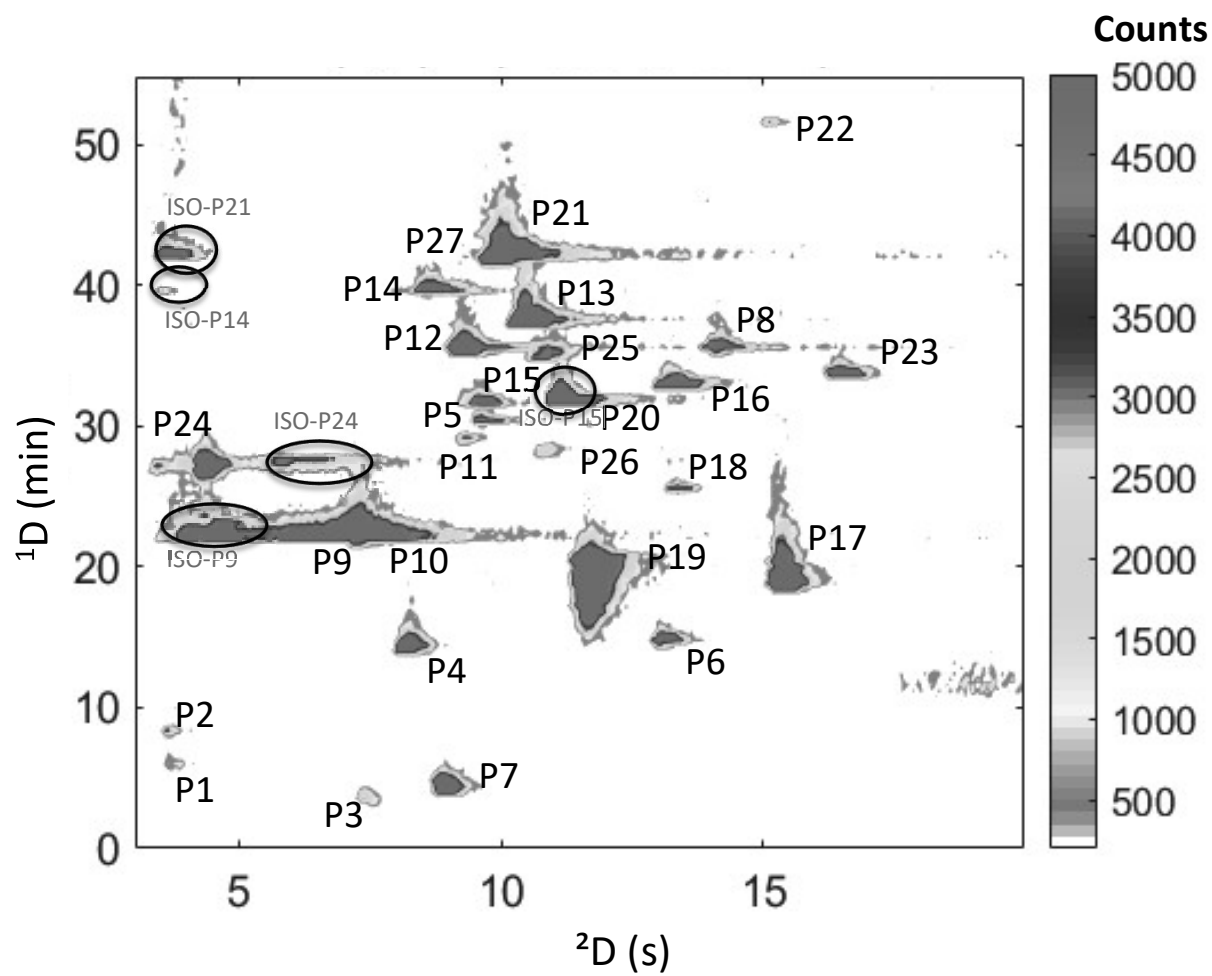


FIG2

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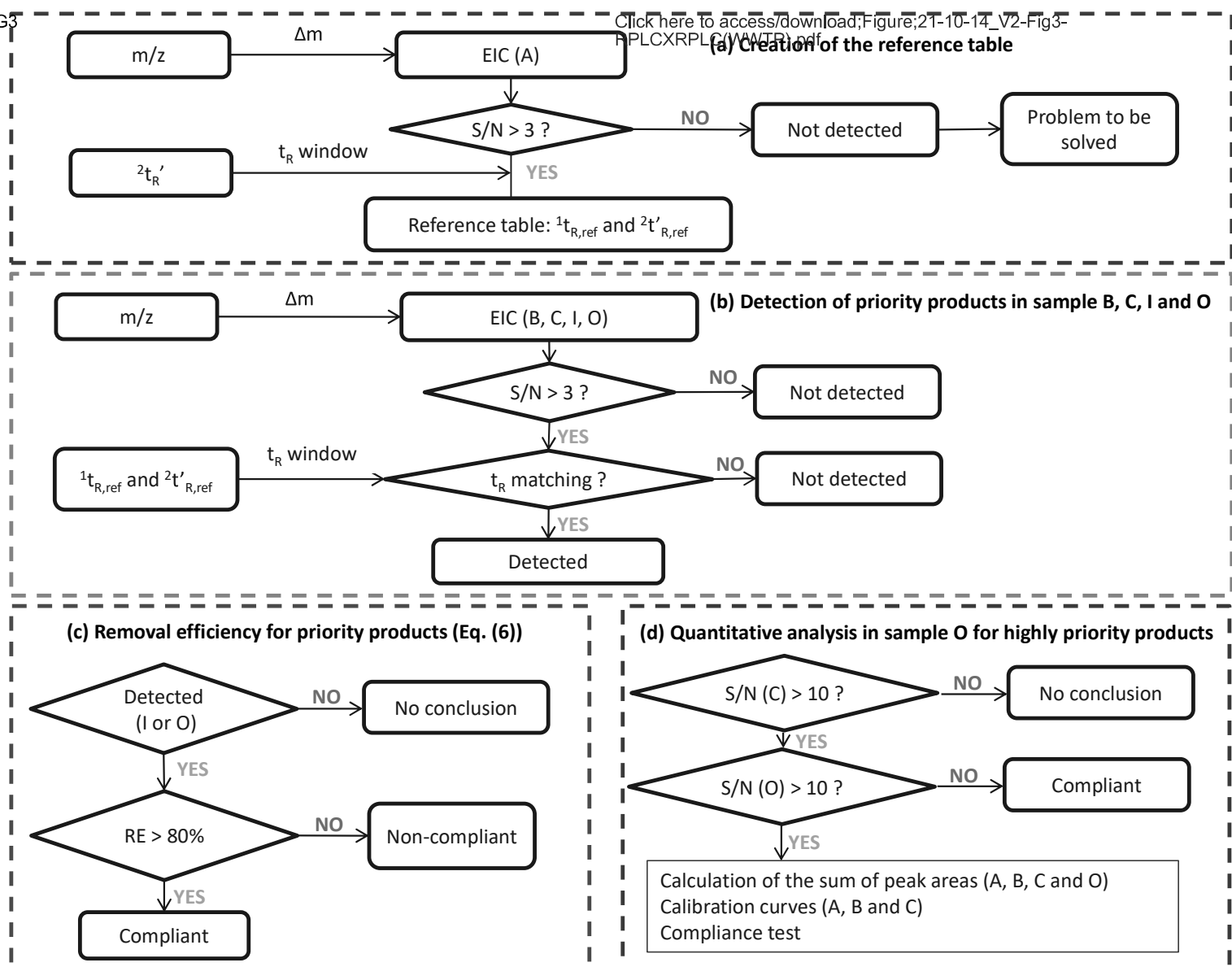
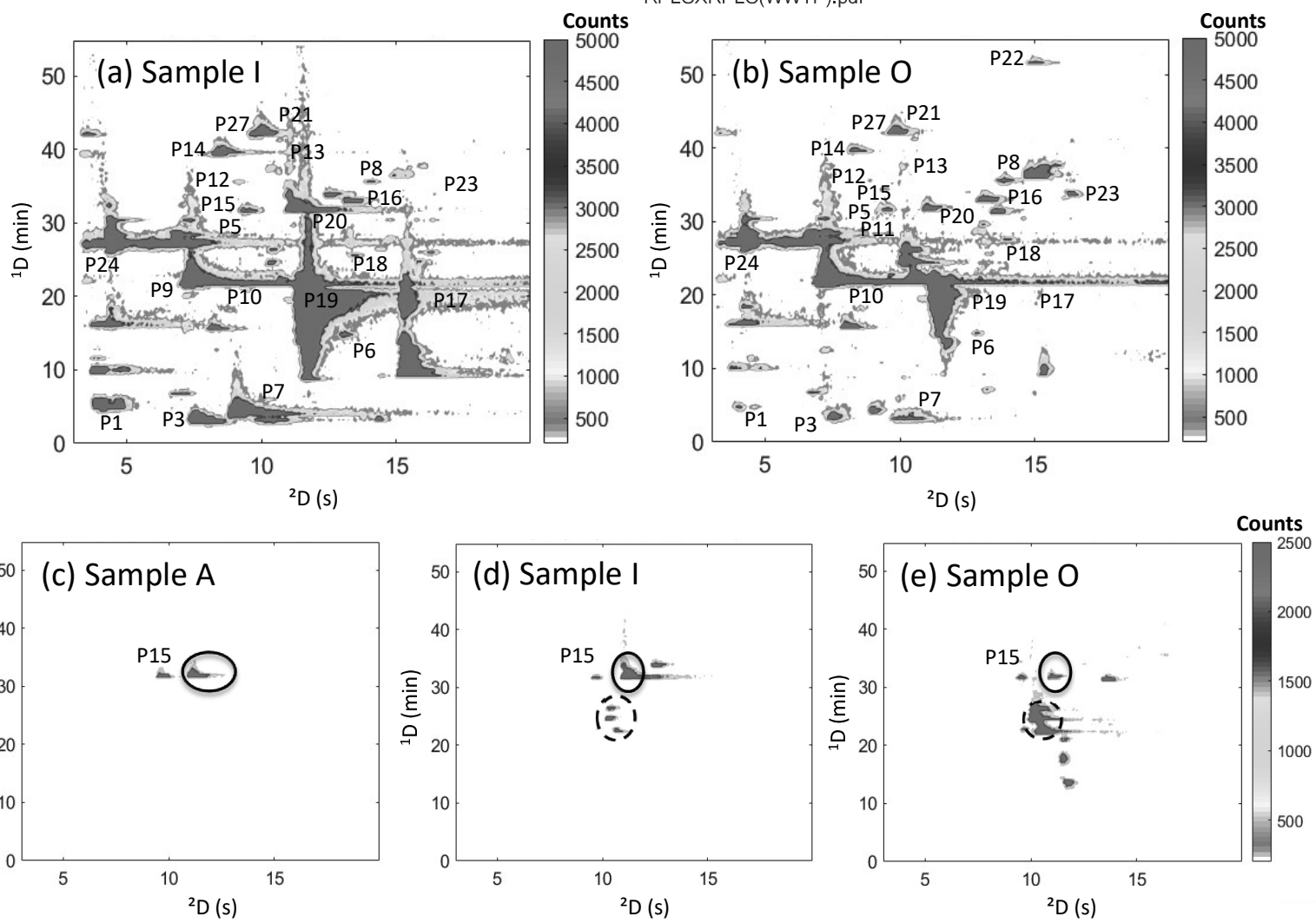


FIG4

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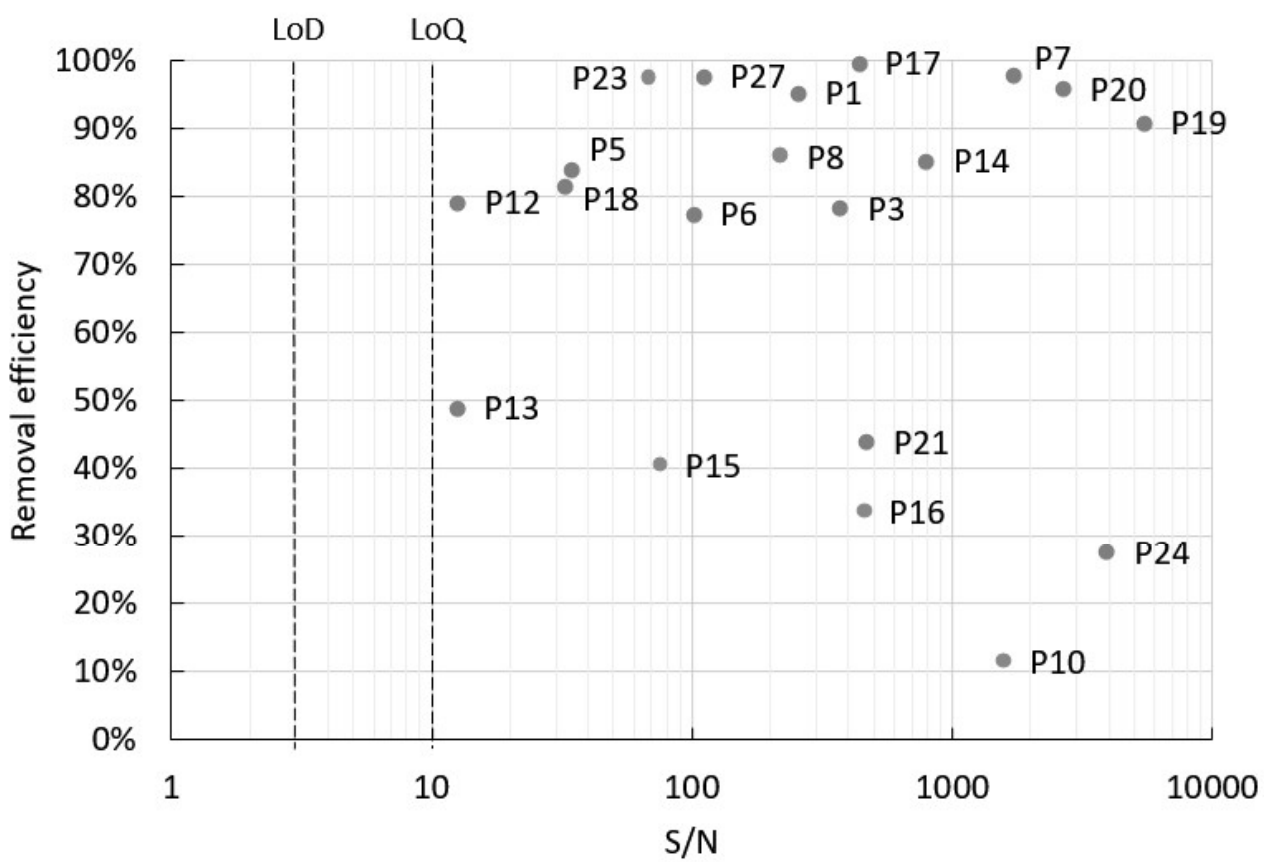
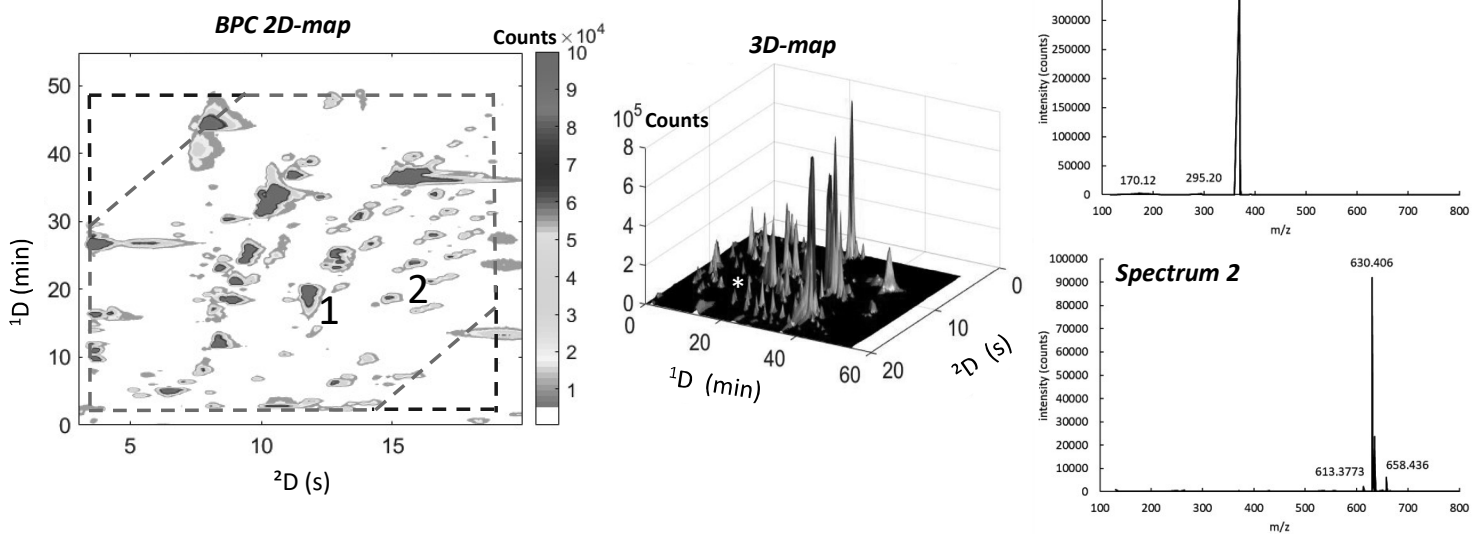


FIG 6

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(a) RPLC x RPLC - HRMS



(b) RPLC - HRMS

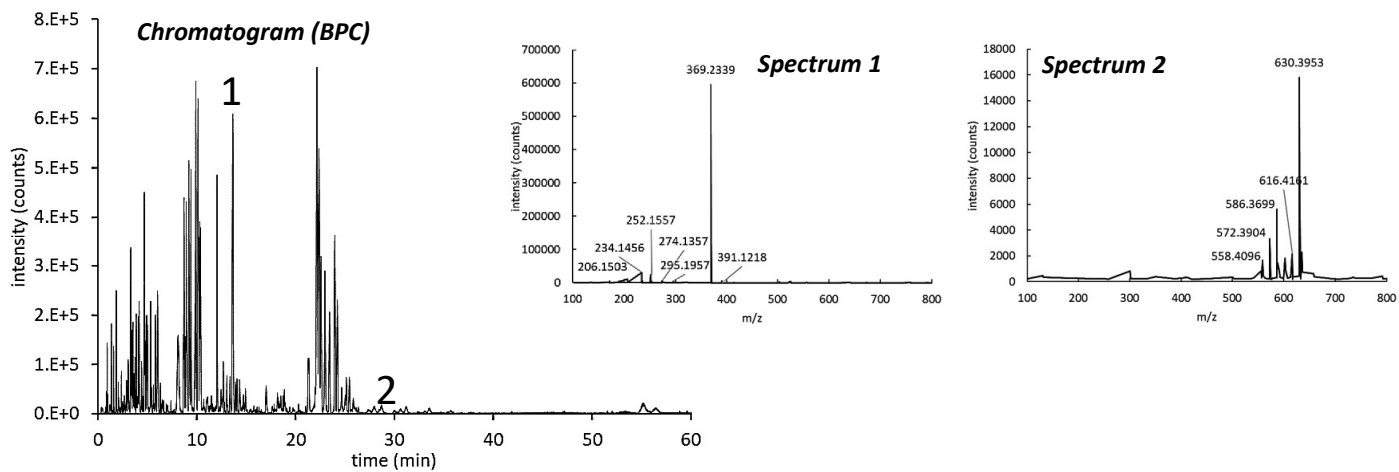
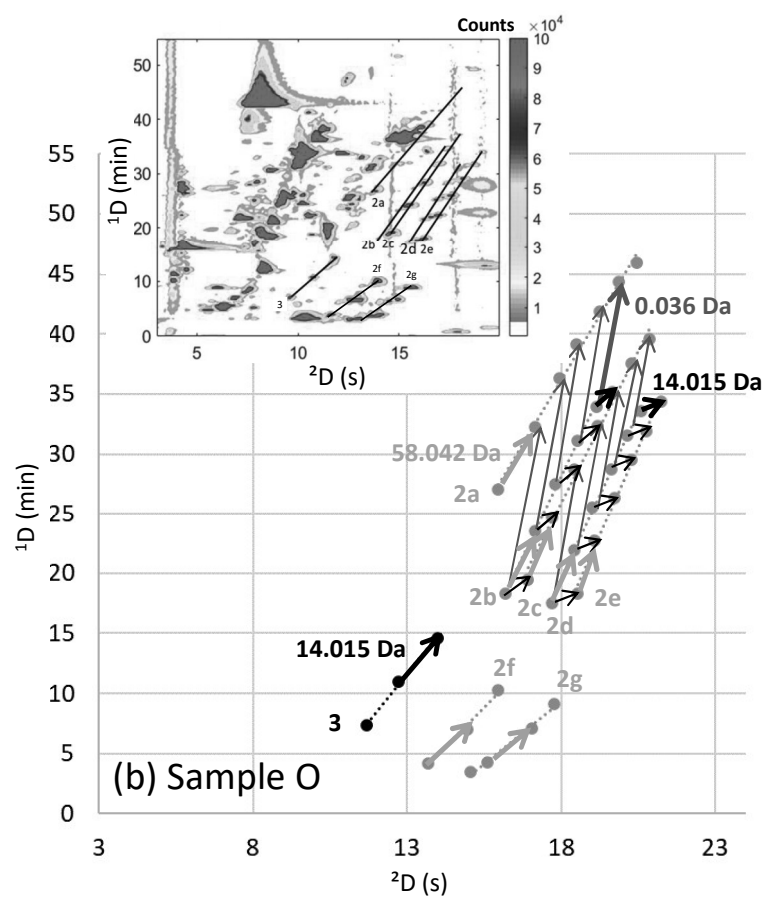
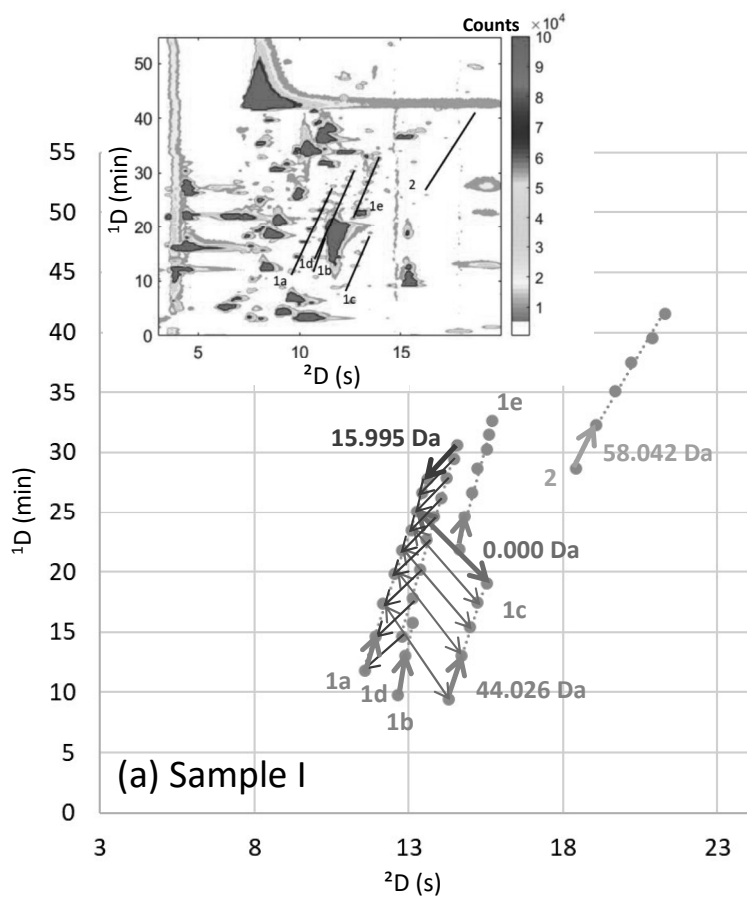


FIG7

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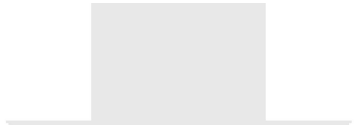
Fleur Marie Saint-Germain: Investigation; Methodology; Formal Analysis; Writing - original draft;

Karine Faure: Investigation; Writing - original draft.

Estelle Corbel : Conceptualization; Writing - review & editing

Jean-Michel Lerestif: Conceptualization; Resources ; Writing - review & editing

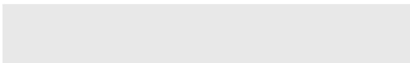
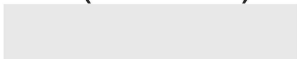
Sabine Heinisch: Conceptualization; Supervision; Formal Analysis; Funding acquisition; Writing - original draft; Writing - review & editing

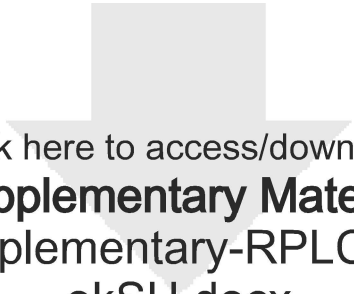


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