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1 **Off-line two-dimensional separation involving supercritical fluid chromatography for the**
2 **characterization of the wastewater from algae hydrothermal liquefaction**

3

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11

12 **Keywords**

13 Two-dimensional liquid chromatography;

14 supercritical fluid chromatography;

15 Off-line LC x SFC separation;

16 Microalgae;

17 Hydrothermal liquefaction

18

19

20 **Abstract**

21 An off-line multidimensional method involving liquid chromatography combined with supercritical
22 fluid chromatography was developed for the characterization of the wastewater of hydrothermal
23 liquefaction of microalgae *Chlorella sorokiniana*. The first dimension consisted of a phenyl hexyl
24 column operated in reversed-phase mode, whereas the second dimension was performed on a diol
25 stationary phase. Optimization of the kinetic parameters of the first and second dimensions were
26 performed, taking into account the fraction collection system. The beneficial effect of working at high
27 flow rate in both dimensions, as well as the need to work with short columns (50 mm) in the second
28 dimension was evidenced. Injection volume was also optimized in both dimensions. The first
29 dimension benefited from on-column focusing, while in the second dimension, untreated water-rich
30 fractions could be injected without peak deformation. The performances of offline LCxSFC were
31 compared to LC-HRMS, SFC-HRMS and LCxLC-HRMS for the analysis of the wastewater. Despite a long
32 analysis time of 3.3h, the off-line separation coupled to high-resolution mass spectrometry exhibited
33 a very large orthogonality with 75 % occupation rate of the separation space, reaching an effective
34 peak capacity of 1050. While other evaluated techniques were faster, one-dimensional techniques
35 failed to separate the numerous isomers while LCxLC exhibited lower orthogonality (45% occupation
36 rate).

37

38 1. Introduction

39 Microalgae conversion is a promising technology for biofuel production. Among various
40 thermochemical methods applied for converting algae biomass, hydrothermal liquefaction (HTL) is one
41 possible conversion process that has the advantage of using wet raw material and moderate
42 temperatures without the need for solvents. Therefore, it possesses a lower energy consumption than
43 pyrolysis [1]. Among cultivated algae for biofuel production, *Chlorellaceae* are very attractive species
44 considering their resistance and fast-growing strains [2]. During the HTL process, the content of algae
45 cells (lipids, proteins, and carbohydrates) is subjected to hydrolysis, deamination, and decarboxylation,
46 leading to the production of compounds of interest for biofuel production. Furthermore,
47 intermolecular reactions such as condensation and Maillard reaction lead to the formation of many
48 polar compounds. The conversion results in two phases: a bio-oil that undergoes catalytic upgrading
49 to produce paraffins, and an aqueous wastewater. This wastewater is rich in dissolved organic
50 compounds, especially in N-containing molecules. It can be valorized further as a nutrient source for
51 cultivating new algae [3-5], which reduces the economic footprint of algae conversion. However, the
52 utilization of HTL wastewater has a dual effect on algae. It may slow down the algae growth but
53 simultaneously increase the lipid content [6]. *Chlorella sorokiniana* showed the best adaptation to HTL
54 wastewater as a growth medium [7]. The characterization of the HTL wastewater is of major
55 importance to i) confirm the conversion pathways and optimize the HTL parameters, ii) select the algae
56 strains and optimize their growth conditions, and iii) identify which compounds serve as nutrients and
57 which compounds may have inhibitory effects on growth or the lipid yield.

58 The complex chemical composition of HTL wastewater has been demonstrated by detecting a large
59 number of compounds with GC x GC-MS: cyclic nitrogen compounds such as pyrroles, indoles,
60 pyrazines, and furans from amino acid condensation; phenols, ketones, and carboxylic acids from the
61 decomposition of polysaccharides, piperazines and pyrrolidones from Maillard reaction between
62 carbohydrates and basic amino acids [8-10]. While most of these molecules can be identified by using
63 the NIST library, only 30 molecules have been quantified so far using GC-FID. Besides, the polarity of
64 the organic acids or N-containing compounds and the aqueous nature of the wastewater may orientate
65 the analyst towards liquid chromatography analysis. Thus, Lazzari et al. [11, 12] used LC x LC-HRMS to
66 analyze the aqueous phase obtained from the pyrolysis of different biomasses, illustrating the power
67 of two-dimensional separation for this purpose. However, the RPLC x RPLC setup showed some
68 limitations in the orthogonality it could provide. Besides, to the best of our knowledge, the use of two-
69 dimensional (2D) LC techniques has not yet been reported for the aqueous phase from HTL.

70 In this work, we evaluated the potential of using supercritical fluid chromatography (SFC) in
71 combination with RPLC to analyze HTL wastewater from algae conversion. While RPLC in the first
72 dimension is fully adapted to the separation of organic compounds dissolved in water, SFC on polar
73 stationary phase shall provide an orthogonal separation mechanism, increasing the overall peak
74 capacity compared to the RPLC x RPLC method.

75 The combination of LC and SFC has been scarcely implemented despite its high orthogonality, mainly
76 because of the unavailability of commercial online instrumentation [13, 14]. Proof of concept online
77 LCxSFC has been experienced in our group on the aqueous phase of pyrolytic oil [15] but it requires
78 mobilizing simultaneously a LCxLC and a SFC instrument. On the other hand, off-line configuration can
79 easily be implemented using two distinct instruments and providing a large set of information in just a
80 few hours. The conditions used in both dimensions were optimized, using analysis time and effective

81 peak capacity as criteria. Careful considerations were brought on the subject of injecting LC fractions
82 into the SFC so that there would be no need to modify the fractions between the two dimensions. The
83 developed off-line LC x SFC-HRMS was then applied to the aqueous wastewater of the HTL conversion
84 of *Chlorella sorokiniana*.

85

86 **2. Material and methods**

87 **2.1 Chemicals and sample preparation**

88 Acetonitrile (ACN) of HPLC grade was purchased from Honeywell Riedel-de-Haën (Seelze, Germany),
89 formic acid (FA) and methanol (MeOH) (HPLC grade) were purchased from Merck (Darmstadt,
90 Germany), and ethanol (EtOH) of HPLC grade was obtained from Fisher Scientific (Loughborough, UK).
91 Water was obtained from an Elga water purification system (Veolia water STI, Le Plessis Robinson,
92 France). Pressurized liquid CO₂ (99.995%) was obtained from Air Liquide (Pierre Bénite, France).

93 *Chlorella sorokiniana* was grown under controlled conditions for 10 days and harvested by Dr. Delrue
94 at CEA Cadarache (CTREG/DPACA, France). It was converted into bio-oil using the HTL process by Dr.
95 Roubaud at CEA Grenoble (Biomass Technology Lab, LITEN, France). The aqueous phase was separated
96 from the organic phase, then filtered through a Chromaphil Xtra PES 0.2 µm membrane filter, and
97 stored at 5 °C.

98

99 **2.2 Instruments**

100 Agilent Infinity II 1260 LC system was equipped with a binary solvent delivery pump, an autosampler
101 with a 900 µL loop, a column oven compatible with temperatures up to 90 °C, a diode array detector
102 (DAD) with a 1 µL flow-cell, and a fraction collector with a capacity of 80 fractions. The maximum
103 allowable flow rate was 4 mL/min and the maximum allowable pressure was 400 bar. Data acquisition
104 was performed using Chemstation software (Agilent Technologies, Waldbronn, Germany). The extra-
105 column variance and dwell volume were determined experimentally and found to be 36 µL² and 2.26
106 mL, respectively.

107 Agilent Infinity II 1260 SFC system was equipped with a binary solvent delivery pump, an Infinity II
108 autosampler with a 100 µL loop, a column oven compatible with temperatures up to 85 °C, a DAD
109 detector with a 2 µL flow-cell withstanding a pressure up to 400 bar, a back pressure regulator (BPR),
110 and a make-up pump. The maximum allowable pressure was 400 bar. The dwell volume was measured
111 in quasi-liquid conditions at 600 µL. Feed injection conditions were as follows: overfeed volume 4 µL,
112 feed speed 1000 µL/min, and heptane as the feed solvent.

113 As the HRMS, an Agilent 6560 Ion-Mobility Quadrupole Time-of-Flight LC/MS equipped with a Jet
114 Stream electrospray ionization (ESI) source (Agilent Technologies) was used.

115 Data acquisition was performed using Chemstation software (Agilent Technologies) and treated using
116 MassHunter Qualitative 10.0. The 2D chromatograms were plotted using an in-house developed
117 Matlab program from S. Heinisch and F. Rouvière.

118

119 **2.3 Methods**

120 The LC method was performed using a XSelect CSH Phenyl-Hexyl column (150 mm x 4.6 mm, 5 μ m) on
121 the Agilent Infinity II 1260 LC. The flow rate was 2.8 mL/min and the temperature was 30 °C. Final
122 gradient conditions are displayed in Table 1. The injection volume was 500 μ L. A set of 80 fractions
123 were collected from the first dimension during the cycle time of 11.55 min, comprising gradient time,
124 dwell time and dead time. Each fraction was collected every 0.14 min, providing a fraction volume of
125 392 μ L, using the fraction collector on the Agilent Infinity II 1260 LC.

126 Four SFC columns purchased from Waters were screened: Torus 2-picolamine (2-PIC), 1-
127 aminoanthracene (1-AA), diol (DIOL), and diethylamine (DEA) (all with dimensions of 100 mm x 3 mm,
128 1.7 μ m), using a generic gradient elution (2% - 50% of methanol or ethanol as the co-solvent,
129 normalized gradient slope of 5%). The temperature, the BPR, and the wavelengths were set
130 respectively at 40 °C, 140 bar, 210 nm and 254 nm for all the experiments. The maximum flow rate
131 allowed for all four columns was determined to be 1.1 mL/min when using 50 % co-solvent (generating
132 around 400 bars). The final method was performed on a shorter DIOL column (50 mm x 3 mm, 1.7 μ m)
133 with a flow rate of 1.8 mL/min, as summarized in Table 1.

134 The 50-mm long SFC column was hyphenated to the MS using a “preBPR splitter with a sheath pump”
135 configuration [16]. Briefly, the end of the SFC column was connected to a make-up pump delivering 2
136 mL/min of MeOH + 0.1% FA using a T-union. Then, the resulting flow was split using a second T-union.
137 A low restriction tubing (170 μ m, 90 cm) led most of the flow to the BPR, while a high restriction tubing
138 (9 μ m, 100 cm) decreased the flow rate entering the ESI source. The stainless steel tubing kit was
139 provided by Agilent Technologies. In the final configuration, an additional pump was connected via a
140 third T-union, delivering MeOH + 0.1 % FA to the MS at 200 μ L/min.

141 The ESI source settings were the following: drying gas temperature, 300 °C; drying gas flow rate, 11
142 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 350 °C; sheath gas flow rate, 11 L/min;
143 fragmentor voltage 185 V; capillary voltage, 3500 V. Mass spectra data were collected in full scan mode
144 with a mass range of 100-1700 m/z (with 18920 FWHM resolution at $m/z = 322$) and an acquisition
145 rate of 10 spectra/s. No collision energy was employed during the MS data collection. The accurate
146 mass of the precursor ion was recorded with a mass tolerance of ± 2 ppm.

147

148 **3. Results and discussion**

149

150 The off-line configuration allowed the use of commercially available instruments and decoupled both
151 separation times and mobile phase flow rates between the two dimensions. This means that the
152 stationary and mobile phases of each dimension could be screened individually and that the geometry
153 of the columns can also be very different. Hence, the first dimension should be set on a column with a
154 large volume, facilitating the work of fraction collection systems while limiting the impact of the
155 external variances of the instrument on the chromatographic performances. The second dimension
156 geometry has to be much smaller so that the flow would be adjusted for the hyphenation with MS
157 while the overall separation time is kept reasonable.

3.1 Development of the first dimension (¹D) separation method

Based on a previous study [15], the phenyl hexyl stationary phase was selected according to its theoretical orthogonality with SFC stationary phases investigated here [17]. A scouting analysis of the sample was performed at 1% gradient steepness over the range 1%- 80% ACN, on a 150 mm x 4.6 mm column to facilitate fraction collection. It was revealed that the detected compounds eluted in a range of 1% to 30% ACN, indicating that the molecules present in the aqueous phase of HTL exhibit medium to high polarity. The ¹D operating conditions were optimized so that the overall peak capacity of the 2D separation was maximized. According to the linear solvent strength theory [18], the theoretical peak capacity in the first dimension n_{th} can be estimated by

$$n_{th} = 1 + \frac{\sqrt{N}}{4} \frac{1}{2.3 sS+1} \ln\left(\frac{2.3 sS+1}{2.3 sS} e^{2.3 S\Delta C} - \frac{1}{2.3 sS}\right) \quad (1)$$

Where N is the column efficiency, S the slope of the retention model, ΔC the elution composition range, and s the ¹D gradient steepness.

However, the first dimension undergoes sampling after the elution, which has deleterious effects on the resolution of peaks. It is generally conceived that a peak should be sampled 3 to 5 times. But in most online LC x LC separations, this recommendation is usually not followed. Indeed, in online configuration the sampling time equals the second dimension duty cycle. Reducing the sampling period would induce a reduction in the second dimension gradient time that limits the peak capacity of the second dimension. In off-line configuration, there is no such constraint on sampling time as the two dimensions are decoupled in time and the number of fractions is only limited by the instrumental constraints. Using Monte Carlo simulation of Gaussian peaks, Davis et al. evaluated the impact of undersampling on the peak capacity obtained after collection (n_s) as Equation 2 [19]

$$n_s = \frac{n_{th}}{\sqrt{1+0.21\left(\frac{t_s}{\sigma_t}\right)^2}} \quad (2)$$

With σ_t the average peak standard deviation in time units and t_s the sampling time, corresponding to the time required to collect a fraction in a vial.

When approximating that the peaks are homogeneously distributed along the gradient time t_g , the theoretical peak capacity n_{th} can be expressed as:

$$n_{th} = \frac{t_g}{4\sigma_t} \quad (3)$$

In a comprehensive mode, the collection has to be performed along the whole separation space, here during t_g . The number of fractions n_f required in a comprehensive mode is therefore:

$$n_f = \frac{t_g}{t_s} \quad (4)$$

Combining equations (2), (3), and (4), the effective peak capacity after sampling in off-line configuration can be expressed as a function of the theoretical peak capacity and the number of fractions (5)

$$n_s = \frac{n_{th}}{\sqrt{1+3.36\left(\frac{n_{th}}{n_f}\right)^2}} \quad (5)$$

192 By plotting the percentage of the remaining peak capacity $\frac{n_s}{n_{th}}$ vs. the ratio of the number of fractions
193 over the theoretical peak capacity $\frac{n_f}{n_{th}}$ (Figure 1a), it can be observed that a goal of 80% remaining peak
194 capacity requires that the number of fractions exceeds the theoretical peak capacity by a factor 2.5.
195 This objective is far from being reached, as off-line LC x SFC publications often report collection of only
196 40-50 fractions [20, 21], a number that is surprisingly low since fraction collection systems offer
197 between 80 and 120 vials, depending on the manufacturer. The undersampling effect is often not
198 taken into account for determining the effective peak capacity of the off-line 2D approach while it is
199 systematically done in the optimization of online LC x LC [22, 23].

200 The theoretical peak capacity on a typical HPLC column geometry of 4.6 x 150 mm; 5 μ m particle size,
201 can be estimated using equation 1 (Figure 1b). It relates both to the square root of efficiency and to
202 the gradient steepness, and so depends on flow rate and gradient time. Increasing the flow rate from
203 0.7 mL/min (optimal flow rate) to 2.8 mL/min (maximal flow rate) on the LC column led to an expected
204 loss of observed efficiency, from 10400 plates to 6600 plates, while for the same gradient time,
205 increasing the flow rate decreased the gradient steepness. Theoretical peak capacity for the two flow
206 rates was plotted as a function of gradient time (Figure 1b) and showed that using the 4.6 mm x 150
207 mm; 5 μ m column geometry, it was more beneficial to work at large flow rate. Theoretical peak
208 capacity was estimated between 50 and 140 for a gradient time between 3 min and 60 min (Figure 1b).
209 Keeping 80% effective peak capacity (Equation 5) would require between 125 and 700 fractions, that
210 would not only being difficult to handle technically, but also would result in an unreasonable ²D
211 analysis time. Figure 1b reports the effective peak capacity obtained after sampling when using a
212 collection system with 80 fractions. The method development in online LC x LC tends to suggest that
213 the ¹D gradient time should be quite long, usually 30 to 60 min, so that the peak broadening in the first
214 dimension would allow a longer sampling time corresponding to the ²D cycle time. On the contrary, in
215 off-line mode, the maximal peak capacity obtained after sampling is reached for short gradient times
216 (Figure 1b). In the present work, using a collection system of 80 fractions, a 10 min gradient performed
217 at maximal flow rate was sufficient to reach the maximal peak capacity of 41 (Figure 1b). Using another
218 collection system allowing 100 fractions would increase the peak capacity after undersampling to a
219 value of 50. With these selected gradient time and flow rate, the gradient range was adjusted to 1%-
220 40% ACN, so that according to the new normalized gradient slope, all the compounds eluted during
221 the gradient. The composition at elution of the last peak was evaluated to be 33 % ACN in these
222 conditions.

223 Thanks to the aqueous nature of the algal HTL waste and the use of an RPLC method in the first
224 dimension, on-column focusing allowed large injection volumes, thus increasing sensitivity without
225 compromising the chromatographic performances. Injection compression factor is related to the ratio
226 between two solute retention factors: k_s , obtained with the injection solvent, here 100 % water, and
227 k_e , obtained with the mobile phase at elution. [24]. With a sample in 100% water and a gradient
228 starting at 1 % ACN, the compression factor is expected to be over 1 and increasing with the solute
229 retention. Three distinct peaks distributed in the first half of the chromatogram were monitored using
230 UV detection at 254 nm (Figure 2a). The peaks eluting before Peak 1 were not sufficiently resolved to
231 measure their peak width. The peak capacity calculated for these three peaks when injecting increasing
232 volumes was compared to the peak capacity obtained when injecting 100 μ L, corresponding to 5.8%
233 of the column volume (Figure 2b). Peak broadening when injecting over 200 μ L resulted in a peak
234 capacity decrease as expected. While Peak 1 (9% ACN at elution) and Peak 3 (17% ACN at elution)

235 followed the same variation, with a loss of only 20% in peak capacity, Peak 2 (16% ACN at elution)
236 exhibited a much larger loss. An injection volume of 500 μL (30% of the column dead volume) was
237 selected, hence keeping more than 70% of the peak capacity for compounds eluting with over 9% ACN.
238 The broadening of compounds with a composition at elution lower than 9% ACN could not be
239 monitored using the ^1D LC-UV system because of co-elution, but the 2D chromatograms developed
240 thereafter showed their peak width remained similar to more retained compounds.

241 The variability of the first dimension separation was assessed in the final conditions, stated in Table 1,
242 by monitoring the peak width and retention time of 10 peaks spread over the gradient. A sequence of
243 5 injections was performed on three different days. The intra-day standard deviation of retention time
244 was below 0.01 min for all 10 peaks. The inter-day standard deviation of retention time was below
245 0.05 min for all peaks, reaching only 0.01 min for 80% of the compounds.

246 The average peak width at 4σ was measured at a value of $0.11 \text{ min} \pm 0.02 \text{ min}$, so the experimental
247 peak capacity before fraction collection was estimated using equation 3 and the experimental peak
248 width, at a value of 67. Using an 80-vials fraction collector, the ^1D effective peak capacity corrected by
249 the undersampling (Equation 5) was 37, quite close to the value of 41 calculated with theoretical
250 performances (Figure 1b). The difference may be explained by the instrumental variance generated by
251 the collector that was not taken into account in the theoretical calculation.

252 **3.2 Development of the ^2D SFC method**

253 The initial screening was performed on four 100 mm long columns with various stationary phases,
254 namely 2-PIC, 1-AA, DIOL and DEA, using methanol or ethanol as co-solvent B, a generic gradient (2-
255 50% B), and a normalized slope of 5% (Figure S1). The sample was initially diluted in 85:15 water/ACN
256 (v/v) to simulate the composition of a fraction collected after the first dimension. The columns based
257 on 1-aminoanthracene and diethylamine were found to be too retentive, leading to uneluted
258 compounds that were removed from the column after flushing it with 100% organic solvent. We
259 suppose that the non-eluting compounds are acidic, as an excessive retention on amino-based
260 stationary phases has already been reported for this class of compounds [25]. The chromatographic
261 profile obtained using 2-PIC and DIOL columns were similar, with a slightly higher retention on DIOL
262 column. Using a lower gradient steepness of 1% and an initial composition of 2 % organic solvent, the
263 separation space offered by the DIOL column was investigated for various co-solvent compositions
264 (Figure S2). Compounds were detected in the range of 2% - 29% using MeOH as the co-solvent and 2%
265 - 37% using EtOH as the co-solvent (Figure S2a). The effect of additives in the EtOH co-solvent such as
266 0.1% formic acid or 2% water was found to have minor impact on the quality of the separation (Figure
267 S2b). Therefore, ethanol was selected as the co-solvent.

268 The performances of the off-line 2D separation were simulated for various sets of LC and SFC
269 conditions. The LC flow rate was 2.8 mL/min. The ^1D gradient time was allowed to vary between 3 min
270 and 60 min. The calculations were performed for 60, 80 or 100 fractions. The ^2D column length was
271 either 50 mm or 100 mm. The ^2D flow-rate was fixed at the maximal allowable column pressure drop
272 (i.e. 400 bars) when reaching the maximal co-solvent content, corresponding to 1.8 mL/min on a 50
273 mm long column and 1.1 mL/min on a 100 mm long column. The ^2D gradient time was allowed to vary
274 between 1 min and 10 min. Other conditions were as reported in Table 1.

275 The theoretical peak capacity of the 2D off-line separation $n_{2\text{D},\text{theo}}$ and the overall analysis time $t_{2\text{D}}$ were
276 the selected criteria.

277 The theoretical peak capacity $n_{2D,theo}$ is the result of the product of the theoretical peak capacities from
278 each dimension, corrected by the undersampling after the ¹D collection (Equation 6). In a first
279 approach, the injection effects and the instrumental variances were not included in the calculation.

$$280 \quad n_{2D,theo} = n_s * n_{SFC} \quad (6)$$

281 The theoretical peak capacity of the LC separation, after fraction collection n_s , was calculated using
282 equation 5. The theoretical peak capacity of the SFC separation n_{SFC} is difficult to model as the density
283 increase that undergoes the mobile phase during the gradient may impact the efficiency. For example,
284 working at a flow rate of 1.8 mL/min on a 50 mm column, the column efficiency was measured at 7400
285 plates for 2% EtOH as co-solvent and at 4700 plates when the mobile phase contained 50% EtOH. For
286 a first estimation, Equation 1 was used for calculation, with an average efficiency. Better
287 approximation should require a deeper investigation on the modeling of kinetic performances of SFC
288 in gradient mode.

289 The duration of the offline 2D analysis t_{2D} is the result from the ¹D gradient time and the sum of the ²D
290 cycle times for analyzing the LC fractions in a comprehensive mode (Equation 7).

$$291 \quad t_{2D} = ({}^1t_{dwell} + {}^1t_0 + {}^1t_g) + n_f \times ({}^2t_{inj} + {}^2t_{dwell} + {}^2t_0 + {}^2t_g) \quad (7)$$

292 With ${}^1t_{dwell}$, 1t_0 and 1t_g the dwell time, the dead time and the gradient time of dimension i , respectively.
293 The injection duration in second dimension ${}^2t_{inj}$ is a fixed value depending only on the SFC injection
294 design. The Agilent SFC instrument incorporates a feed injector, aimed at pressurizing the sample
295 before introduction along the mobile phase flow [26]. The duration of the injection process ${}^2t_{inj}$ using
296 this instrument was estimated to be 0.56 min (average of 10 measurements).

297 The calculated performances of the offline LCxSFC separation are represented in Figure 3. As could be
298 expected, an increase in theoretical peak capacity may be achieved at the expense of longer analysis
299 time, whether increasing the first or the second dimension gradient time. However, the ¹D gradient
300 time had a limited impact on the performances with an optimum at 10 min whatever the set of
301 conditions, as observed previously, whereas the ²D gradient time (colors in Figure 3) had a major
302 impact on both peak capacity and analysis time. For example for 80 fractions, the optimized theoretical
303 peak capacity was 3359 for 159 min (2t_g 1 min), 3866 for 234 min (2t_g 2 min) and 4994 for 479 min (2t_g
304 5 min). Secondly, the number of fractions collected had a major impact on both performances criteria.
305 Finally, it appeared that the shortest column, i.e. 50 mm (circle symbols in Figure 3) provided better
306 results than the longest one for short 2t_g . This may due to the beneficial impact of shallow gradients
307 despite a loss in column efficiency. Nonetheless, the two trends were very similar for 2t_g equal 10
308 min. Using a longer SFC column should be advantageous when peak capacity higher than 5000 are
309 required, but at an expense of analysis time over 16 hours.

310 In the light of the above, the LC gradient time was set to 10 min and the number of fractions to the
311 maximum allowed by the available instrument. The second dimension was performed on a 50 mm long
312 column at a maximal flow rate of 1.8 mL/min. For an analysis time of 200 min, the gradient time of the
313 second dimension was set at 1.34 min (equation 7) considering an injection time of 0.56 min, a step to
314 return to initial composition in 0.12 min (corresponding to one column dead time) and a hold time at
315 initial composition for 0.33 min (corresponding to the instrument dwell time). In these conditions, the
316 theoretical peak capacity was estimated at 3587.

317

318 The SFC instrument was then coupled with HRMS using a commercial pre-BPR splitter with a make-up
319 pump interface that has been proven optimal for the ESI source [27]. Methanol + 0.1% FA was added
320 as the make-up solvent to further improve the MS signal. The solvent flow rate entering the MS source
321 can be calculated using the equations described by Guillarme et al. [16], adapted in our work to the
322 viscous binary mixture of ethanol and methanol. Simulations were performed for the initial mobile
323 phase composition (2% EtOH in CO₂) and for the final mobile phase composition (50% EtOH). When
324 the make-up flow was below 600 μL/min, the flow was directed entirely to the MS and the BPR system
325 experienced an issue with maintaining stable pressure. Increasing the make-up flow resulted in an
326 increase in the split ratio. At initial composition, the flow of solvent entering the MS slightly increased
327 with the make-up flow while the flow of CO₂ drastically decreased (Figure S1). Using a make-up flow
328 of 2000 μL/min resulted in an initial flow of solvent entering the MS at 760 μL/min. The overall flow,
329 including SFC eluent and make-up flow was splitted by 2.7, from 3800 μL/min to 1419 μL/min into the
330 MS. Unfortunately, the MS signal was not repeatable at the beginning of the gradient in these
331 conditions, despite using a caloratherm at 60 °C to avoid freezing the tubing. Using an additional pump
332 delivering 200 μL/min as proposed by Crepier [28] prevented solute precipitation by reducing the CO₂
333 content to 40%. At the final gradient composition, the viscosity of the eluent was much higher, leading
334 to a more important split of flow, estimated at a value of 4.6 (Figure S3). The flow of CO₂ entering the
335 MS tubing was low, calculated at 196 μL/min and no freezing or precipitation was noted. With a final
336 2000 μL/min make-up flow and a 200 μL/min additional flow, the amount of organic solvent entering
337 the MS source remains almost constant through the gradient (960 μL/min at initial gradient conditions
338 and 850 μL/min at final gradient conditions). Meanwhile, the composition of the organic solvent
339 entering the source evolved with the chromatographic gradient, with a ratio 4:96 EtOH/MeOH (v/v) at
340 initial composition and 53:47 EtOH/MeOH (v/v) at final conditions, which had no major influence on
341 the MS response.

342 The peaks were spread over the whole range of co-solvent (Figure S4) with very different profiles
343 according to detection modes. The molecules responding in positive ESI-MS mode eluted between 0.6
344 min and 2 min, whereas peaks detected in ESI-MS negative mode eluted mostly between 0.4 min to
345 1.2 min, and peaks eluting between 0.2 min and 0.4 min were only detected in UV. This observation
346 clearly illustrated the diversity of molecules and the need for three detection modes.

347

348 Because isolated peaks could not be distinguished using this short SFC separation, the maximal volume
349 that could be injected into the SFC had to be determined using extracted ion chromatograms (EIC). The
350 fractions collected from the first dimension were in a composition ranging from 99% to 60% water. As
351 the sample was not soluble in aprotic solvents, it was decided to inject the fractions without any
352 treatment. With the objective of increasing the sensitivity, increasing volumes of sample diluted in
353 water/ACN with either 80% or 50% water were injected in the ²D conditions. Two peaks were
354 monitored, one eluting in the early part of the gradient, at 8% co-solvent (*m/z* 184) and the other one
355 eluting at 18% co-solvent (*m/z* 151), after the water peak, which eluted at 15% co-solvent (1.01 min).
356 The retention time of the early eluting peak increased linearly with the injected water volume (Figures
357 4a and 4b). Nonetheless, the peak width was not affected by the content of water and the peak
358 capacity was found to be higher when the diluent contained more water (Figure 4e). This phenomenon
359 resulted from the higher retention of water molecules than of early eluting compounds on the polar
360 stationary phase of SFC [29]. As expected, the compounds eluting after water molecules were much

361 more affected by the content of water in the diluent. The content of 80% water in the diluent
362 (corresponding to the fractions eluting at the beginning of the ¹D gradient) did not produce any
363 retention shift of the highly retained compound. However, the increase in volume had a detrimental
364 effect on peak width, with peaks broadening when 10 μL were injected and peaks splitting when 20 μL
365 were injected (Figure 4c). With a lower water content in the diluent (50%, corresponding to the
366 composition at the end of the ¹D gradient), peaks were broadening in a more typical way, without any
367 splitting (Figure 4d). Therefore, to maintain 70% of the peak capacity in SFC, whatever the variation of
368 diluent of the fractions eluting from the ¹D gradient, it was decided to limit the injection volume in the
369 second dimension to 10 μL. Because the peak widths had to be measured on EIC, the experimental
370 peak capacity took into account the dispersion generated by the electrospray source. Under these
371 injection conditions, the average peak width at 4σ was measured 5 times for the peak eluting before
372 water, i.e., before 1.0 min at a value of 0.039 min and for 5 peaks eluting after water at a value of 0.050
373 min. Hence, the experimental peak capacity reached between dead time and 1.0 min was estimated
374 at 22, while the experimental peak capacity in the second part of the SFC chromatogram was estimated
375 at 16.

376

377 **3.3 LC x SFC-HRMS analysis of the HTL wastewater**

378 Applying the final conditions (Table 1), the wastewater from the HTL of *Chlorella sorokiniana* was
379 analysed by off-line LC x SFC-HRMS. The total time could be divided into a 10 min ¹D separation and 80
380 times 2.35 min (²D cycle time), yielding an overall analysis time of 3.3 h. The 80 resulting
381 chromatograms were compiled in an in-house developed Matlab program to plot a 2D chromatogram
382 (Figure 5). The final chromatographic profiles that were obtained in the range 100-1700 *m/z* in negative
383 ionization mode (Figure 5a) and positive ionization mode (Figure 5b) were quite dissimilar, suggesting
384 the interest in keeping both ionization modes. This was expected given the wide diversity of molecular
385 structures present in algae wastewater, with N-containing compounds and O-containing compounds
386 having very different ionization responses in the ESI source.

387 The peak broadening due to injection effects during transfer was confirmed by monitoring the peak
388 width (expressed here as FWHM) of 4 isolated peaks detected in negative mode, noted 1 to 4 in Figure
389 5a. Peaks eluting in the left part of the contour plot, i.e., with ²D retention time below 1.2 min (Peaks
390 1 and 2), exhibited low peak width whatever their position in ¹D, measured at 0.022 min and 0.023 min
391 for peak 1 and peak 2, respectively. Peaks eluting in the first part of the ¹D LC (¹t_R below 5 min, i.e.,
392 fraction 30) and with ²t_R over 1.2 min (bottom-right in Figure 5) were very broad due to the transfer of
393 diluent with high water content. For instance, peak 3 exhibited a width of 0.036 min. Finally, the
394 molecules retained in both LC and SFC (top-right in Figure 5) were well resolved, with peak 4 displaying
395 a width of 0.024 min. Overall, it can be concluded that when fractions contained high amounts of
396 water, only one-quarter of the elution space (bottom-right in Figure 5) exhibited broader peaks, for
397 which the impact on peak capacity was limited to around 30% of loss compared to the other peaks.

398

399 The resulting 2D plots show that the developed LC x SFC method has large orthogonality, with the
400 peaks spread over the entire separation space. The high orthogonality is caused by highly different
401 retention mechanisms, as LC retention is based on dispersive and π-π interactions while SFC retention
402 is based on dipole-dipole interaction. In negative mode, a downward diagonal was observed (Figure

403 5a), implying that the retention mechanisms are of opposing forces on the compounds responding in
404 this ionization mode. However, the molecules responding in positive ionization mode (Figure 5b) were
405 distributed evenly over the separation space. The orthogonality performances were calculated using
406 the asterisk method [30], taking into account all detection modes. The results in Table 2 show that
407 each dimension was well used, with peak spread over 90%. As already observed in Figure 5, there was
408 no direct correlation between the two dimensions, as demonstrated by the absence of clustering along
409 the upward diagonal. On the other hand, a certain degree of clustering could be detected along the
410 downward diagonal, but its extent seemed quite low, with a 75% peak spread around this vector.
411 According to the asterisk method, the overall occupation rate γ was 75%, while a convex hull method
412 [31], much simpler to implement, slightly overestimated this value to be 78%.

413 The predicted effective peak capacity was estimated (equation 8) as the theoretical peak capacity
414 corrected by *i*) the external variances due to the injection effects in LC, β_{LC} , and in SFC, β_{SFC} , both
415 values being set at 70%, and *ii*) the overall occupation rate γ . The predictive effective peak capacity
416 was calculated at 1318.

$$417 \quad n_{2D,pred.} = n_{2D,theo} * \beta_{LC} * \beta_{SFC} * \gamma \quad (8)$$

418 Taking into account experimental peak widths in both dimension, undersampling with 80 fractions,
419 and orthogonality, the experimental effective peak capacity of this method was estimated at around
420 1050 for an overall analysis time of 200 min (3.3 h). The discrepancy between prediction and
421 experimental value may come from the effect of external variances and the bias from LSS hypothesis
422 on SFC separation.

423 This experimental value was compared to one-dimensional techniques, either LC-HRMS or SFC-HRMS
424 or to the two-dimensional online LCxLC-HRMS, all in optimized conditions. Both one-dimensional
425 separations were carried out on 100 mm long column at a 1% gradient steepness to maximize peak
426 capacity while limiting dilution. The best LC separation was achieved in 15 min (Figure S5a) and
427 provided 122 in peak capacity whereas the best SFC separation was achieved in 30 min (Figure S5b)
428 and provided 250 as experimental effective peak capacity. Online LCxLC was considered in two
429 different configurations. The first one consisted in the use of fluorophenyl stationary phase in the first
430 dimension while the second configuration used phenylhexyl in the first dimension. Both configurations
431 used ACN and formic acid as ¹D mobile phase. The ²D column was a Cortecs C18 with MeOH and
432 ammonium acetate as 2D mobile phase. While these configurations were selected to maximize
433 orthogonality, there was still a large correlation of the retention behaviour in both dimensions (Figure
434 S6) as expected from RPLC x RPLC combinations. Peaks responding in ESI + occupied at best 45% of the
435 available separation space (calculated on phenylhexyl x C18 combination), very similar to the
436 occupation rate found by Lazzari et al. [11] for the analysis of aqueous phases using other LCxLC
437 combination. Nonetheless, the effective peak capacity generated by LCxLC reached 1190 in 30 min,
438 but required a specific instrumentation.

439 The developed LC x SFC-HRMS technique could be used for a non-target analysis to compare the
440 wastewater composition resulting from different HTL processes or from different algae strains.
441 However, this kind of study would require a large number of replicates to get statistically
442 representative results due to the versatility of plant demography, in which case a faster online strategy
443 could be preferred. On the other hand, a two-dimensional separation hyphenated to high-resolution
444 mass spectrometry is of interest for target analysis by reducing matrix effects and improving separation

445 of isomers. An off-line configuration offers a quick access, without dedicated instrumentation, to a
446 highly discriminative analysis. Once the position of the target is elucidated using a comprehensive
447 analysis, the separation can be restricted to elution zones of interest, and fraction analysis can be
448 completed *a posteriori* if necessary. The interest of the LC x SFC method over a one-dimensional
449 method can be illustrated through the monitoring of molecules whose content in the wastewater used
450 for algae cultivation is suspected to have an impact on either the algae's growth or lipid yield. For
451 example, Figure 6 shows the extracted chromatograms of ion m/z 247.1447. This ion corresponds to
452 the molecular ion of 3-benzyl-6-isopropyl-2,5-piperazinedione. Piperazinediones are generated from
453 the recombination of amino acids present in the algae during the hydrothermal liquefaction. They
454 represent a beneficial source of nitrogen for algae cultivation on wastewater. However, after reaching
455 the optimal growth, a prolonged N-starvation induces the production of starch and lipids for energy
456 storage in the cells. If the N-starvation is prolonged, then lipids accumulate while the starch content
457 decreases [32]. Consequently, the intake of piperazinediones should be strongly controlled during N-
458 starvation. The one-dimensional LC-HRMS analysis exhibited a cluster and two distinct peaks with a
459 signal-to-noise ratio above 10 (Figure 6a), while the SFC-HRMS analysis retrieved two main peaks in a
460 much noisier baseline (Figure 6b), that would not allow any quantitative analysis. The two-dimensional
461 LCxLC analysis (Figure 6c) exhibited 9 spots with a signal-to-noise ratio over 10, demonstrating two-
462 dimensional separation reduced matrix effects that strongly inhibited MS signal when only one
463 separation dimension was used. Nonetheless, a cluster was still present (around 10 min in the first
464 dimension) with a diagonalization suggesting that despite a larger peak capacity, the peaks with the
465 same m/z ratio were not fully resolved. The 2D LCxSFC chromatogram with its higher orthogonality
466 compared to LCxLC (Figure 6d) exhibited 14 spots with a signal-to-noise ratio over 10 at this specific
467 m/z ratio, offering a better characterization of these potential isomers or compounds with similar
468 fragments.

469

470 **Conclusion**

471 In this study, an off-line 2D RPLC x SFC-HRMS method was developed and proved to be a powerful
472 tool for the characterization of HTL wastewater of algae.

473 The wastewater was directly introduced into the first RPLC dimension without any sample treatment.
474 Injection volume was maximized up to 30% of the column dead volume. Repeatability and
475 intermediate precision of the ¹D separation were assessed. The theoretical estimation of fraction
476 collection highlighted the importance of the number of fractions while underlining the limitation in
477 peak capacity performances of an off-line approach. The ²D SFC separation was optimized in terms of
478 stationary phase, mobile phase, and hyphenation to HRMS. The comprehensive LC x SFC-HRMS
479 analysis of HTL wastewater from *Chlorella sorokiniana* illustrated the large orthogonality of the 2D
480 method, with a large occupation of the separation space. Using 80 fractions, the overall peak capacity
481 reached 1000.

482 The developed off-line LC x SFC-HRMS method could be used for non-target analysis but also to
483 facilitate target analysis thanks to the ability to decrease matrix effects and separate the numerous
484 isomers present in HTL wastewater.

485

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490 in HTL wastewater. The authors also thank Dr. Anne Roubaud and Lucie Matricon from CEA Grenoble
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495 References

- 496 1. D. López Barreiro, W. Prins, F. Ronsse, and W. Brillman, Hydrothermal liquefaction (HTL) of
497 microalgae for biofuel production: State of the art review and future prospects. *Biomass and*
498 *Bioenergy*, 2013. 53: 113-127.DOI: <https://doi.org/10.1016/j.biombioe.2012.12.029>.
- 499 2. L. Krienitz, E.H. Hegewald, D. Hepperle, V.A.R. Huss, T. Rohr, and M. Wolf, Phylogenetic
500 relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae).
501 *Phycologia*, 2004. 43(5): 529-542.DOI: 10.2216/i0031-8884-43-5-529.1.
- 502 3. U. Jena, N. Vaidyanathan, S. Chinnasamy, and K.C. Das, Evaluation of microalgae cultivation
503 using recovered aqueous co-product from thermochemical liquefaction of algal biomass.
504 *Bioresource technology*, 2011. 102: 3380-7.DOI: 10.1016/j.biortech.2010.09.111.
- 505 4. C. Hognon, F. Delrue, J. Texier, M. Grateau, S. Thiery, H. Miller, and A. Roubaud, Comparison
506 of pyrolysis and hydrothermal liquefaction of *Chlamydomonas reinhardtii*. *Growth studies on*
507 *the recovered hydrothermal aqueous phase*. *Biomass and Bioenergy*, 2015. 73: 23-31.DOI:
508 <https://doi.org/10.1016/j.biombioe.2014.11.025>.
- 509 5. L. Leng, J. Li, Z. Wen, and W. Zhou, Use of microalgae to recycle nutrients in aqueous phase
510 derived from hydrothermal liquefaction process. *Bioresource Technology*, 2018. 256: 529-
511 542.DOI: <https://doi.org/10.1016/j.biortech.2018.01.121>.
- 512 6. M. Tsarpali, N. Arora, J.N. Kuhn, and G.P. Philippidis, Beneficial use of the aqueous phase
513 generated during hydrothermal carbonization of algae as nutrient source for algae
514 cultivation. *Algal Research*, 2021. 60: 102485.DOI:
515 <https://doi.org/10.1016/j.algal.2021.102485>.
- 516 7. M.J. Stablein, D.H. Baracho, J.T. Watson, J.C. Silva, Y. Zhang, and A.T. Lombardi, Microalgal
517 photosynthetic inhibition and mixotrophic growth in Post Hydrothermal Liquefaction
518 Wastewater (PHW). *Algal Research*, 2021. 60: 102548.DOI:
519 <https://doi.org/10.1016/j.algal.2021.102548>.
- 520 8. P. SundarRajan, K.P. Gopinath, J. Arun, K. GracePavithra, A. Adithya Joseph, and S. Manasa,
521 Insights into valuing the aqueous phase derived from hydrothermal liquefaction. *Renewable*
522 *and Sustainable Energy Reviews*, 2021. 144: 111019.DOI:
523 <https://doi.org/10.1016/j.rser.2021.111019>.
- 524 9. B. Maddi, E. Panisko, T. Wietsma, T. Lemmon, M. Swita, K. Albrecht, and D. Howe,
525 Quantitative Characterization of Aqueous Byproducts from Hydrothermal Liquefaction of
526 Municipal Wastes, Food Industry Wastes, and Biomass Grown on Waste. *ACS Sustainable*
527 *Chemistry & Engineering*, 2017. 5(3): 2205-2214.DOI: 10.1021/acssuschemeng.6b02367.
- 528 10. M. Pham, L. Schideman, J. Scott, N. Rajagopalan, and M.J. Plewa, Chemical and Biological
529 Characterization of Wastewater Generated from Hydrothermal Liquefaction of *Spirulina*.
530 *Environmental Science & Technology*, 2013. 47(4): 2131-2138.DOI: 10.1021/es304532c.
- 531 11. E. Lazzari, K. Arena, E.B. Caramão, P. Dugo, L. Mondello, and M. Herrero, Comprehensive
532 two-dimensional liquid chromatography-based quali-quantitative screening of aqueous

- 533 phases from pyrolysis bio-oils. ELECTROPHORESIS, 2021. 42(1-2): 58-67.DOI:
534 <https://doi.org/10.1002/elps.202000119>.
- 535 12. E. Lazzari, K. Arena, E.B. Caramão, and M. Herrero, Quantitative analysis of aqueous phases
536 of bio-oils resulting from pyrolysis of different biomasses by two-dimensional comprehensive
537 liquid chromatography. Journal of Chromatography A, 2019. 1602: 359-367.DOI:
538 <https://doi.org/10.1016/j.chroma.2019.06.016>.
- 539 13. A.S. Kaplitz, M.E. Mostafa, S.A. Calvez, J.L. Edwards, and J.P. Grinias, Two-dimensional
540 separation techniques using supercritical fluid chromatography. Journal of Separation
541 Science, 2021. 44: 426-437.DOI: <https://doi.org/10.1002/jssc.202000823>.
- 542 14. M. Burlet-Parendel and K. Faure, Opportunities and challenges of liquid chromatography
543 coupled to supercritical fluid chromatography. TrAC Trends in Analytical Chemistry, 2021.
544 144: 116422.DOI: <https://doi.org/10.1016/j.trac.2021.116422>.
- 545 15. M. Sarrut, A. Corgier, G. Cretier, A. Le Masle, S. Dubant, and S. Heinisch, Potential and
546 limitations of on-line comprehensive reversed phase liquid chromatography x supercritical
547 fluid chromatography for the separation of neutral compounds: An approach to separate an
548 aqueous extract of bio-oil. Journal of Chromatography A, 2015. 1402: 124-133.DOI:
549 10.1016/j.chroma.2015.05.005.
- 550 16. D. Guillarme, V. Desfontaine, S. Heinisch, and J.-L. Veuthey, What are the current solutions
551 for interfacing supercritical fluid chromatography and mass spectrometry? Journal of
552 Chromatography B, 2018. 1083: 160-170.DOI:
553 <https://doi.org/10.1016/j.jchromb.2018.03.010>.
- 554 17. C. West, E. Lemasson, S. Bertin, P. Hennig, and E. Lesellier, An improved classification of
555 stationary phases for ultra-high performance supercritical fluid chromatography. Journal of
556 Chromatography A, 2016. 1440: 212-228.DOI: 10.1016/j.chroma.2016.02.052.
- 557 18. L.R. Snyder and J.W. Dolan, *High-Performance Gradient Elution: The Practical Application of*
558 *the Linear-Solvent-Strength Model*, ed. Wiley. 2006, New York, NY.
- 559 19. J.M. Davis, D.R. Stoll, and P.W. Carr, Effect of First-Dimension Undersampling on Effective
560 Peak Capacity in Comprehensive Two-Dimensional Separations. Analytical Chemistry, 2008.
561 80(2): 461-473.DOI: 10.1021/ac071504j.
- 562 20. P.G. Stevenson, A. Tarafder, and G. Guiochon, Comprehensive two-dimensional
563 chromatography with coupling of reversed phase high performance liquid chromatography
564 and supercritical fluid chromatography. Journal of Chromatography A, 2012. 1220: 175-
565 178.DOI: <https://doi.org/10.1016/j.chroma.2011.11.020>.
- 566 21. W. Wei, J. Hou, C. Yao, Q. Bi, X. Wang, Z. Li, Q. Jin, M. Lei, Z. Feng, W. Wu, and D. Guo, A high-
567 efficiency strategy integrating offline two-dimensional separation and data post-processing
568 with dereplication: Characterization of bufadienolides in Venenum Bufonis as a case study.
569 Journal of Chromatography A, 2019. 1603: 179-189.DOI:
570 <https://doi.org/10.1016/j.chroma.2019.06.037>.
- 571 22. G. Vivó-Truyols, S. van der Wal, and P.J. Schoenmakers, Comprehensive Study on the
572 Optimization of Online Two-Dimensional Liquid Chromatographic Systems Considering Losses
573 in Theoretical Peak Capacity in First- and Second-Dimensions: A Pareto-Optimality Approach.
574 Analytical Chemistry, 2010. 82(20): 8525-8536.DOI: 10.1021/ac101420f.
- 575 23. M. Sarrut, A. D'Attoma, and S. Heinisch, Optimization of conditions in on-line comprehensive
576 two-dimensional reversed phase liquid chromatography. Experimental comparison with one-
577 dimensional reversed phase liquid chromatography for the separation of peptides. Journal of
578 Chromatography A, 2015. 1421: 48-59.DOI: 10.1016/j.chroma.2015.08.052.
- 579 24. S.R. Groskreutz and S.G. Weber, Quantitative evaluation of models for solvent-based, on-
580 column focusing in liquid chromatography. Journal of Chromatography A, 2015. 1409: 116-
581 124.DOI: <https://doi.org/10.1016/j.chroma.2015.07.038>.
- 582 25. J. Prothmann, M. Sun, P. Spégel, M. Sandahl, and C. Turner, Ultra-high-performance
583 supercritical fluid chromatography with quadrupole-time-of-flight mass spectrometry
584 (UHPSFC/QTOF-MS) for analysis of lignin-derived monomeric compounds in processed lignin

- 585 samples. *Analytical and Bioanalytical Chemistry*, 2017. 409(30): 7049-7061. DOI:
586 10.1007/s00216-017-0663-5.
- 587 26. K. Vanderlinden, G. Desmet, and K. Broeckhoven, Effect of the feed injection method on
588 band broadening in analytical supercritical fluid chromatography. *Journal of Chromatography*
589 *A*, 2020. 1630: 461525. DOI: <https://doi.org/10.1016/j.chroma.2020.461525>.
- 590 27. A.G.-G. Perrenoud, J.-L. Veuthey, and D. Guillarme, Coupling state-of-the-art supercritical
591 fluid chromatography and mass spectrometry: From hyphenation interface optimization to
592 high-sensitivity analysis of pharmaceutical compounds. *Journal of Chromatography A*, 2014.
593 1339: 174-184. DOI: 10.1016/j.chroma.2014.03.006.
- 594 28. J. Crepier, A. Le Masle, N. Charon, F. Albrieux, P. Duchene, and S. Heinisch, Ultra-high
595 performance supercritical fluid chromatography hyphenated to atmospheric pressure
596 chemical ionization high resolution mass spectrometry for the characterization of fast
597 pyrolysis bio-oils. *Journal of Chromatography B*, 2018. 1086: 38-46. DOI:
598 <https://doi.org/10.1016/j.jchromb.2018.04.005>.
- 599 29. M. Batteau and K. Faure, Effect of the injection of water-containing diluents on band
600 broadening in analytical supercritical fluid chromatography. *Journal of Chromatography A*,
601 2022. 1673: 463056. DOI: <https://doi.org/10.1016/j.chroma.2022.463056>.
- 602 30. M. Camenzuli and P.J. Schoenmakers, A new measure of orthogonality for multi-dimensional
603 chromatography. *Analytica Chimica Acta*, 2014. 838: 93-101. DOI:
604 <http://dx.doi.org/10.1016/j.aca.2014.05.048>.
- 605 31. W. Nowik, S. Héron, M. Bonose, M. Nowik, and A. Tchaplá, Assessment of Two-Dimensional
606 Separative Systems Using Nearest-Neighbor Distances Approach. Part 1: Orthogonality
607 Aspects. *Analytical Chemistry*, 2013. 85(20): 9449-9458. DOI: 10.1021/ac4012705.
- 608 32. N. Nordin, N. Yusof, T. Maeda, N.A. Mustapha, M.Z. Mohd Yusoff, and R.F. Raja Khairuddin,
609 Mechanism of carbon partitioning towards starch and triacylglycerol in *Chlorella vulgaris*
610 under nitrogen stress through whole-transcriptome analysis. *Biomass and Bioenergy*, 2020.
611 138: 105600. DOI: <https://doi.org/10.1016/j.biombioe.2020.105600>.

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616 Table 1: Off-line LC x SFC - HRMS conditions

	First dimension (¹ D)	Second dimension (² D)	HRMS	
Injection volume	500 µL	10 µL	Mode	ESI +/ ESI -
Injection mode	Loop injection	Feed solvent heptane (volume 4 µL; speed 1000 µL/min)	Mass range (m/z)	100- 1700
Column parameters	Xselect Phenyl hexyl (150 x 4.6 mm, 5 µm)	Torus diol (50 x 3 mm, 1.7 µm)	Acquisition rate (spectra/s)	10
Temperature	30 °C	40 °C	Transient	761
BPR	N/A	140 bars	Gas temp (°C)	300
Flow rate	2.8 mL/min	1.8 mL/min	Drying gas (l/min)	11
Mobile phase A	A : H ₂ O	A : CO ₂	Nebulizer (psi)	40
Mobile phase B	B : ACN	B : EtOH	Sheat gas temp (°C)	350
Gradient conditions	1-40 % B in 10 min, 40-1 % B in 0.8 min, 1 % B for 1.2 min	2-50 % B in 1.34 min, 50-2 % B in 0.14 min, 1 % B for 0.46 min	Sheat Gas Flow (l/min)	11
Sampling time	0.14 min	N/A	Capillary voltage (V)	3500
Make up solvent	N/A	MeOH + 0.1 % FA; 2000 µL/min	Nozzle voltage (V)	300
Additional solvent	N/A	MeOH + 0.1 % FA; 200 µL/min	Fragmentor voltage (V)	185
Split SFC to MS	N/A	2.7	Oct 1 RF (Vpp)	750

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620 Table 2. Orthogonality performances using the asterisk equations [27]

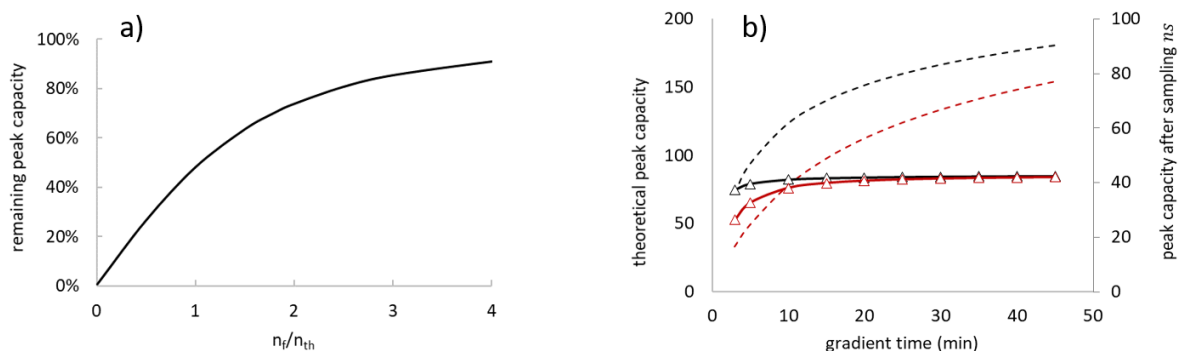
Dimension	Asterisk equation	Peak spread
LC dimension	Z1	89%
SFC dimension	Z2	96%
Upward diagonal	Z-	87%
Downward diagonal	Z+	75%
Overall occupation rate	A0	75%

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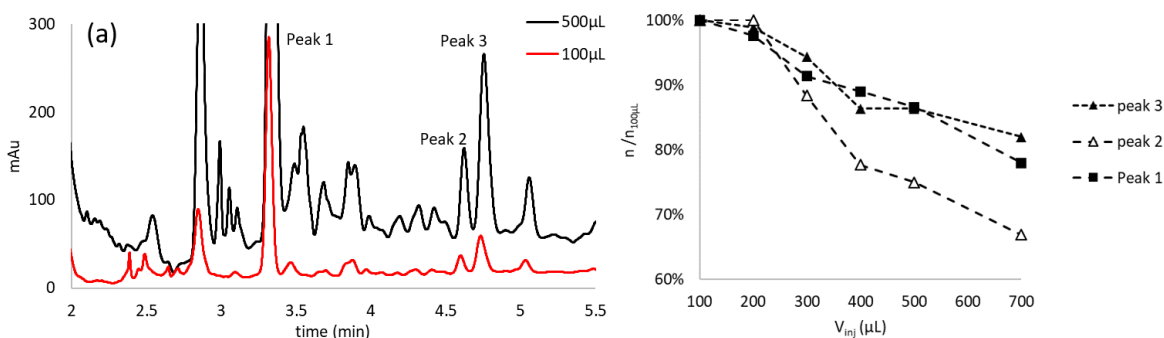
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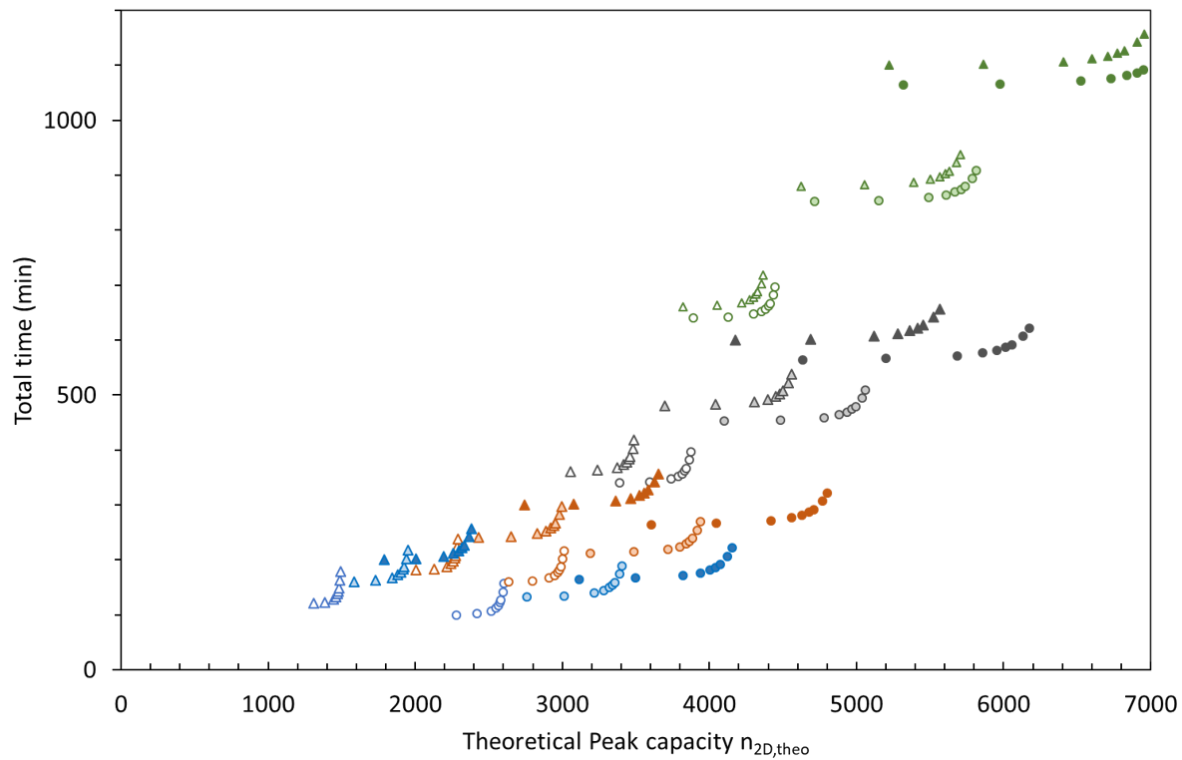
626 Figure 1. Theoretical performances of the LC dimension. a) plot of the remaining peak capacity vs. the
627 ratio of the number of fractions over theoretical peak capacity $\frac{n_f}{n_{th}}$ (Equation 5); b) plot of the peak
628 capacity as a function of gradient time, for a column with geometry 4.6 x 150 mm, 5 μ m, with a gradient
629 running from 1 % to 40 % B, using a flow rate at its optimal value (0.7 mL/min, red marks) or maximal
630 value (2.8 mL/min, black marks), with 80 fractions collected. Left axis is the theoretical peak capacity
631 (dashed lines) and right axis is the effective peak capacity after sampling n_s (plain lines).

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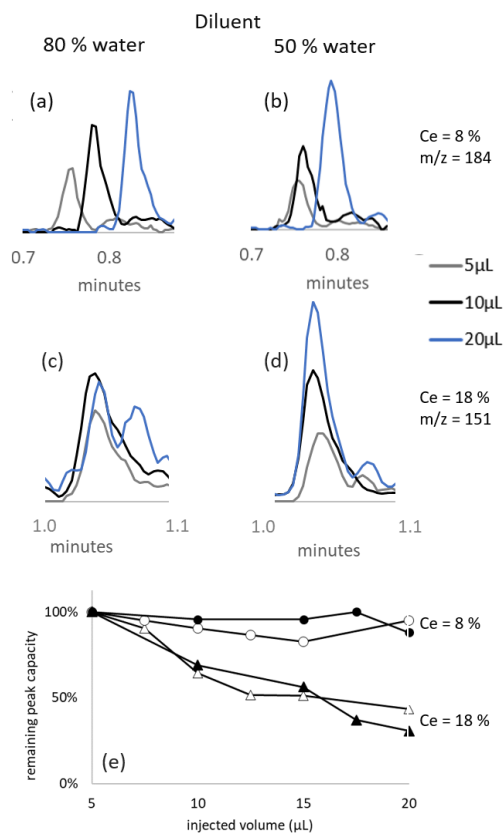
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634 Figure 2. Effect of the injection volume on the RPLC separation of the aqueous phase (a) Overlaid
635 chromatograms with injection volumes of 100 μ L (red) and 500 μ L (black). (b) Peak capacity loss as a
636 function of injection volume for three different peaks (shown in the top figure). Peak width measured
637 at half peak height. Flow rate: 2.8 mL/min; gradient time: 10 min. UV-detection at 254 nm. Other
638 conditions given in Table 1 (1D conditions).

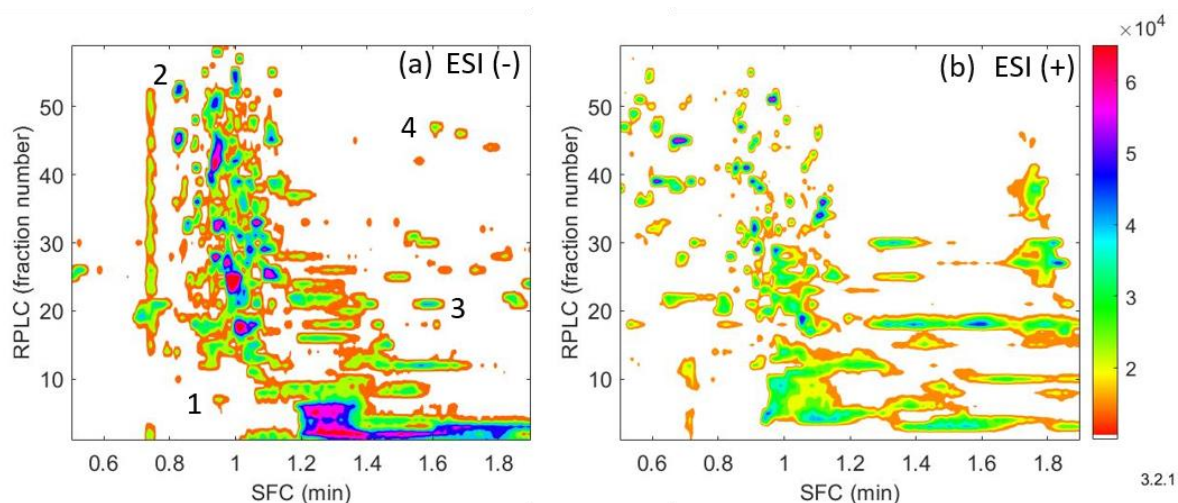


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640 Figure 3. Theoretical performances of the LC x SFC separation, expressed by a plot of the total time as
 641 a function of the 2D theoretical peak capacity. LC flow rate 2.8 mL/min, LC gradient range 3 to 60 min.
 642 Fraction number: 60, 80 and 100 (white, light colored and deep colored marks, respectively). SFC
 643 gradient time 1 min (blue), 2 min (orange), 5 min (grey) and 10 min (green). SFC column length 50 mm
 644 (circle marks) and 100 mm (triangle marks)

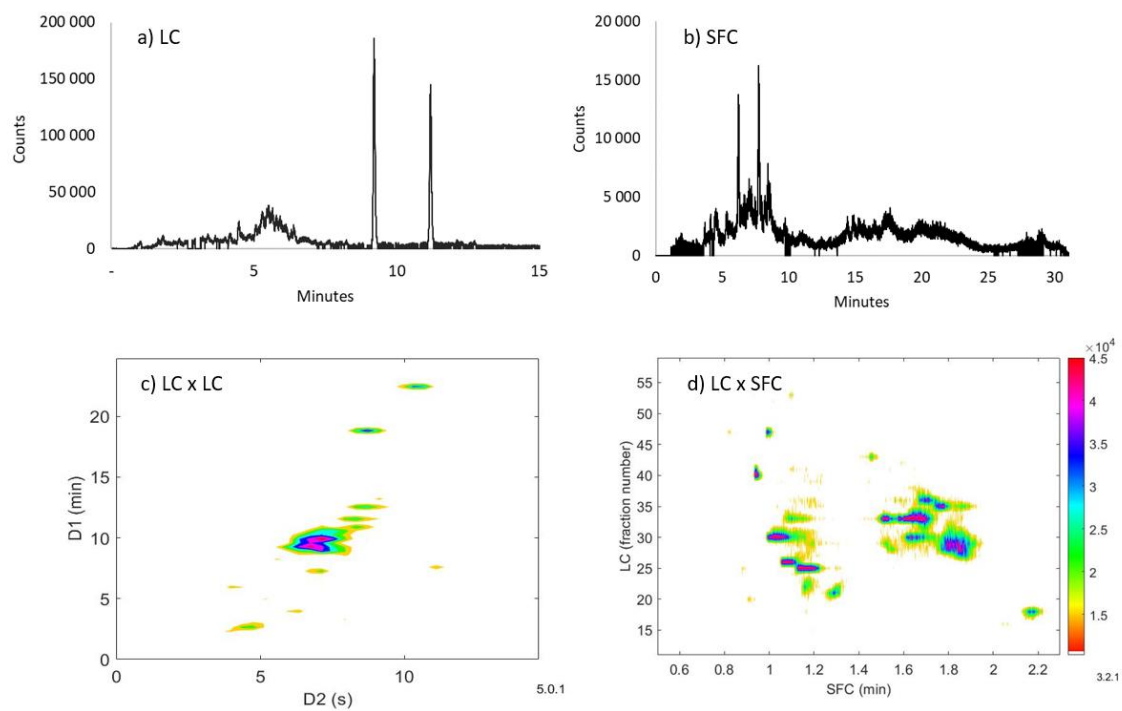


645
 646 Figure 4. Effect of the injection in ²D SFC. EIC chromatograms of (a-b) an early eluting peak and (c-d) a
 647 late eluting peak when the water/ACN diluent contains (a;c) 80% water or (b;d) 50% water. (e) Loss in
 648 peak capacity as a function of injection volume for a diluent 80:20 H₂O/ACN (v/v) (full symbols) and
 649 50:50 H₂O/ACN (v/v) (open symbols). Peak width measured at half peak height (FWHM). SFC conditions
 650 given in Table 1 (²D conditions). Wastewater was diluted by 10 in the corresponding diluent.



651
 652 Figure 5. Base peak chromatograms from the LC x SFC-ESI-HRMS analysis of the wastewater sample,
 653 using ESI source in a) negative mode and b) positive mode. Peaks noted 1 to 4 are the monitored
 654 isolated peaks spread over the separation space. The experimental conditions are in Table 1.

655



656
 657 Figure 6. Extracted ion chromatograms at m/z 277.1447 for the analysis of *Chlorella sorokiniana* HTL
 658 wastewater using a) LC-HRMS, b) SFC-HRMS, c) LCxLC-HRMS and d) LCxSFC -HRMS

659

660

661 **Supplementary material**

662

663 Figure S1. SFC chromatogram of the aqueous phase of algae HTL using various column chemistries.
664 Generic gradient A: CO₂ B: MeOH, 40°C, BPR 140 bar, gradient: 5-50% B, normalized gradient slope 5%,
665 column dimensions 100x3mm, 1.7µm, 1.1 mL/min, V_{inj} 5µL, injection feedsolvent 80/20 Heptane/IPA,
666 UV detection (254nm)

667 Figure S2. SFC chromatograms of the aqueous phase of algae HTL using a DIOL column and various co-
668 solvents. Gradient A: CO₂ B: co-solvent, 40°C, BPR 140 bar, gradient: 2-50% B, normalized gradient
669 slope 1%, column dimensions 100 x 3 mm, 1.7µm, 1.1 mL/min, V_{inj} 5µL, injection feedsolvent 80/20
670 Heptane/IPA, UV detection (254 nm)

671 Figure S3: Theoretical variation of the flow entering the MS, at initial mobile phase composition of 2 %
672 ethanol (blue marks) and final mobile phase composition of 50 % ethanol (grey mark), as a fonction of
673 the flow rate of the make-up pump. Make-up solvent is methanol. Open marks are CO₂ flows, plain
674 marks are organic solvent flows. Triangle marks represent the organic solvent flow when no additional
675 pump is used, circle marks are flow values when the additional pump delivers 200 µL/min methanol.

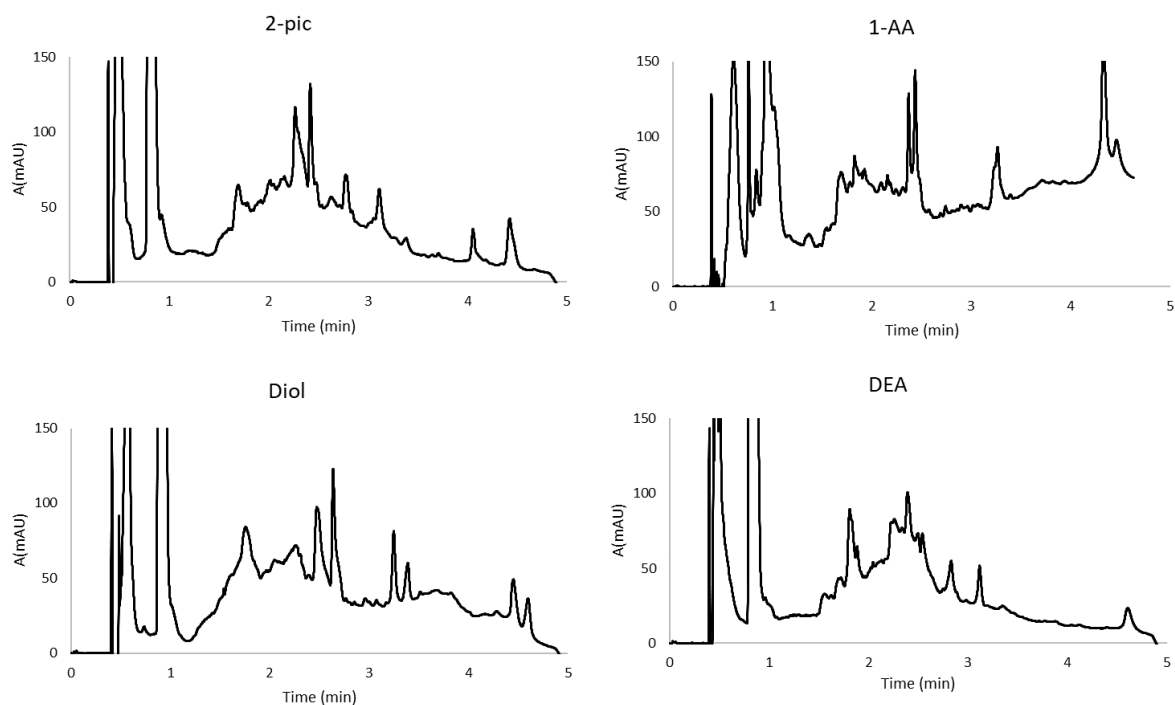
676 Figure S4 Base peak chromatogram of the aqueous phase of HTL from Sorokiniana Algae, SFC-HRMS
677 obtained with an electrospray source in a) positive mode, b) negative mode, c) UV at 254 nm. Diol
678 column (3 x 50 mm; 1.7 µm), gradient 2% to 50 % EtOH in 1.34 min, 1.8 mL/min, other conditions in
679 Table 1

680 Figure S5 Base peak chromatogram (ESI+) of the aqueous phase of HTL from Sorokiniana Algae using
681 a) LC phenyl hexyl column, isocratic step at 1 % ACN + 0.1 % formic acid for 1.5 min, then gradient 1%-
682 30% ACN + 0.1 % formic acid in 15 min, 0.5 mL/min, column geometry 2.1 x 100 mm; 1.7 µm, sample
683 diluted by 10 in water, V_{inj} 10 µL. b) SFC Diol column gradient 2% to 50 % EtOH in 30 min, 1.0 mL/min,
684 column geometry 3 x 100 mm; 1.7 µm, BPR 140 bars, 40°C. sample diluted by 3 in ACN/H₂O 50/50. V_{inj}
685 7 µL. HRMS conditions as in Table 1.

686

687 Figure S6. Online LCxLC - Base peak chromatogram of the aqueous phase of HTL from *Chlorella*
688 *sorokiniana*. First dimension: column geometry 100 x 2.1 mm, 1.7 µm. 30°C, A: water and B: ACN,
689 gradient 1% to 31 % B in 30 min, flow rate 150 µL/min, V_{inj} 5 µL. Second dimension: column
690 geometry 30 x 2.1 mm, 1.6 µm. 40°C, A: water + 10 mM ammonium acetate and B: MeOH + 10 mM
691 ammonium acetate, gradient 20% to 100% B in 0.21 min, flow rate 2600 µL/min, V_{inj} 16 µL. Split
692 between first and second dimension by 3. Detection as in Table 1

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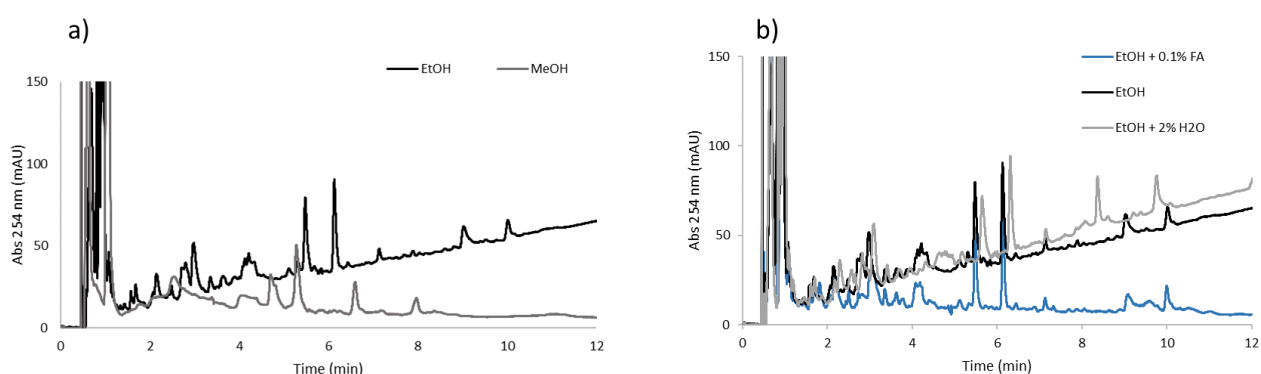
695

696 Figure S1. SFC chromatogram of the aqueous phase of algae HTL using various column chemistries.
 697 Generic gradient A: CO₂ B: MeOH, 40°C, BPR 140 bar, gradient: 5-50% B, normalized gradient slope
 698 5%, column dimensions 100x3mm, 1.7µm, 1.1 mL/min, V_{inj} 5µL, injection feedsolvent 80/20
 699 Heptane/IPA, UV detection (254nm)

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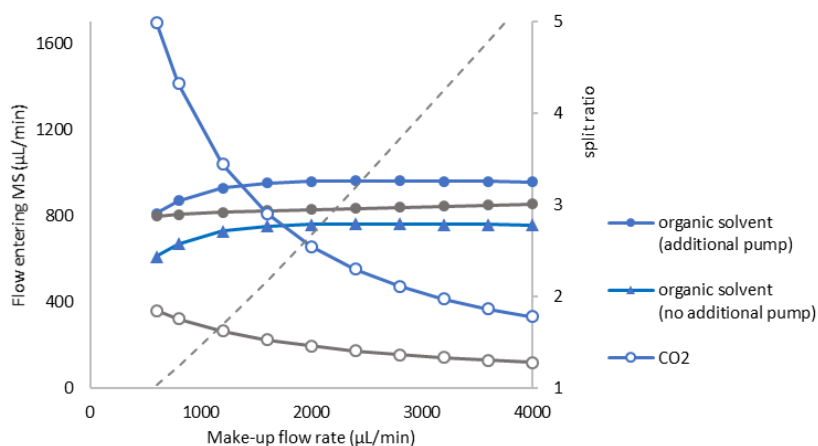
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703

704 Figure S2. SFC chromatograms of the aqueous phase of algae HTL using a DIOL column and various
 705 co-solvents. Gradient A: CO₂ B: co-solvent, 40°C, BPR 140 bar, gradient: 2-50% B, normalized gradient
 706 slope 1%, column dimensions 100 x 3 mm, 1.7µm, 1.1 mL/min, V_{inj} 5µL, injection feedsolvent 80/20
 707 Heptane/IPA, UV detection (254 nm)

708

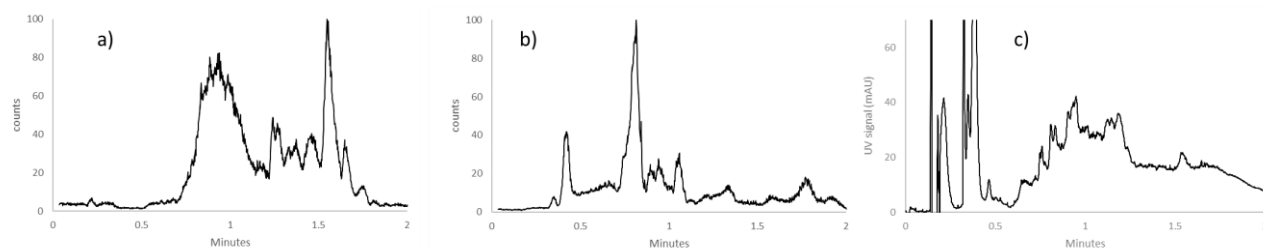


709

710 Figure S3: Theoretical variation of the flow entering the MS, at initial mobile phase composition of 2
 711 % ethanol (blue marks) and final mobile phase composition of 50 % ethanol (grey mark), as a function
 712 of the flow rate of the make-up pump. Make-up solvent is methanol. Open marks are CO₂ flows,
 713 plain marks are organic solvent flows. Triangle marks represent the organic solvent flow when no
 714 additional pump is used, circle marks are flow values when the additional pump delivers 200 µL/min
 715 methanol.

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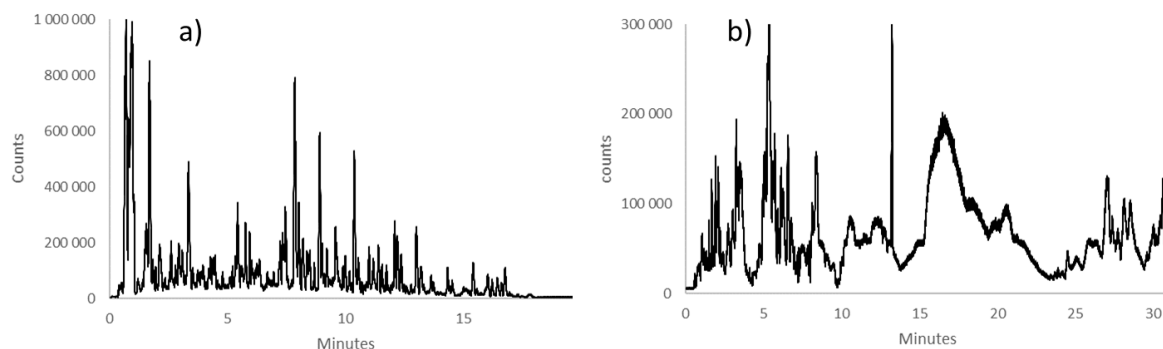
718

719 Figure S4 Base peak chromatogram of the aqueous phase of HTL from Sorokiniana Algae, SFC-HRMS
 720 obtained with an electrospray source in a) positive mode, b) negative mode, c) UV at 254 nm. Diol
 721 column (3 x 50 mm; 1.7 µm), gradient 2% to 50 % EtOH in 1.34 min, 1.8 mL/min, other conditions in
 722 Table 1

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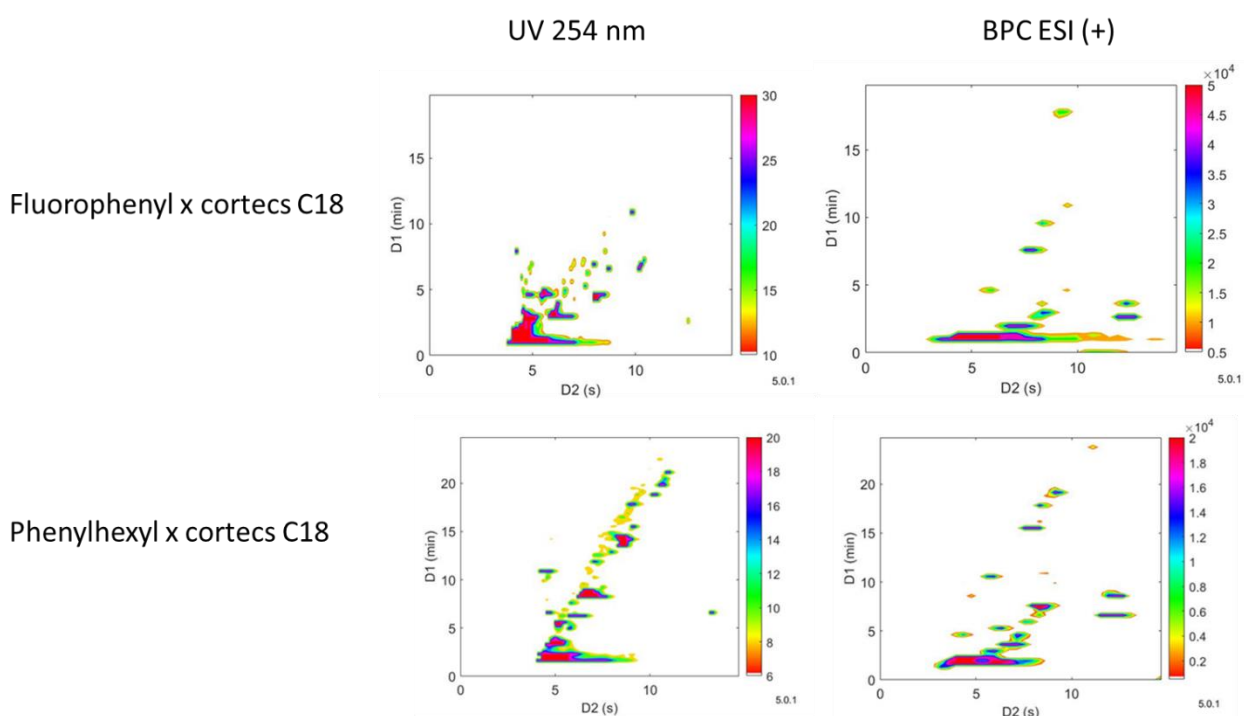


726

727 Figure S5 Base peak chromatogram (ESI+) of the aqueous phase of HTL from Sorokiniana Algae using
728 a) LC phenyl hexyl column, isocratic step at 1 % ACN + 0.1 % formic acid for 1.5 min, then gradient
729 1%-30% ACN + 0.1 % formic acid in 15 min, 0.5 mL/min, column geometry 2.1 x 100 mm; 1.7 μ m,
730 sample diluted by 10 in water, V_{inj} 10 μ L.

731 b) SFC Diol column gradient 2% to 50 % EtOH in 30 min, 1.0 mL/min, column geometry 3 x 100 mm;
732 1.7 μ m, BPR 140 bars, 40°C. sample diluted by 3 in ACN/H₂O 50/50. V_{inj} 7 μ L. HRMS conditions as in
733 Table 1.

734



735

736 Figure S6. Online LCxLC - Base peak chromatogram of the aqueous phase of HTL from *Chlorella*
737 *sorokiniana*. First dimension: column geometry 100 x 2.1 mm, 1.7 μ m. 30°C, A: water and B: ACN,
738 gradient 1% to 31 % B in 30 min, flow rate 150 μ L/min, V_{inj} 5 μ L. Second dimension: column
739 geometry 30 x 2.1 mm, 1.6 μ m. 40°C, A: water + 10 mM ammonium acetate and B: MeOH + 10 mM
740 ammonium acetate, gradient 20% to 100% B in 0.21 min, flow rate 2600 μ L/min, V_{inj} 16 μ L. Split
741 between first and second dimension by 3. Detection as in Table 1

742

743