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1 **Separation of unsaturated C18 fatty acids using**
2 **perfluorinated-micellar electrokinetic chromatography: I)**
3 **Optimization and separation process.**

4
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18
19
20 **Keywords:** micellar electrokinetic chromatography, ammonium perfluorooctanoate,
21 fatty acids, surfactant, self-assembly

22
23 **Abbreviations:** alpha linolenic acid ALA, ammonium perfluorooctanoate APFOA,
24 elaidic acid EA, fatty acids FAs, linoleic acid LA, perfluorooctanoic acid PFOA, oleic
25 acid OA, trifluoroethanol TFE.

26 **Abstract**

27 Ammonium perfluorooctanoate (APFOA) was used as surfactant for the separation of
28 free unsaturated C18 fatty acids by micellar electrokinetic chromatography. A simple
29 background electrolyte of 50 mM APFOA water/methanol (90:10, v/v) at pH = 10
30 enabled repeatable separation of oleic acid, elaidic acid, linoleic acid, and alpha-
31 linolenic acid in less than 20 min. Separation conditions were optimized regarding
32 various parameters (organic solvent, counterion, APFOA concentration, and pH).
33 Because the repulsive interactions between fluorocarbon chains and hydrogenated
34 chains are known to lead to segregation and phase separation, the choice of
35 perfluorinated micelles to separate such perhydrogenated long-chain acids could
36 appear astonishing. Therefore, the cmc, the charge density, and the mobility of the
37 micelles have been determined, resulting in a first description of the separation
38 process.

39 **1 Introduction**

40 Fatty acids (FAs) represent one of the major lipid classes and are involved in many
41 biological processes. Among the analytical techniques suitable for the separation of
42 FAs, gas chromatography (GC) is the mainly used one. GC-based protocols have
43 been very well optimized especially for the separation of FA methyl esters (FAMES),
44 and several applications have been described in the literature [1–3]. Capillary
45 electrophoresis (CE) is another leading technique, now well described for the
46 separation of free FAs [4,5]. In most of the reported separation conditions, the use of
47 sodium dodecyl sulfate (SDS) is necessary, in combination with organic solvents or
48 cyclodextrins. This ensures a good solubilization of FAs, which are poorly water-
49 soluble and can themselves form micelles.

50 Micellar electrokinetic Chromatography (MEKC) can be used for the separation of FAs
51 [6–8], including detection in real matrixes [9–11], as well as non-aqueous capillary
52 electrophoresis (NACE) [12]. In the latter case, the separation is performed using
53 organic solvents (acetonitrile, methanol, isopropanol) in the presence of aqueous
54 ammonia or ammonium acetate. These volatile electrolytes have the advantage to be
55 useable when CE is coupled to electrospray ionization mass spectrometry (in CE/ESI-

56 MS experiments), while MEKC using SDS is not compatible with ESI-MS due to its
57 low volatility. To overcome this major drawback and enable MS detection, volatile
58 surfactants have to be used in replacement of SDS.

59 Two decades ago, ammonium perfluorooctanoate (APFOA) has been used as
60 surfactant in MEKC experiments [13]. It was found to be a compatible volatile
61 surfactant for MEKC-ESI/MS analysis of polar compounds such as N-
62 methylcarbamates [14,15], charged and uncharged phenyl compounds (more or less
63 hydrophobic) [13], pyrene and benzopyrene [16], benzimidazoles [17], amino acids
64 [18,19], etc. However, its use for FAs separation in MEKC-ESI/MS has not been
65 reported so far. Moreover, the physical and chemical characteristics of the resulting
66 micelles in the BGE have not been characterized neither.

67 The repulsive interactions between fluorocarbon chains and hydrogenated chains are
68 known to lead to segregation and phase separation [20], due to severe nonidealities.
69 The mixing of SDS and APFOA was found to result in demixing into two kinds of
70 micelles with either a high-level content of SDS or APFOA [21]. This is still under
71 debate since another hypothesis about this kind of mixture is that it would form mixed
72 micelles [22]. However, it seems that small amounts of hydrogenated surfactants can
73 be incorporated into APFOA-rich micelles, and small amounts of fluorinated
74 surfactants into hydrogenated surfactant-based micelles [23]. In this context, could
75 APFOA - used as volatile surfactant - be a good candidate to separate FAs, keeping
76 in mind that it could be later be compatible with MS analysis?

77 As a proof of concept, the latter possibility was positively examined for the separation
78 of low concentrations of fatty acids by APFOA MEKC, keeping simple UV-Vis detection
79 as a first step. Thus, APFOA micelles were used to separate four free unsaturated
80 FAs, namely oleic acid (OA), elaidic acid (EA), linoleic acid (LA), and alpha-linolenic
81 acid (ALA). The interactions underlying the separation of these hydrogenated
82 compounds in the perfluorinated medium were studied, and a first approach of the
83 separation mechanism was proposed. This manuscript will first present the
84 optimization of CE separation. Then, the measurement of the mobility of the APFOA
85 micelles was performed by using a micelle marker we synthesized. The CE instrument
86 was also used to determine the cmc (critical micelle concentration), which was
87 confirmed by NMR experiments. Based on the residence time of FAs and geometric

88 considerations, we propose a preliminary separation mechanism of FAs by APFOA
89 micelles.

90 **2 Materials and methods**

91 **2.1 Chemicals and reagents**

92 Perfluorooctanoic acid (96 %, PFOA), FAs (≥ 99 %, including OA, EA, LA, and ALA),
93 2,2,2-trifluoroethanol (TFE), phenylalanine (99 %), sodium hydroxide, ammonium
94 hydroxide, methanol, ethanol, and acetonitrile (HPLC grade solvents) were purchased
95 from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Perfluorooctanoyl chloride (97
96 %) was supplied from Fisher Scientific-Alfa Aesar (Illkirch, France). Deuterated
97 solvents were supplied from Eurisotop (Saint-Aubin, France).

98

99 **2.2 Preparation of samples and buffers**

100 Stock solutions of separation buffer - the ammonium perfluorooctanoate (APFOA)
101 micellar solutions - were prepared by weighting and dissolving the appropriate
102 amounts of perfluorooctanoic acid (PFOA) in aqueous ammonium hydroxide (NH_4OH).
103 The concentration ratios R , defined as $R = [\text{NH}_4\text{OH}]/[\text{PFOA}]$, were $R = 3.4$ or $R = 5.1$.
104 As a consequence, $[\text{NH}_4\text{OH}]$ was always proportionally related to $[\text{PFOA}]$, depending
105 on the R value. The addition of NH_4OH to increase pH almost did not change the ionic
106 strength because excess NH_4OH remained mostly in a neutral form close to its pK_a
107 (9.2). The formation of NH_4^+ cations only came from the reaction between NH_4OH and
108 PFOA, maintaining $[\text{NH}_4^+]$ equal to the initial $[\text{PFOA}]$. The pH of these stock solutions
109 were measured using a Seven Compact S220 pH-meter (Mettler-Toledo, Viroflay,
110 France). The pH values were 9.5 and 10 for $R = 3.4$ and $R = 5.1$, respectively. The
111 working BGEs used for optimizing the method were prepared by diluting 100 mM
112 APFOA aqueous stock solutions with water and organic modifiers (*i.e.* MeOH, EtOH,
113 or ACN) to get the desired final APFOA concentration and organic solvent content. As
114 an illustrative example, the BGE composed of 50 mM APFOA along with 10% MeOH
115 at pH 10 was prepared by mixing the 100 mM APFOA aqueous stock solution (pH=10,
116 $R=5.1$) with water and MeOH in the ratio 50-40-10 (volume fractions). 30 mM FAs
117 stock solutions were prepared in methanol and kept at 5 °C before use. The injection

118 buffers containing the samples were prepared in 23 mM APFOA water/MeOH (47:53,
119 v/v), to allow solubilization of the four FAs (1 mM).

120

121 **2.3 CE separation parameters**

122 CE analysis was performed on an Agilent Technologies CE7100 system (Waldbronn,
123 Germany). Separation of FAs was carried out on a 50 μm id x 365 μm od x 51 cm
124 effective length (59 cm total length) bare-fused silica capillary (Picometrics - Adelis
125 Technologies, Labège, France).

126 New capillaries were rinsed with 1 M NaOH for 60 min, water (HPLC grade) for 30
127 min, and background electrolyte (BGE) for 15 min. Between two analyses, the capillary
128 was rinsed with methanol for 3 min, 1 M NaOH for 3 min, water for 2 min, and BGE for
129 3 min. At the end of each day, the capillary was rinsed with methanol for 3 min, 1 M
130 NaOH for 15 min, and water for 5 min. The pressure applied for the rinsing procedures
131 was 950 mbar. Samples were hydrodynamically injected at 50 mbar for 5 s. All
132 analyses were carried out at 25 °C and at 25 kV for 30 min; the wavelength of UV
133 direct detection was set at 195 nm.

134

135 **2.4 Cmc determination of APFOA**

136 **2.4.1 CE method**

137 Solutions containing PFOA at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100
138 mM, and 5.1-fold more ammonia were flushed for 10 min in the separation capillary,
139 then a voltage of +25 kV was applied. The currents were recorded and the curve $I^c =$
140 $f([\text{APFOA}])$, which represents the variation of I^c as a function of APFOA concentration,
141 was drawn as reported in [24]. We obtained a curve which allowed the calculation of
142 the cmc. This curve shows two linear portions, the first one at low APFOA
143 concentrations as described in equation 1:

$$144 \quad I^c = a_1 \cdot [\text{APFOA}] \quad (1)$$

145 with $a_1 = 6.67 \cdot 10^{-7} \text{ A} \cdot \text{mM}^{-1}$

146 and the second straight line at high APFOA concentrations as described below in
147 equation 2:

$$148 \quad I^c = a_2 \cdot [\text{APFOA}] + b_2 \quad (2)$$

149 with $a_2 = 4.5 \cdot 10^{-7} \text{ A} \cdot \text{mM}^{-1}$, $b_2 = 4.10 \cdot 10^{-6} \text{ A}$

150 [APFOA] is expressed in mM, and I^c in A. At the intersection of these two lines,
151 [APFOA] is equal to the cmc, which can then be obtained by combining equations 1
152 and 2 as follows:

$$153 \quad \text{cmc} = b_2 / (a_1 - a_2) \quad (3)$$

154 **2.4.2 NMR method**

155 All solutions for NMR experiments were prepared with deuterated water as solvent.

156 Fluorine-19 NMR experiments at the different concentrations of APFOA (5, 7, 10, 15,
157 20, 25, 30, 40, 50, 60, 80, and 92 mM) including TFE as chemical shift reference (2
158 mM) were acquired on a Bruker Avance 400 spectrometer (Bruker Biospin AG,
159 Fallanden, Switzerland) equipped with a 5 mm triple resonance probe (TXO) with ^{19}F
160 direct detection (376 MHz).

161 One dimensional (1D) ^{19}F pulse sequence with power-gated decoupling for ^1H (zgpg
162 in Bruker library) was used at 25 °C with the following parameters: acquisition time of
163 1.74 s with 256K data points and a spectral width of 200 ppm, a relaxation delay of 2
164 s and 16 scans.

165 Diffusion NMR spectra were recorded with a classical stimulated echo NMR
166 experiment using bipolar gradients (stebpgp1s in Bruker library). The acquisition
167 parameters were as follows: 64K data points, acquisition time 1.19 s, spectral width
168 80 ppm, number of scans 16, relaxation delay 3 s, pulse field gradient length 3.6 ms
169 and recovery delay 1 ms, diffusion time 200 ms. The spoiler gradients were 0.6 ms
170 long. Sixteen experiments were recorded with a linear gradient sampling from 5 to
171 95% of the maximum gradient strength.

172 All NMR data were processed using the TOPSPIN 4.0.8 software with one level of
173 zero-filling and an exponential line-broadening function of 0.5 Hz. Spectra were
174 calibrated at -76.96 ppm on the CF_3 signal of TFE. The data sets of diffusion NMR
175 experiments were processed with TOPSPIN software and transferred to the Origin 9.1
176 software. The exponential fit tool was then used to determine the values of the self-
177 diffusion coefficient (D).

178 ^{19}F NMR assignments of APFOA were given at 50 mM (CE conditions) as previously
179 reported by Liu and Goddard [25]: F2 (-117.91 ppm, tt, 3.0 and 12.8 Hz, $-\text{CF}_2\text{-COOH}$),
180 F3 (-122.58 ppm, m, $-\text{CF}_2\text{-CF}_2\text{-COOH}$), F4 (-122.86 ppm, m, $-\text{CF}_2\text{-CF}_2\text{-CF}_2\text{-COOH}$),

181 F5 (-123.49 ppm, m, -CF₂-CF₂-CF₂-CF₃), F6 (-123.65 ppm, m, -CF₂-CF₂-CF₃), F7 (-
182 127.23 ppm, m, -CF₂-CF₃), F8 (-82.34 ppm, tt, 2.2 and 10.1 Hz, -CF₂-CF₃).

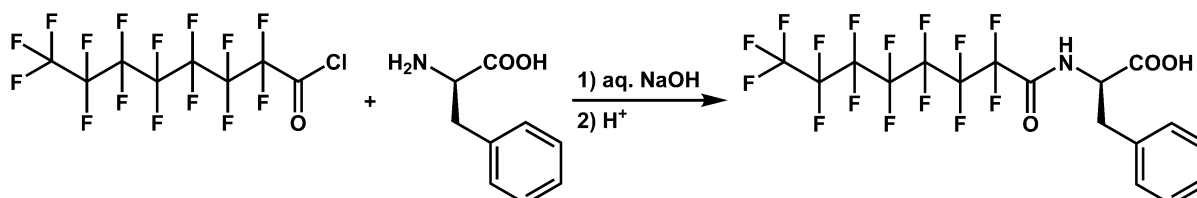
183 The determination of the critical micelle concentration (cmc) was performed as
184 described by Xing et al. [26] using the following equation based on the theory of
185 pseudo-phase transition model:

$$186 \quad \Delta\delta = \frac{cmc}{C} (\delta_{mon} - \delta_{mic}) + \delta_{mic} - \delta_{mon} \quad (4)$$

187 Where $\Delta\delta$ is the chemical shift variation of selected ¹⁹F NMR signals in each solution
188 compared to the solution at a concentration below the cmc (5 mM in the present study),
189 δ_{mon} and δ_{mic} are the chemical shifts of monomers and micelles respectively and C is
190 the concentration of the solution analyzed. The cmc can be obtained based on the
191 curve $\Delta\delta = f(1/C)$. A constant line function is observed at concentrations below the
192 cmc and become a linear function of 1/C in the micellar region. The intercept between
193 these two straight lines defines the cmc.

194

195 2.5 Synthesis of the micelle marker, the N-perfluorooctanoyl 196 phenylalanine



197

198 **Scheme 1.** Synthesis of the micelle marker by direct acylation of phenylalanine

199

200 The target compound was prepared *via* a classical Schotten-Baumann acylation.
201 Phenylalanine (0.215 g, 1.3 mmol) was dissolved in 2 mL aqueous NaOH (0.2 M) and
202 placed at 0 °C. Perfluorooctanoyl chloride (0.433 g, 1 mmol) was added dropwise
203 followed by additional 2 mL aqueous NaOH (0.2 M). The mixture was stirred for 1 h at
204 0 °C then for 4 h at room temperature. The white suspension was acidified till pH = 1
205 using aqueous 6 M HCl. After filtration and thorough washes with water then methanol,
206 the resulting solid was dried under vacuum affording a white powder (0.157 g, 28 %
207 yield). NMR characterizations were in accordance with literature [27,28].

208 High-resolution mass spectrometry (HRMS) analysis was performed by electrospray
209 ionization (ESI) coupled to a Time of Flight (TOF) analyzer, in the negative mode

210 (Xevo™ G2 QTOF, Waters). Mass obtained for C₁₇H₉NO₃F₁₅ (M-H)⁻ are: m/z
211 560.0343 (calculated) and m/z 560.0347 (experimental) (+0.7 ppm).

212 ¹H NMR (DMSO-*d*₆, 400 MHz): (9.76 ppm, d, ³J_{HH} = 8.5 Hz, 1H, NH), (7.34-7.16 ppm,
213 m, 5H, CH_{arom}), (4.57 ppm, ddd, ³J_{HH} = 8.5 Hz, ³J_{HH} = 4 Hz, ³J_{HH} = 12 Hz, 1H, ^αCH),
214 (3.23 ppm, dd, ²J_{HH} = 14 Hz, ³J_{HH} = 4 Hz, 1H, 1/2xCH₂), (3.01 ppm, dd, ²J_{HH} = 14 Hz,
215 ³J_{HH} = 12 Hz, 1H, 1/2xCH₂).

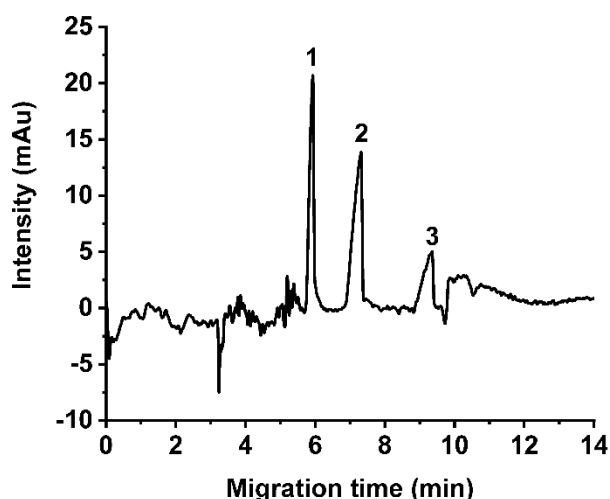
216 ¹³C NMR (DMSO-*d*₆, 100 MHz): (171.3 ppm, -COOH), (156.6 ppm, -CONH), (137.3
217 ppm, C_{q,arom}), (128.9 ppm, CH_{arom}), (128.1 ppm, CH_{arom}), (126.4 ppm, CH_{arom}), (116.6
218 ppm, -CF₃), (110.2 ppm, -CF₂), (110.2 ppm, -CF₂), (110.0 ppm, -CF₂), (109.7 ppm, -
219 CF₂), (108.5 ppm, -CF₂), (107.8 ppm, -CF₂), (54.2 ppm, -CH), (35.4 ppm, -CH₂)

220 ¹⁹F NMR (DMSO-*d*₆, 376 MHz): (-80.3 ppm, 3F), (-118.6 ppm, 2F), (-121.6 ppm, 2F),
221 (-122.0 ppm, 2F), (-122.6 ppm, 4F), (-125.9 ppm, 2F).

222 **3 Results and discussion**

223 **3.1 Optimization of MEKC conditions for the separation of mono-,** 224 **di- and tri-unsaturated free C18 FAs**

225 A standard mixture of three *cis*-isomers free C18 FAs (OA, LA, and ALA) was first
226 analyzed by using a BGE of 50 mM APFOA at pH 9.5. At this initial chosen pH, the
227 ratio between concentrations of ammonium hydroxide and perfluorooctanoic acid
228 ([NH₄OH]/[PFOA]) was R = 3.4. Peak assignment was realized by comparison to
229 reference migration time of each acid, as individually injected. The obtained
230 electropherogram exhibited a noisy baseline due to a strong absorption of the APFOA
231 medium at 195 nm (Figure 1). In addition, all FAs peaks were asymmetric. In order to
232 gain in separation performance, the composition of the BGE was optimized (nature
233 and amount of the added organic solvent, APFOA concentration, [NH₄OH]/[PFOA]
234 ratio), along with the presence of methanol in the sample composition.



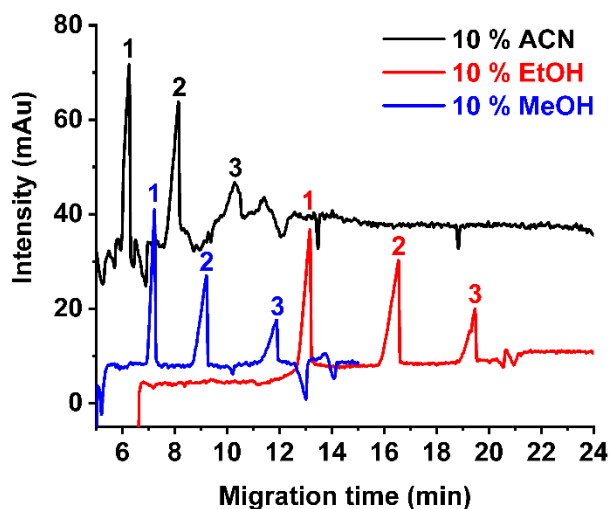
235

236 **Figure 1.** Separation of the three cis-isomers
 237 free FAs C18: (1) alpha linolenic acid (ALA), (2)
 238 linoleic acid (LA), and (3) oleic acid (OA). CE
 239 conditions: fused silica capillary (id: 50 μ m, L_{tot} :
 240 59 cm, L_{eff} : 51 cm), BGE consisting of 50 mM
 241 APFOA at pH 9.5, $[NH_4OH]/[PFOA] = 3.4$;
 242 samples injected at 50 mbar for 5s, +25 kV, 25
 243 $^{\circ}$ C, detection at 195 nm. FA injected
 244 concentration: 1 mM.

245

246 3.1.1 Influence of the organic solvent added in the BGE

247 The presence of organic solvent usually helps dissolving FAs and often allows better
 248 separation. It also has an influence on the viscosity of the BGEs and consequently
 249 affects the separation times. The addition of 10 % (v/v) acetonitrile (ACN), methanol,
 250 or ethanol in the BGE was tested. The corresponding viscosities were reported to be
 251 0.96, 1.07, and 1.20 cP for water/ACN, water/MeOH, and water/EtOH, respectively
 252 [29,30]. The obtained electropherograms (Figure 2) showed that acetonitrile led to the
 253 fastest separation of FAs but also to the noisiest baseline. Electropherograms with
 254 more stable baselines were obtained with methanol and ethanol, and the three FAs
 255 were well separated. However, the presence of ethanol in the BGE lead to the longest
 256 migration times. Thus, methanol was selected as it allows obtaining an efficient
 257 separation in the shortest migration time.



258

259 **Figure 2.** Effect of organic solvents on the
 260 separation of three unsaturated free FAs C18:
 261 (1) alpha linolenic acid ALA, (2) linoleic acid LA,
 262 and (3) oleic acid OA. ACN: acetonitrile, EtOH:
 263 ethanol, MeOH: methanol. CE conditions:
 264 fused silica capillary (id: 50 μm , L_{tot} : 59 cm, L_{eff} :
 265 51 cm). BGE: 50 mM APFOA at pH 9.5
 266 water/organic solvent (90:10, v/v),
 267 $[\text{NH}_4\text{OH}]/[\text{PFOA}] = 3.4$. Samples injected at 50
 268 mbar for 5 s. +25 kV, 25 $^{\circ}\text{C}$, λ : 195 nm. FAs
 269 injected concentration: 1 mM.

270

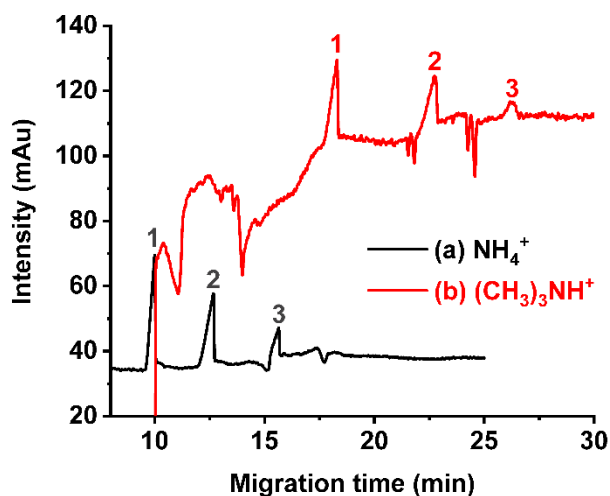
271 The methanol content in the BGE was then optimized. By increasing it from 10 %, 15
 272 % to 20 % (v/v), the viscosity of the separation medium was increased, leading to
 273 longer migration times and peak broadening. At 20 % (v/v) methanol in the BGE, we
 274 did not observe any peak for OA (the most slowly migrating FA) after 70 min of analysis
 275 (data not shown). Thus, the methanol content was set at 10% (v/v) for the next
 276 optimizations.

277

278 3.1.2 Effect of the counter ion

279 It is well-known that the micelle properties not only depend on the nature of the
 280 surfactant but also on the type of counterion present at the micellar surface. In this
 281 study, we used ammonia or trimethylamine to adjust the pH of perfluorooctanoic acid
 282 solutions, resulting in the presence of NH_4^+ or $(\text{CH}_3)_3\text{NH}^+$ as counterions, respectively.
 283 The results showed that, as compared to NH_4^+ , longer migration times were obtained
 284 for ALA, LA, and OA in the presence of $(\text{CH}_3)_3\text{NH}^+$ and that the corresponding
 285 electropherogram presented a very noisy baseline (Figure 3), while the peak

286 symmetries were not improved. Consequently, the ammonium ion NH_4^+ was selected
287 as counterion in the separation electrolyte.



288

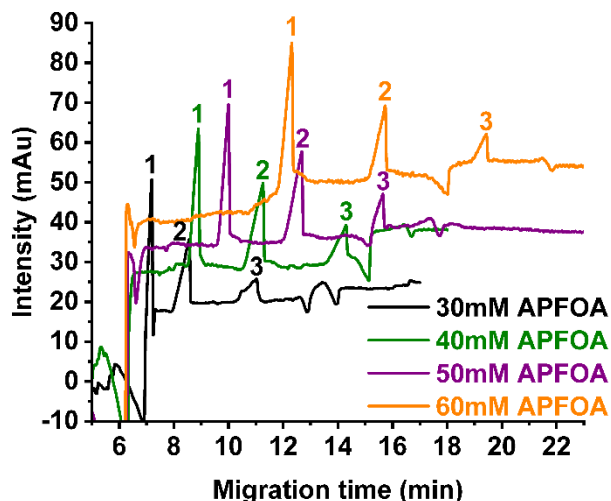
289 **Figure 3.** Effect of counterion on the separation
290 of three unsaturated free FAs C18: (1) alpha
291 linolenic acid ALA, (2) linoleic acid LA, and (3)
292 oleic acid OA. CE conditions: fused silica
293 capillary (id: 50 μm , L_{tot} : 59 cm, L_{eff} : 51 cm). a)
294 BGE: 50 mM ammonium perfluorooctanoate
295 pH 9.5/MeOH (90:10, v/v) and b) BGE: 50 mM
296 trimethylammonium perfluorooctanoate pH 9.5
297 water/MeOH (90:10, v/v). Samples injected at
298 50 mbar for 5 s. +25 kV, 25 $^{\circ}\text{C}$, λ : 195 nm. FAs
299 injected concentration: 1 mM.

300

301 3.1.3 Effects of APFOA concentration and $\text{NH}_4\text{OH}/\text{PFOA}$ ratio

302 The influence of APFOA concentration on the separation of OA, LA, and ALA was also
303 studied (Figure 4). APFOA concentrations ranging from 20 mM to 60 mM were tested
304 ($[\text{NH}_4\text{OH}] = 68\text{-}204$ mM at $R = 3.4$), in the presence of 10% MeOH in the BGE. At 20
305 mM APFOA, the three FAs cannot be separated, while the separation was achieved
306 at 25 mM APFOA but the peak resolutions were too low (data are not shown for those
307 two concentrations). At such low concentrations, just above the cmc, only very small
308 quantities of APFOA micelles are formed in the BGE. In contrast, the three FAs were
309 well separated at 30 mM APFOA and above (Figure 4). Increasing APFOA
310 concentration improved peak resolutions but also led to increased migration times.
311 Moreover, for the highest surfactant concentration, some problems appeared such as
312 unstable current or clogging of the capillary. With a view to analyzing more complex
313 samples containing ALA, LA, and OA, the quality of separation was considered as the
314 most relevant criterion to choose the BGE composition. The quality of separation was

315 evaluated considering peak visibility and separation as well as baseline quality.
316 Compared to 40 mM, the baseline is more stable at 50 mM and the peak 3 is more
317 visible. Therefore, 50 mM APFOA along with 10% MeOH was selected as BGE
318 composition.



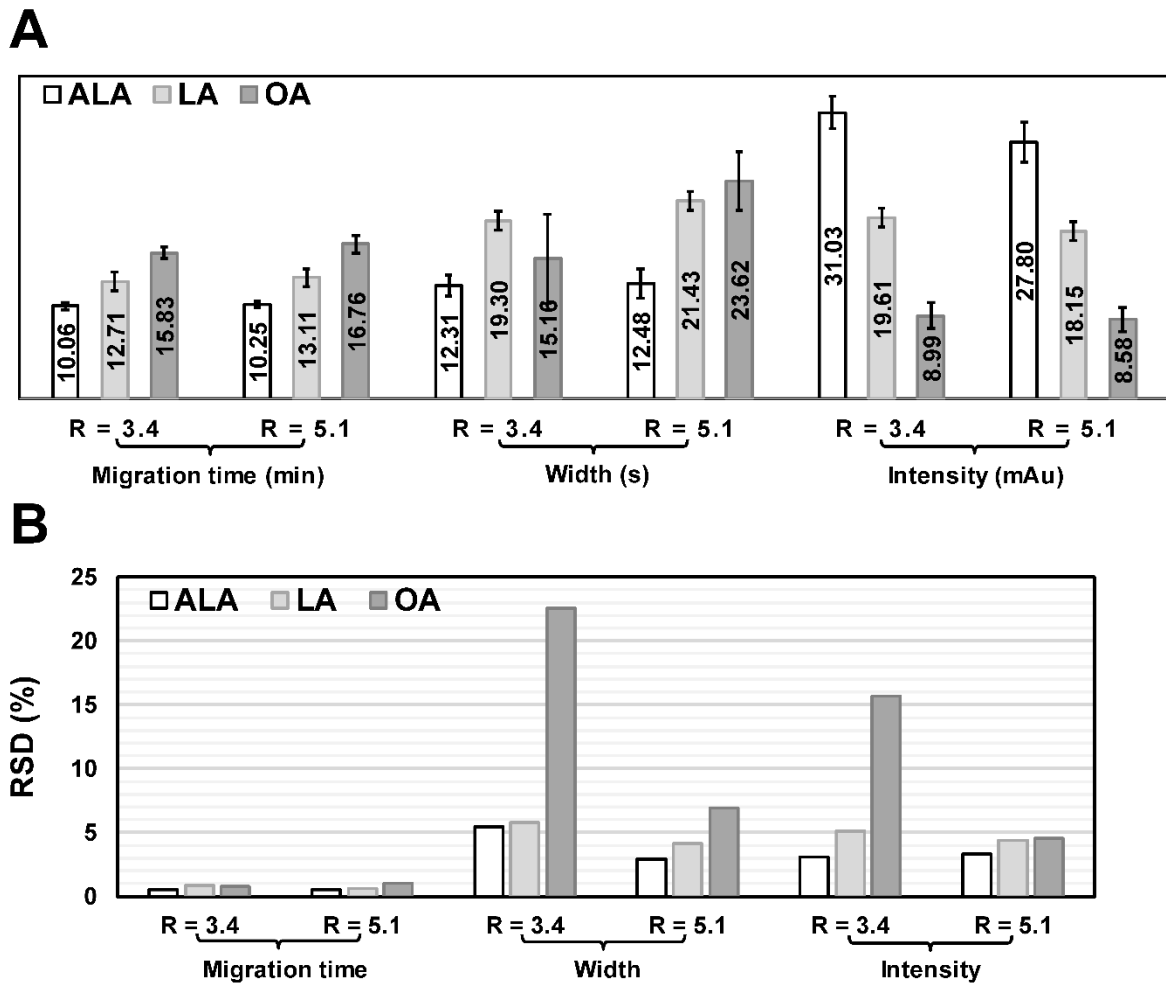
319

320 **Figure 4.** Effect of APFOA concentration on
321 the separation of three unsaturated free FAs
322 C18: (1) alpha linolenic acid ALA, (2) linoleic
323 acid LA, and (3) oleic acid OA. CE conditions:
324 fused silica capillary (id: 50 μ m, L_{tot} : 59 cm, L_{eff} :
325 51 cm). BGE: 50 mM APFOA at pH 9.5
326 water/methanol (90:10, v/v), $[NH_4OH]/[PFOA]$
327 = 3.4. Samples injected at 50 mbar for 5 s. + 25
328 kV, 25 $^{\circ}C$, λ : 195 nm. FAs injected
329 concentration: 1 mM.

330

331 The pH also plays an important role in the separation buffer because it defines the
332 ionization forms of species in solution and thus affects their electrophoretic mobilities.
333 The separation of FAs often requires a buffer at pH > 9 to ensure a strong
334 electroosmotic flow (EOF). In our case, working at high pH also helped to maintain the
335 buffering efficiency of the ammonia solution (pKa ~ 9.2). The influence of the ammonia
336 concentration or the pH was then evaluated. Therefore, the initial ratio $R =$
337 $[NH_4OH]/[PFOA] = 3.4$ (pH = 9.5) was then increased to 5.1 (pH = 10). The separation
338 performances of the three FAs were compared in these two conditions. Different
339 parameters including migration times, peak widths, and intensities were under focus,
340 and the repeatability of the analysis was evaluated. The results in Figure 5A (left)
341 showed that increasing 1.5 times the ammonia concentration in the BGE led to slightly
342 longer migration times of FAs (2 % for ALA, 4 % for LA, and 6 % for OA). At $R = 3.4$,

343 FA peak widths were reduced, and peak intensities were slightly higher than at R =
 344 5.1 (Figure 5A, center and right). In contrast, higher ammonia concentration (R = 5.1)
 345 led to better repeatabilities of peak widths and intensities, in particular for OA, for which
 346 RSDs were 7 % and 5 %, respectively (Figure 5B), instead of 23.5 % and 15.5 %, respectively,
 347 respectively, at R = 3.4. Therefore, the [NH₄OH]/[PFOA] ratio at 5.1 has finally been
 348 preferred and selected for the separation of these three FAs.



349

350 **Figure 5.** A) Effects of R=[NH₄OH]/[PFOA] on the migration times, peak widths, and intensities
 351 of three FAs: alpha linolenic acid (ALA), linoleic acid (LA), and oleic acid (LA). A triplicate
 352 injection has been performed for each sample. All reported values were averaged. B) Effect
 353 of the R ratio on the interdays repeatability (n = 4) of the previous parameters, reported as
 354 relative standard deviation (RSD, %).

355

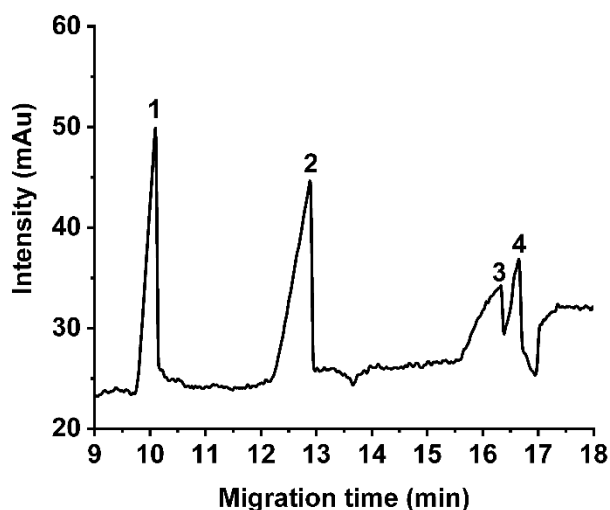
356 3.1.4 Optimization of the methanol content in the sample (injection buffer)

357 The methanol content in the sample was optimized to enable analysis of both *cis*- and
 358 *trans*-isomers of FAs. In a first approach, the sample preparation of FAs consisted in
 359 their dilution in the BGE. The three *cis*-isomers ALA, LA, and OA were found to easily
 360 dissolve in the BGE consisting of 50 mM APFOA with 10 % (v/v) methanol. However,

361 in the same conditions, the *trans*-isomer of OA, namely elaidic acid (EA), was found
362 to form an opaque solution, resulting in an electropherogram with unstable baseline.
363 Therefore, the BGE was gradually diluted with methanol to get an injection buffer
364 containing EA giving satisfying CE analysis. Considering solubility, peak widths, and
365 intensities, the optimal amount was found to be 53 % (v/v) methanol. This composition
366 was then used for all samples. Figure 6 presents the CE analysis a sample containing
367 a mixture of ALA, LA, OA, and EA (1 mM each), using the simple (previously
368 optimized) BGE consisting of 50 mM APFOA ($[\text{NH}_4\text{OH}]/[\text{PFOA}] = 5.1$, pH = 10) with
369 10 % (v/v) methanol. It shows the effective separation of these FAs differing by the
370 number of unsaturations (ALA, LA, and OA) and the *cis/trans* configuration (OA and
371 EA, with resolution peak = 1.6).

372 Cyclodextrins and derivatives are often added in the BGE to improve the separation
373 of isomer mixtures. Thus, we tried adding 5 to 15 mM β -cyclodextrin in the BGE to get
374 a better OA vs EA separation. However, in these conditions, the resolution of those
375 two latter peaks was not improved.

376 Despite the optimization of all above-mentioned parameters, we always observed
377 asymmetric peaks. Only the dilution of FA samples improved peak symmetry.
378 However, APFOA has a high UV absorption which severely limits FA detection upon
379 dilution and imposes working at high analyte concentration (1mM) in sample.



380
381 **Figure 6.** Separation of four unsaturated free
382 FAs C18: (1) alpha linolenic acid ALA, (2)
383 linoleic acid LA, (3) oleic acid OA, and (4)
384 elaidic acid EA. CE conditions: fused silica
385 capillary (id: 50 μm , L_{tot} : 59 cm, L_{eff} : 51 cm).
386 BGE: 50 mM APFOA at pH=10 water/MeOH
387 (90:10, v/v), $[\text{NH}_4\text{OH}]/[\text{PFOA}] = 5.1$. Samples

388 injected at 50 mbar for 5 s. +25 kV, 25 °C, λ :
389 195 nm. Concentration of each FA: 1 mM.

390

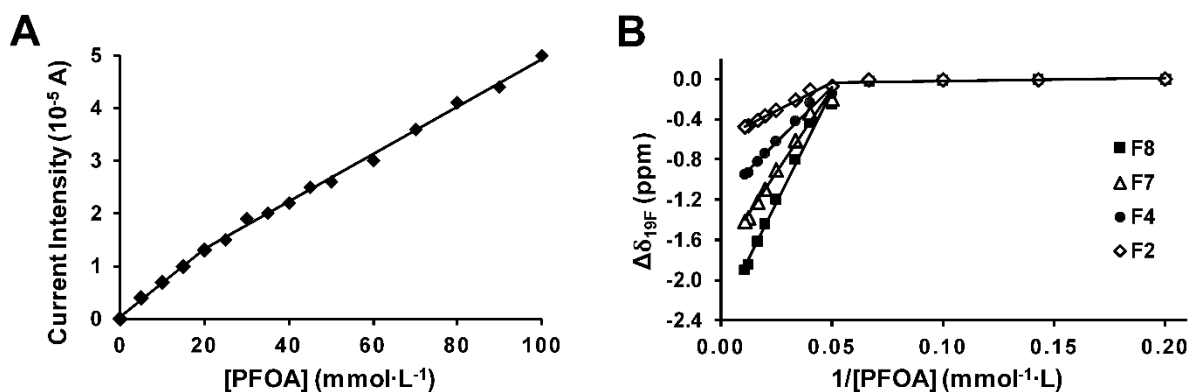
391 **3.2 Characterization of APFOA micelles (50 mM APFOA, R = 5.1 in** 392 **10 % methanol v/v)**

393 The mechanism allowing the separation between FAs using APFOA micelles remains
394 unclear: how the poor interactions between perfluorinated chains and
395 perhydrogenated chains could assist the separation of these unsaturated C18 acids?
396 To understand this point, we initiated the characterization of APFOA micelles. We first
397 conducted experiments using the CE instrument to determine the cmc and the charge
398 density of the micelles. Then, we also determined the mobility of the perfluorinated
399 micelles by introducing a PFOA analogue marker, namely the N-perfluorooctanoyl
400 phenylalanine.

401

402 **3.2.1 Determination of the cmc of APFOA**

403 The cmc of APFOA in the presence of 10 % (v/v) methanol and at R = 5.1
404 ($[\text{NH}_4\text{OH}]/[\text{PFOA}]$) was determined. The cmc is the minimal concentration at which
405 APFOA micelles start to form, and above which the concentration of free APFOA
406 (C^{RCOO^-}) remains constant and equal to the cmc. The formation of micelles induces a
407 change in the conductivity of the medium. The conductivities of APFOA solutions were
408 obtainable *via* the current intensities in the capillary, which were plotted as a function
409 of PFOA concentration (Figure 7A). The curve consisted in two linear parts with
410 different slopes: the cmc was obtained at the intersection of these two parts.



411

412 **Figure 7.** Determination of PFOA critical micellar concentration in the presence of 10 % (v/v)
413 methanol by (A) CE and (B) NMR. (A) Current intensity in the capillary as a function of PFOA

414 concentration. CE conditions: fused silica capillary (id: 50 μm , L_{tot} : 59 cm, L_{eff} : 51 cm). BGEs:
415 x mM APFOA at pH 9.5 water/methanol (90:10, v/v), x = 5 to 50 mM, $[\text{NH}_4\text{OH}]/[\text{PFOA}] = 5.1$.
416 +25 kV, 25 $^\circ\text{C}$, λ : 195 nm. (B) Chemical shift changes of Fluorine ($\Delta\delta_{19\text{F}}$) measured at 376
417 MHz, 25 $^\circ\text{C}$ as a function of the reciprocal of PFOA concentration. F2, F4, F7, and F8 refer to
418 ^{19}F NMR assignments of APFOA as reported in the experimental section.

419

420 As proposed by Cifuentes *et al* [24] we can measure the cmc of the APFOA in such
421 solution (Figure 7A). Here, the cmc was determined to be 19 +/- 0.9 mM. The
422 confirmation of the cmc value was performed by NMR experiments by following the
423 chemical shift variations of ^{19}F , and by plotting them as a function of the reciprocal
424 PFOA concentration (Figure 7B). The resulting curve consisted in two linear parts, the
425 second having an almost zero slope. The cmc was obtained at the intersection of these
426 two parts, and found to be 19 +/- 0.9 mM. Thus, the NMR data also confirmed this
427 value.

428

429 **3.2.2 Degree of counterion dissociation of the perfluorinated micelles**

430 The degree of counterion dissociation can be determined as the ratio of the slopes
431 above cmc (a_2) and below cmc (a_1) in the $I = f(C^f)$ representation (Figure 7A) [31], as
432 described in the experimental section. This ratio gives the degree of counterion
433 dissociation, β , and subsequently the degree of counterion association α : $\alpha = (1-\beta)$. In
434 our case $\beta = 0.68$, which is a very high value as compared to the one obtained for SDS
435 micelles ($\beta=0.34$ [27], or $\beta=0.28$ [32]). In another study, Kancharla *et al.* have found a
436 β value of 0.47, in the absence of MeOH in the BGE. The value was found to greatly
437 increase in the presence of urea [33]. Short-chain alcohols and urea have similar
438 effects on the cmc of APFOA as its value decreases when these compounds are
439 added to the APFOA solutions [33,34]. Therefore, both MeOH and urea could have a
440 similar effect on the counterion dissociation: the presence of MeOH in the BGE could
441 explain the high β -value observed in our experimental conditions. Such a significant
442 value makes the micelles clearly negatively charged and not neutral.

443

444 **3.2.3 Measurements of the mobilities of the micelles and the free APFOA**

445 To measure the mobility of the APFOA micelles (μ^{mic}), an aromatic analog of PFOA
446 was synthesized by grafting PFOA on phenylalanine *via* an amide link. The obtained
447 compound, which will have an important affinity for APFOA micelles, will allow

448 measuring the migration time of these aggregates by UV-detection at 260 nm
 449 (absorption of phenylalanine). We obtained a migration time of 146 ± 5 min ($n = 3$),
 450 which is much higher than the one of the measured EOF, $t_{EOF} = 3.25$ min (injection of
 451 water/MeOH 47/53, v/v). In MEKC, the negatively charged micelles normally have a
 452 negative mobility compared to the EOF. Moreover, the velocity of the micelles in
 453 absolute value is very close to the one of the EOF: the calculated values are $\mu^{EOF} =$
 454 $61.7 \cdot 10^{-9} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ and $\mu^{mic} = -60.4 \cdot 10^{-9} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$. Thus, there is almost no movement
 455 of the micelles inside the capillary and the separation of FAs in these conditions
 456 depends on their interaction and residence time in the micelles.

457 3.2.4 Capacity factor (k') and residence time (RT) of the FAs in the micelles

458 To better understand the mechanism underlying the separation of FAs in our
 459 experimental conditions, their capacity factor and their residence time in the micelles
 460 have been studied. Terabe *et al* [35,36] proposed the measurement of k' to appreciate
 461 the interaction between the solutes (here the FAs) and the micelles, for neutral
 462 species. Khaledi *et al* completed this study for acidic solutes [37]. k' is defined as:

$$463 \quad k' = n_{mic}/n_{aq} = (\mu^{ag} - \mu^{FA})/(\mu^{mic} - \mu^{ag}) \quad (5)$$

464 where n_{mic} and n_{aq} are the molar quantities of the solute in the micelles and in the
 465 aqueous media, respectively, μ^{ag} is the mobility of the FAs using a micellar medium
 466 (*i.e.* corresponding to the fatty acid migration time), while μ^{FA} is their mobility in the
 467 absence of micelles. μ^{FA} is not easy to determine because FAs are not soluble in 10%
 468 methanol and 255 mM ammonia. μ^{FA} was thus estimated at the cmc of APFOA,
 469 considering that, at the cmc, the concentration of micelles is very low and can be
 470 neglected. In these conditions, we found an average value of $\mu^{FA} = -20.8 \cdot 10^{-9} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$.
 471

472 The residence time RT_{mic} of the solute in the micelle is defined as:

$$473 \quad RT_{mic} = t_r \cdot k' / (1 + k') \quad (6)$$

474 where t_r and k' are the migration time and the capacity factor, respectively. Table 1
 475 reports the values obtained for the migration time, capacity factor, and residence time
 476 for the three FAs ALA, LA, and OA.

477

478 **Table 1.** MEKC parameters of the perfluorinated micelles. Migration time t_r , capacity factor k' ,
 479 residence time in the micelles RT_{mic} , of the three FAs in the perfluorinated micelles.

t_{mc} 146.0 min, t_0 3.25 min, [APFOA] 50 mM			
	t_r (min)	k'	RT_{mic} (min)
ALA	10.8	1.3	6.1
LA	13.2	1.9	8.6
OA	16.9	2.8	12.4

480

481 The residence times of FAs are ranging from 6.1 min to 12.4 min in the micelles,
 482 suggesting that FAs spend 56 % to 73 % of their migration time into the micelles. Such
 483 high values were not expected since interactions are usually not favored between
 484 perhydrogenated molecules and perfluorinated micelles. In addition, the residence
 485 time decreases as the number of unsaturations of the FAs increases: OA (1
 486 unsaturation) is more prone to insert into micelles than ALA (3 unsaturations). As a
 487 consequence, reminding that the micelles almost do not move inside the capillary, FAs
 488 that reside less in these micelles will migrate first in the order ALA>LA>OA. In this
 489 system, the separation mechanism originates directly from the interactions between
 490 the analytes and the micelles. From these observations, we can suggest a separation
 491 process similar to what is usually observed in the case of SDS used in MEKC, which
 492 remains quite surprising considering the repulsive interactions between
 493 perhydrogenated and perfluorinated chains. Complementary structural analysis at the
 494 *meso* scale will provide more in depth characterization of the separation mechanism
 495 [38].

496

497 **4 Concluding remarks**

498 In this article we have shown that perfluorinated micelles of APFOA in the presence
 499 of 10% methanol and a ratio $[NH_4OH]/[PFOA] = 5.1$ allow the separation of four
 500 unsaturated C18 FAs by MEKC. The relatively high electrophoretic mobility ($\mu^{mic} =$
 501 $60.4 \cdot 10^{-9} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$) of the micelles is certainly due to the high degree of counterion
 502 dissociation of the perfluorinated micelles ($\beta = 0.68$). This mobility is much higher than
 503 the micelles of SDS ($-22.2 \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$) [35]. In the literature it is reported that the mixture
 504 of perfluorinated micelles with perhydrogenated ones exhibits severe non idealities
 505 [21]. However, the successful separation of the different FAs indicates that there is a
 506 specific interaction with the micelles, which constitute a pseudo-chromatographic
 507 phase, because their displacements are very slow, *i.e.* their electrophoretic mobilities

508 are almost compensated by the EOF. Indeed, the residence times of FAs in the
509 micelles are long, more than 56% of their full migration times. In this study we show
510 that it is possible to separate FAs thanks to their number of double bounds or the
511 double bond position in the carbon chain. However, the characterization of the
512 interactions between the FAs and the micelles is not accessible with CE. Therefore
513 we conducted other complementary studies involving scattering experiments which
514 are described in a complementary article [38].

515 **Conflict of interest**

516 The authors have declared no conflict of interest.

517 **Data Availability Statement**

518 The data that support the findings of this study are available from the corresponding
519 author upon reasonable request.

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