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1 **Studying the real-time interplay between triglyceride digestion and lipophilic**
2 **micronutrient bioaccessibility using droplet microfluidics. 1 Lab on a chip method**

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5

6 **Abstract**

7 This article is the first part of a series reporting on real-time digestion kinetics of triglyceride
8 droplets containing different lipophilic micronutrients. This part focuses on the design,
9 fabrication, and operation of a polydimethylsiloxane microfluidic device which enables the
10 generation and digestion of oil droplets. The micro-channels were made hydrophilic to obtain
11 oil droplets in an aqueous continuous phase. Optimized chip design and outlet control were
12 implemented to provide efficient oil droplet generation, manipulation, and immobilization on a
13 single chip. Highly monodisperse oil droplets were generated, immobilized in an array of
14 traps and monitored in real time by fluorescence using a confocal microscopy method. The
15 device was used to study the kinetics of beta-carotene release during tricaprylin digestion
16 (intestinal lipolysis and micellar solubilization). The effect of the gastric phase on beta-
17 carotene degradation was also investigated using the same method.

18

19 **1. Introduction**

20 Over the past decades, the development of technologies based on microfluidics has
21 expanded in analysis and research domains (Huebner et al., 2009; Šalić, Tušek, & Zelić,
22 2012). Indeed, the use of micro-scale experimental devices involves small sample volumes
23 with a high surface-volume ratio that allows reduction of costs and rapid kinetics study. In
24 addition, the optical transmission of the materials commonly used in microfluidics (mostly
25 polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), and glass) provides high
26 flexibility to use external light-based analysis techniques for real-time monitoring (Desai &
27 Zaman, 2015; Heus et al., 2010; Mongersun, Smeenk, Pratz, Asuri, & Abbyad, 2016;
28 Windbergs & Weitz, 2011). With the trend of further reducing the sample volume, droplet
29 microfluidics was developed. In this technique, each droplet is used as an independent
30 micro-reactor of pico- to nano- litre scale (Huebner et al., 2009; Huebner, Abell, Huck,
31 Baroud, & Hollfelder, 2011; Mongersun et al., 2016). However, most of the studies are based

32 on water droplets, while oil droplets are rarely explored. In particular, lipid droplets containing
33 lipophilic micronutrients or hydrophobic drugs were rarely investigated.

34 On the contrary, *in vitro* digestion of lipid and lipophilic bioactive molecules is extensively
35 carried out using emulsions (Marze, 2015; Li, Kim, Park, & McClements, 2012).
36 Nevertheless, even in a “simple” system such as emulsion, studying mechanisms is still
37 challenging due to many interactions involved simultaneously. Emulsion digestion is indeed
38 influenced by many physicochemical characteristics which are difficult to control. Moreover,
39 real-time kinetics studies of lipid/lipophilic molecules digestion were rarely achieved using the
40 conventional emulsion approach.

41 Those issues can be solved using droplet microfluidics. In that context, the use of the lipid
42 droplet microfluidic digestion system recently developed by Marze et al. showed equivalent
43 results to those obtained from static *in vitro* digestion of emulsions (Marze, Algaba, &
44 Marquis, 2014). Then, Scheuble et al. reported a similar approach based on lipid droplet
45 digestion with multiple oil droplets trapping to study the coalescence effect (Scheuble et al.,
46 2017). Using various lipid droplets in a microfluidic device can be seen as a potential
47 screening approach not only for the digestion of lipids but also for the release of lipophilic
48 bioactive molecules. However, several difficulties needed to be solved to make droplet
49 microfluidics an experimental standard for lipid studies in micro-reactors. First, the micro-
50 channel surface has to be hydrophilic to enable oil droplet generation and manipulation. As
51 most microfluidic materials are natively hydrophobic, a chemical treatment is required to
52 obtain a persistent hydrophilic surface (He et al., 2011; Marze et al., 2014; Tan, Xu, Li, &
53 Luo, 2008; Wang, Lu, Xu, & Luo, 2009). The second difficulty is the control of the oil droplet
54 generation, which may undergo flow instabilities due to the high viscosity of edible oils
55 compared to that of water (Marze et al., 2014). Finally, a proper optical setup is needed to
56 quantify the kinetics of lipophilic molecules in real time.

57 In this article, we present an optimized microfluidic platform to overcome these limitations. A
58 single PDMS chip based on a microfluidic device with hydrophilic surface modification was
59 developed to generate and manipulate monodisperse oil droplets that are immobilized in an
60 array of traps. The hydrophilic treatment and device storage were optimized to obtain a long
61 hydrophilicity persistency of the channel surface. The oil droplet generation and flow were
62 stabilized by an open-close procedure of the outlets, with no extra devices or valves
63 required. The use of this platform is illustrated by examples of single and multiple droplet
64 trapping. Then, the implementation of a confocal fluorescence microscope setup for real-time
65 monitoring is illustrated by the kinetics of beta-carotene release from tricaprylin droplets

66 during digestion. The kinetics of beta-carotene degradation in gastric conditions is also
67 presented, monitored in real time using this setup as well.

68

69 **2. Experimental Section**

70 **2.1. Materials**

71 The negative photoresist (SU8-2100) was from MicroChem Corp, PDMS (RTV615) was from
72 Eleco Produits, polytetrafluoroethylene (PTFE) tubes (11919445) were from Fisher Scientific,
73 stainless steel tubes (Coop 23G/15 mm) and Luer lock needles (LS22) were from Phymep.
74 The other chemicals were provided by Sigma-Aldrich: propylene glycol methyl ether acetate
75 (PGMEA), benzophenone, acrylic acid, pancreatic lipase (L3126, lipase from porcine
76 pancreas type II, 1.6-8.3 U mg⁻¹), sodium glycodeoxycholate (G9910), tricaprylin (T9126),
77 beta-carotene (22040), Amano lipase A (534781, fungal lipase from *Aspergillus niger*, 12 U
78 mg⁻¹, protease activity \leq 2.5 U mg⁻¹), pepsin (P7012, from porcine gastric mucosa, 2500 U
79 mg⁻¹). β -lactoglobulin was purified from whey protein isolate in our laboratory. Milli-Q water
80 with an electrical resistivity of 18.2 M Ω was used for all solution preparations.

81 **2.2. Microfluidic Device**

82 The preparation of the PDMS microfluidic device is based on soft lithography techniques
83 using silicon wafer for the master (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001).

84 **2.2.1. Photomask design.** We used the Adobe Illustrator software to draw the mask design
85 which was then printed out as a photo mask by high resolution printing.

86 **2.2.2. Master fabrication.** The master was made using photolithography techniques. A thin
87 layer of negative photoresist was coated on a silicon wafer using a spin-coater (SPIN150,
88 SPSEurope). This was prebaked for 5 min at 65 °C followed with 20 min at 95 °C before
89 being exposed to UV light (365 nm) for 40 s through the photomask by a UV LED masker
90 (UV-KUB 2, Kloé). The post-baking was done for 5 min at 65 °C followed by 10 min at 95 °C.
91 Finally, the master with microstructures of 120 μ m in height was obtained by development
92 with a solution of PGMEA for 20-30 min. Propanol was used to wash excess products of
93 development, resulting in a clean master.

94 **2.2.3. PDMS chip.** The device was fabricated using similar techniques found in Marquis et
95 al. (Marquis, Renard, & Cathala, 2012). One device is composed of two PDMS parts bonded
96 by the gradient technique. First, two PDMS/crosslinker mixtures (10% or 5% crosslinker)
97 were poured on the master and in a Petri dish, respectively. Then, both parts were degassed
98 in a vacuum chamber (50 mbar). After the degassing step, both parts were cured at 70 °C for
99 30 min. The cured PDMS (10% crosslinker) was cut and peeled off the master before access

100 holes for inlets and outlets were punched through PDMS. Then, this PDMS part was cleaned
101 and assembled with the 5% crosslinker PDMS in the Petri dish by curing at 70 °C for 1 hour.
102 Stainless steel tubes were inserted in the access holes, reinforced by plastic rings filled with
103 cured PDMS (5% of crosslinker). Finally, the device was cured overnight at 70 °C and the
104 stainless steel tubes were replaced by new ones for the inlets/outlets of the device. The
105 design of the chip is shown in fig. 1a.

106 **2.2.4. Hydrophilic treatment.** PDMS surface is natively hydrophobic (water contact angle >
107 100°) (Mata, Fleischman, & Roy, 2005). In this work, the PDMS device was used to generate
108 and trap oil droplets in an aqueous continuous phase. Thus, the surface of PDMS channel
109 needed to be modified. A hydrophilic treatment was achieved by UV-initiated graft
110 polymerization of acrylic acid as proposed by Schneider et al. (Schneider, Willaime, Tran,
111 Rezgui, & Tabeling, 2010). The first step was the injection of a 10% benzophenone in
112 acetone at a flow rate of 200 $\mu\text{L min}^{-1}$ for 10 min. Then, the remaining solution was blown out
113 by air flow and the device was placed under vacuum (85 mbar) for 35 min before the
114 injection of a 20% acrylic acid aqueous solution at a flow rate of 200 $\mu\text{L min}^{-1}$ for 5 min. Next,
115 the acrylic acid solution was sealed into the device by closing access holes. The device was
116 illuminated with UV for 5 min using the UV LED masker. Finally, the device was cleaned by
117 successive flow of ethanol and water (pH 11) at 200 $\mu\text{L min}^{-1}$ for 1 hour. After the hydrophilic
118 treatment, the device was put inside a plastic bottle filled with distilled water (pH 11) and
119 stored at 4 °C to maintain the hydrophilicity of the channel surface.

120 **2.3. Droplet Generation and trapping**

121 All fluid flows were generated and controlled by syringe pumps 11 elite (Harvard Apparatus)
122 with glass syringes connected to the inlets of device by a Luer lock needle and PTFE tubes.
123 During the device operation, the outlets and inlets of the device were temporarily blocked by
124 a piece of PTFE tube filled with cured PDMS.

125 For droplet generation, the microfluidic device was placed under an IX51 inverted
126 microscope (Olympus) with a 4x objective. First, the continuous phase (7.5 mg mL⁻¹ β -
127 lactoglobulin in 10 mM NaH₂PO₄ adjusted to pH 7.0) and the oil phase (tricaprylin) were
128 injected into the micro-channels via inlet 1 at a flow rate of 150 $\mu\text{L min}^{-1}$, and via inlet 2 at a
129 flow rate of 4 $\mu\text{L min}^{-1}$, respectively. Then, the flow rate was decreased to 50 $\mu\text{L min}^{-1}$ for the
130 continuous phase and to 1 $\mu\text{L min}^{-1}$ for the oil phase to generate oil droplets of 100 μm .
131 Initially, outlet 4 was blocked, leading the first oil droplets to the waste tank via outlet 3.
132 When the desired oil droplet size was reached (100 μm), outlet 4 was opened and outlet 3
133 was blocked, leading the oil droplets to the chamber. When most of the chamber traps were
134 filled with one oil droplet, the oil flow was stopped and the aqueous continuous phase flow
135 was increased to 100 $\mu\text{L min}^{-1}$ to wash out any untrapped droplets of the chamber. Next,

136 outlet 3 was opened and outlet 4 was blocked to prevent any undesirable large oil droplets
137 from entering the chamber.

138 **2.4. Droplet digestion**

139 Initially, monodisperse tricaprylin droplets (100 μm in diameter) with added 0.2 wt% beta-
140 carotene were generated and trapped in the microfluidic device as described above. Then,
141 all four access tubes were blocked and the device was stored inside an aluminium box in
142 order to avoid chemical degradation due to ambient lights. The device containing the droplets
143 was then moved to a hot plate set on a confocal microscope stage (fig. 1b). The temperature
144 of the hot plate was set to 56 $^{\circ}\text{C}$ to maintain a measured temperature of 37 $^{\circ}\text{C}$ inside the
145 chamber. Using PTFE tubes, inlet 1 and outlet 3 were connected to the aqueous continuous
146 phase syringe (the same one used for droplet generation) and the digestive fluid syringe,
147 respectively. Outlet 4 was connected to the waste tank. Before thermal equilibrium was
148 reached, the continuous phase was injected into the chamber at a flow rate of 50 $\mu\text{L min}^{-1}$ to
149 prevent air bubble development due to temperature rising. Then, the flow of the continuous
150 phase was stopped and the reaction in the droplets was initiated by injecting digestive fluid
151 via outlet 3 at a flow rate of 50 $\mu\text{L min}^{-1}$. This flow rate was kept constant throughout the
152 reaction so that the digestive fluid in the chamber was theoretically renewed every 6 s. In
153 practice, we measured that a steady-state concentration was typically reached after 1 min
154 when replacing one solution by another one (results not shown).

155 Digestion was carried out with an intestinal phase or a gastric phase. For the intestinal
156 phase, 10 mL of buffer solution (100 mM NaH_2PO_4 adjusted to pH 7.0) was mixed with
157 pancreatic lipase at 4 mg mL^{-1} and a bile salt (sodium glycodeoxycholate) at 5 mg mL^{-1} to
158 prepare a fresh intestinal digestive fluid forming an aqueous micellar solution due to the bile
159 salt. This fluid was centrifuged at 1000g for 15 min to remove large residues before injection
160 into the chamber.

161 The gastric phase experiments were run for 2 hours with a gastric digestive fluid containing
162 0.03 mg mL^{-1} lipase from *Aspergillus niger* (lipase AN), and 0.6 mg mL^{-1} pepsin in a 100 mM
163 KCl buffer adjusted to pH 3.0. In order to get insights into the mechanisms of BC degradation
164 during the gastric phase, three compositions of the gastric fluid were tested: i) lipase AN and
165 pepsin, ii) lipase AN without pepsin, iii) only buffer with no enzymes.

166 **2.5. Lipid monitoring**

167 Tricaprylin (TC) droplets containing beta-carotene (BC) were observed during the digestion
168 using a confocal microscope (Nikon A1+) with a 10 \times objective. The pinhole was set so that
169 the thickness of the optical section was larger than the droplet initial diameter. Seven trapped
170 droplets were monitored simultaneously in the field of view. A laser with an excitation
171 wavelength of 488 nm and an emission window of 500-530 nm was used to obtain the

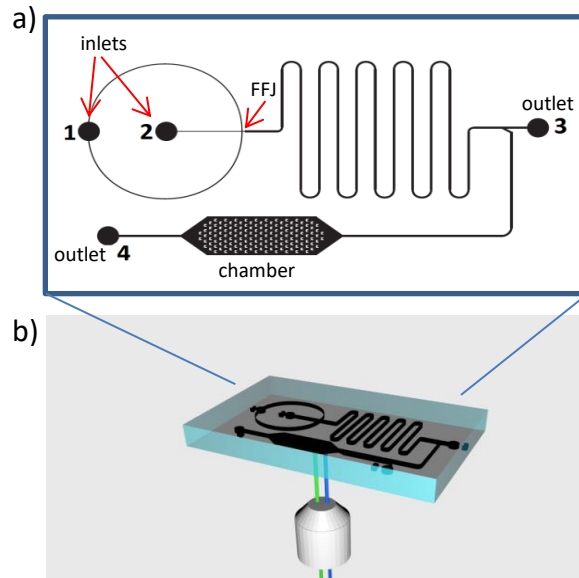
172 autofluorescence image of BC contained in the oil droplets. A transmitted light image for the
173 droplet size was obtained simultaneously using the same excitation beam (Paddock, 2000).
174 For quantitative analysis, a calibration curve was constructed with five points. TC droplets
175 with various BC concentrations (0 wt% as the negative control, 0.05, 0.2, 0.4, 0.5 wt%) were
176 trapped in five different microfluidic devices to measure the fluorescence intensity due to BC
177 autofluorescence (no dye is used in the experiments). The degradation of BC was also
178 checked as a function of time for TC droplets containing BC. In those tests, the conditions
179 were the same than for intestinal digestion, except only the buffer solution (pH 7.0) was
180 injected instead of the digestive fluid. The measured fluorescence intensity was found to be
181 proportional to the BC concentration inside the oil droplets, independently on the droplet size,
182 and the degradation was found to be negligible (supplementary material S1).
183 Images were recorded automatically with an interval of 2 min or 5 min for intestinal or gastric
184 digestion, respectively. Then, image analysis was performed to measure the size of the
185 seven droplets in the field of view and their average fluorescence intensity. The droplet size
186 was converted to the droplet volume, and the average fluorescence intensity was converted
187 to BC concentration inside the oil droplet.

188 Note that the analysis was developed using pure chemicals (pure tricaprylin and a single bile
189 salt) to avoid autofluorescence of undesirable molecules. As lipophilic micronutrients have
190 specific fluorescent properties, it was found that real edible oils could be used with no
191 fluorescence overlapping. In contrast, bile extract was difficult to use because it contains
192 many unidentified molecules, resulting in fluorescence over a wide wavelength range (see
193 the second article of this series).

194 For each system, two to three independent digestions were conducted with the monitoring of
195 seven individual droplets for each digestion. A distinct microfluidic device was used for each
196 digestion to ensure identical initial conditions. The variability of the measurements was very
197 low between the seven droplets monitored during one digestion, so the error bar (plotted as
198 the standard deviation) represents the variability of the two to three independent digestions.

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218 Fig. 1 a) Design of the PDMS microfluidic device for the generation, immobilization, and
219 digestion of oil droplets. b) Real-time reactivity monitoring using confocal fluorescence
220 microscopy.

221

222 3. Results and discussion

223 3.1. PDMS device fabrication

224 The PDMS device was constructed using two PDMS layers with different crosslinker
225 concentrations (gradient technique). This technique resulted in a better association between
226 the two PDMS parts compared to the plasma treatment method, eliminating the leakage
227 during device operation while simplifying device fabrication. Moreover, this enabled the
228 fabrication of 100% PDMS devices, which are lighter and easier to shape compared to
229 devices made of PMMA, or using glass as the bottom part (Desai & Zaman, 2015; Huebner
230 et al., 2009). In addition, PDMS has a broader transmission range (transmittance > 85%)
231 than that of glass and PMMA, what is an advantage for the fluorescence analysis
232 (Žukauskas et al., 2014).

233 3.2. PDMS surface modification

234 For the generation of oil droplets, the PDMS surface was rendered hydrophilic using the
235 method proposed by Schneider et al. (Schneider et al., 2010). In this method, the
236 benzophenone molecules diffuse into the PDMS matrix and play the role of the photoinitiator
237 of UV polymerization, turning acrylic acid (pre-absorbed in the PDMS) into grafted hydrophilic
238 poly(acrylic acid). The advantages of this method are to keep the geometry of the channel

239 unchanged and to provide a long persistency of the surface channel hydrophilicity. The post-
240 treated devices still have an operational hydrophilic surface after one month of storage in pH
241 11 water at 4 °C. On the contrary, the layer coating technique changed the PDMS device
242 geometry (Abate, Lee, Do, Holtze, & Weitz, 2008), and the hydrophilicity obtained via the
243 plasma treatment method was only stable within 24 hours (Marze et al., 2014). The
244 hydrophilic persistency we obtained enabled a systematic preparation of microfluidic devices
245 that could be stored for later experiments.

246 **3.3. Oil droplet immobilization**

247 Oil droplets were generated in the flow focusing junction (FFJ) and trapped in the chamber of
248 the microfluidic device (see fig. 1a). At the FFJ, the aqueous continuous phase entered
249 perpendicularly to the oil disperse phase, facilitating the formation of oil droplets
250 (supplementary video 1). Due to fluid handling limitation and instabilities, oil droplet size
251 could not be modulated in our previous work (Marze et al., 2014). Here, the flow of initial
252 polydisperse large oil droplets was led to the waste tank. When the desired droplet size was
253 reached, the flow was switched to the chamber. Supplementary video 2 shows that this flow
254 switch does not change the droplet size and the monodispersity. Thus, our new design with
255 the chamber part and the FFJ part on the same chip leads to stable flows and enables
256 droplet sorting so that only size-controlled ones enter the chamber. Moreover, its operation is
257 more reliable, as the connection between the FFJ device and the chamber was a source of
258 air/liquid leakage, flow disturbance, and droplet coalescence in the previous design (Marze et
259 al., 2014). Nevertheless, one aspect needs to be discussed for this droplet generation
260 approach. During the flow switch, the difference of hydrodynamic resistances between the
261 two paths could disturb flow equilibrium. We optimized the geometry of the chip so that no
262 droplet flow interruption was observed. Then, the continuous phase flow rate is only 50-fold
263 higher than the oil flow rate, what is low compared to typical values required for viscous oils
264 (200-fold higher). Indeed, the disturbance of the flow equilibrium comes from the disturbance
265 of the pressure $\Delta P = R_h \cdot Q$, (ΔP : applied pressure, R_h : hydrodynamic resistance, Q : flow rate).
266 Thus, in the case of a low flow rate Q , the change in R_h will only have a small effect on the
267 pressure ΔP . On the contrary, in the case of a high flow rate, the flow equilibrium would be
268 more susceptible to the change in the hydrodynamic resistance. So the geometry (width,
269 height and length of the channel) of the two branches has to be calculated carefully to
270 suppress any hydrodynamic resistance difference.

271 Supplementary video 3 shows the trapping process of the oil droplets in the chamber. Most
272 of the traps are filled with oil droplets. The number of traps was made large enough (150
273 traps) so that several droplet-free traps do not compromise the experiment. The average
274 diameter of the oil droplets was manipulated to be $100 \pm 5 \mu\text{m}$ in the different experiments.

275 The monodispersity of the droplet size in the same experiment is about 0.8%, crucial for
276 repeatable measurements in the case of surface-dependent reactions. The total volume of
277 the oil droplets represents approximately 0.7% of the total volume of the chamber. The oil
278 droplets kept their spherical shape with no sign of surface deformations that could be caused
279 by local defects of the hydrophilic treatment at the internal surface of the traps (Marze et al.,
280 2014). The absence of hydrophilicity defects minimizes the contact between the oil droplets
281 and the trap, maximizing the accessible droplet surface area. This validates this passive
282 immobilization method by obstacles, which gives a high accessible droplet surface area and
283 a high trapping efficiency compared to active trapping methods (optical tweezers,
284 dielectrophoresis, acoustic trapping), which give a full accessible surface area but a low
285 trapping efficiency (Hunt, Issadore, & Westervelt, 2007; Lee et al., 2009; Park & Chiou,
286 2011).

287 During device operation, the order of opening and blocking of outlets 3 and 4 must be
288 respected to achieve monodisperse oil droplets trapping in the chamber. In this condition,
289 neither air bubbles nor droplet coalescence are observed inside the device during oil droplet
290 generation and trapping. This open-close procedure for the outlets is a very efficient method
291 to control the flow of oil droplets while simplifying the device structure, avoiding the use of
292 pneumatic valves (Unger, Chou, Thorsen, Scherer, & Quake, 2000), or torque-actuated
293 valves as proposed by Weibel et al. (Weibel et al., 2005). Also, our method does not require
294 infusion-withdraw pumps to control the droplet flow (Huebner et al., 2009).

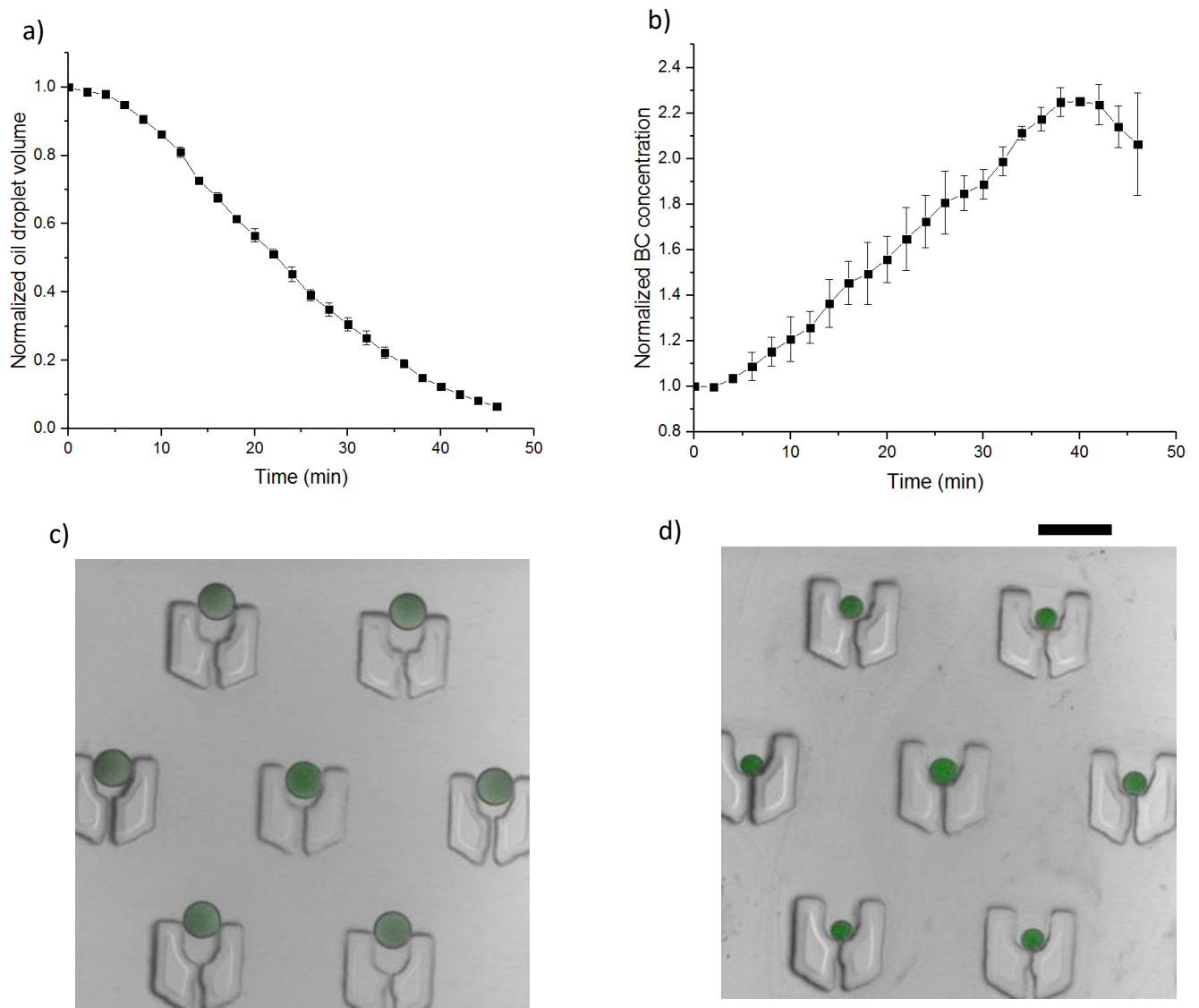
295 Our device was also used to obtain multiple droplets trapped in a single trap for coalescence
296 studies. Monodisperse TC droplets of 90 μm diameter were generated and trapped. In this
297 case, the droplet size was smaller than that of the trapping space, resulting in two droplets
298 per trap. This configuration was used to study the effect of droplet coalescence during the
299 gastric phase on the subsequent intestinal droplet digestion (supplementary material S2).

300 Similar PDMS devices were proposed to immobilize aqueous droplets for enzymatic
301 reactions (Huebner et al., 2009), or oil droplets for lipid digestion (Marze et al., 2014). In this
302 work, oil droplets containing BC were used to study the release (or degradation) kinetics of
303 BC during the intestinal phase (or the gastric phase), as reported in the following section.

304 **3.4. Kinetics of beta-carotene release during intestinal digestion**

305 In order to illustrate the application of the device for release kinetics of lipophilic molecules,
306 immobilized TC droplets with added 0.2 wt% BC were generated (single droplet trapping)
307 and then submitted to intestinal digestion conditions. The kinetics of lipid digestion and beta-
308 carotene release were monitored simultaneously in real time with a confocal fluorescence
309 microscope setup.

310 Figs. 2 and supplementary video 4 show the evolution of oil droplet size and BC
 311 concentration as a function of digestion time. The droplet volume and BC concentration are
 312 reported as normalized values (relative to the initial values) in order to simplify the
 313 comparisons. Droplet volume is reduced over time (fig. 2a) because triglyceride lipolysis
 314 produces fatty acids and monoglycerides that exit the droplet as they are soluble in the
 315 aqueous micellar phase. The kinetics is similar to the one reported by Marze et al. (Marze et
 316 al., 2014). A discussion of the effect of lipase concentration on digestion kinetics is found in
 317 the supplementary material S3.
 318



319
 320 Fig. 2 a) Evolution of the normalized volume of TC droplets during intestinal digestion. b)
 321 Evolution of the normalized beta-carotene concentration inside TC droplet during intestinal
 322 digestion. c) d) Images of TC droplets containing beta-carotene at digestion times: 0, 24 min,
 323 respectively. The scale bar represents 200 μ m.

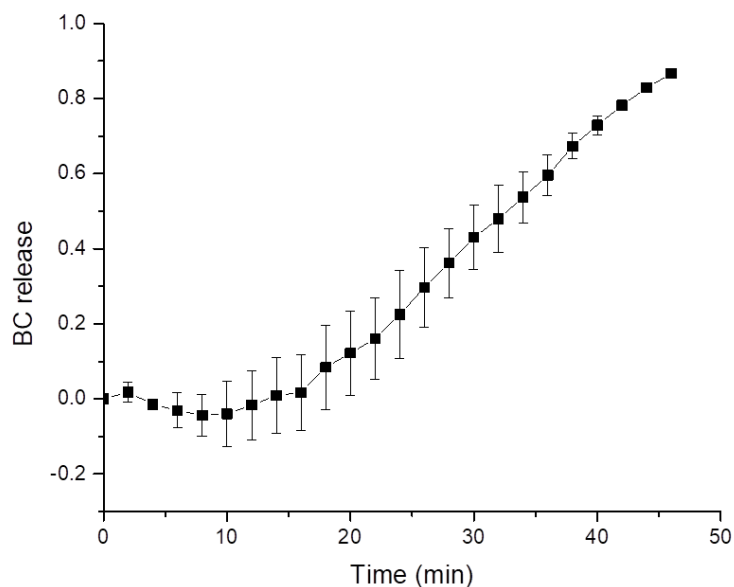
324

325 As evidenced in fig. 2b, a trend for BC concentration in oil droplets is observed. Indeed, BC
326 concentration mainly increases during digestion, although it reaches a maximum and then
327 decreases near the end of the digestion. This means that the reduction rate of oil droplet
328 volume is faster than the solubilizing rate of BC out of the droplets. Thus, BC concentrates
329 inside the reducing droplets. At the end of the digestion, triglyceride digestion rate slows
330 down but not the BC release rate (fig. 3), resulting in the decreasing trend for BC
331 concentration.

332 From the data presented in figs. 2a and 2b, BC quantity released out of the droplets
333 (incorporated in the micellar phase) can be calculated for quantitative analysis, using the
334 mass balance:

335
$$m_{RL} = m_{Di} - m_D \quad \text{with} \quad m_D = V_D C \quad (1)$$

336 Here m_{RL} is the mass of BC released from the oil droplet, m_{Di} is the initial mass of BC inside
337 the oil droplet, m_D is the mass of BC inside the oil droplet, V_D is the volume of the oil droplet
338 and C is the concentration of BC inside the oil droplet, determined from the fluorescence
339 intensity using the calibration curve. All values are presented in normalized form.



340

341 Fig. 3 Evolution of the BC proportion released from TC droplets during intestinal digestion
342 (bioaccessibility).

343

344 Kinetics of BC release during intestinal digestion is shown in fig. 3. Note that negative values
 345 can be obtained at the beginning of the digestion where both kinetics are slow. Due to these
 346 initial low releases, the determination of BC concentration is indeed sensitive to the precision
 347 of the calibration curve, and to fluctuations of the laser intensity. Nevertheless, the error bars
 348 in fig. 3 show that these values are not significantly different from zero.

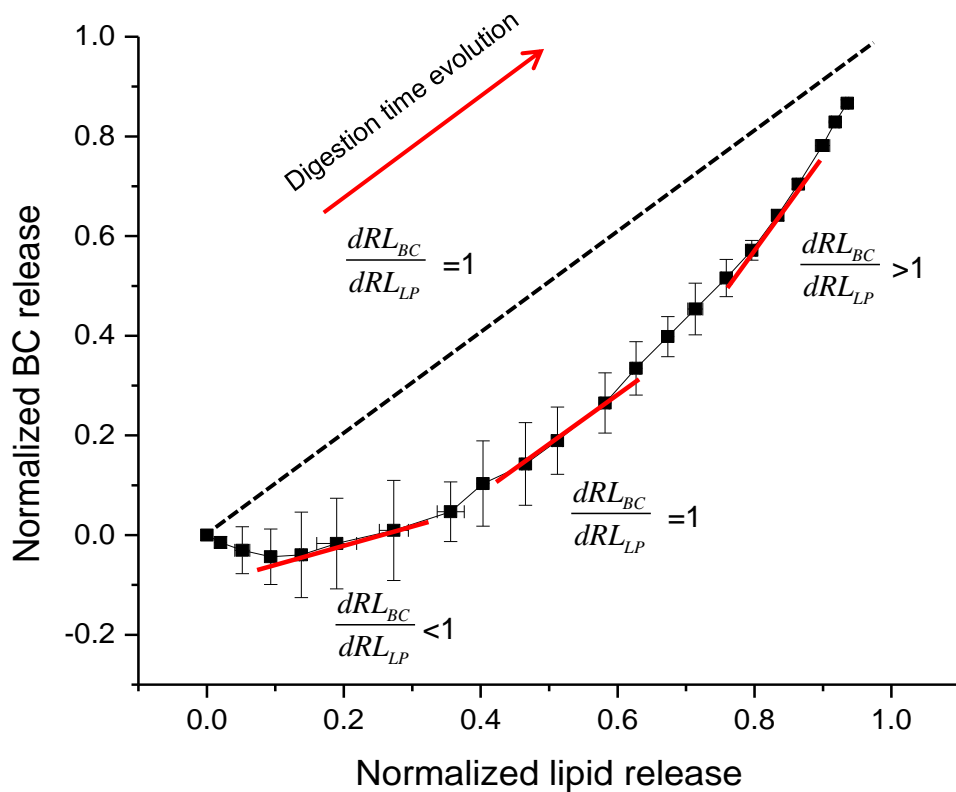
349 In the current non-static digestion conditions with continuous renewal of the intestinal
 350 digestive fluid, bile salt micelles come in large excess compared to the digestion products
 351 and BC to solubilize. That explains the higher release of BC (almost 90% bioaccessibility)
 352 than those typically reported for static digestion of emulsions in the literature (Nik, Corredig,
 353 & Wright, 2011; Mutsokoti et al., 2017; Salvia-Trujillo et al., 2017).

354 Fig. 4 shows the relation between the micellar solubilization of BC and of the lipolytic
 355 products during intestinal digestion, providing a better view of their kinetic interplay. The
 356 black dash line represents the “balance” case of identical BC and lipid release rate. In order
 357 to analyze the curves in fig. 4 in terms of relative kinetics, three tangent lines are added,
 358 representing different BC release regimes. The first order derivative of the curve $\frac{dRL_{BC}}{dRL_{LP}}$
 359 (slope of the tangent line) is equal to the release rate ratio between BC and lipids:

$$360 \quad \frac{dRL_{BC}}{dRL_{LP}} = \frac{\frac{dRL_{BC}}{dt}}{\frac{dRL_{LP}}{dt}} = \frac{\text{BC release rate}}{\text{Lipid release rate}} \quad (2)$$

361 In which, $\frac{dRL_{BC}}{dt}$ and $\frac{dRL_{LP}}{dt}$ are the first order derivative of BC and lipid release as a
 362 function of digestion time, respectively.

363 The slopes on the non-linear curve reveal three different kinetic regimes. In the first part
 364 (beginning of the digestion), the slope of the tangent line is smaller than 1 ($\frac{dRL_{BC}}{dRL_{LP}} < 1$),
 365 showing that BC release rate is slower than that of lipids. This caused the increase of BC
 366 concentration inside the oil droplets. In the middle part, a balanced regime can be observed.
 367 In the third part (end of the digestion), the release rate of BC becomes faster than that of the
 368 lipids ($\frac{dRL_{BC}}{dRL_{LP}} > 1$), explaining the decrease trend of BC concentration observed near the end
 369 of the digestion (fig. 2b).

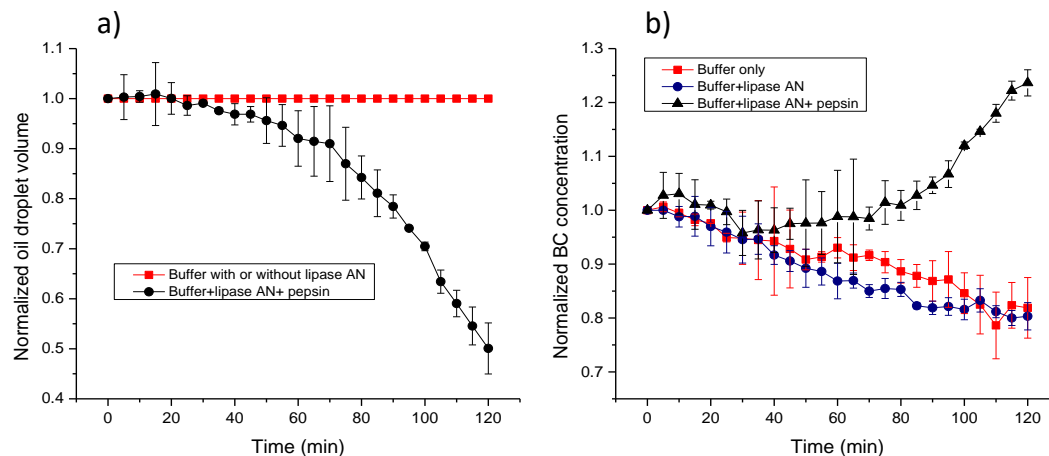


370
371 Fig. 4 Relation between the normalized mass release of BC and of lipids.

372

373 3.5. Beta-carotene degradation in gastric conditions

374 For this study, the same protocols (as in section 3.4.) were used except the intestinal fluid
 375 was replaced by a gastric fluid. Fig. 5a shows the evolution of the droplet volume in gastric
 376 conditions using various gastric fluid compositions. The decrease of the TC droplet volume
 377 was only seen with the gastric fluid containing both lipase AN and pepsin. This decrease
 378 reached up to 50% of the initial droplet volume, what is higher than the usual lipolysis degree
 379 (10-30%) measured during the gastric phase *in vivo* (Favé, Coste, & Armand, 2004). This
 380 difference could be explained by the absence of droplet coalescence, that has an essential
 381 effect in the stomach *in vivo* (Li et al., 2012). Marze et al. reported 10-20% TC droplet
 382 volume reduction (initial size of 137 μm) obtained after 55 min of gastric digestion using a
 383 similar device (Marze et al., 2014). The larger initial droplet size, shorter duration of the
 384 gastric phase, and the absence of pepsin in the gastric fluid could explain the lower lipolysis
 385 degree of tricaprylin. Fig. 5a also shows the role of pepsin, as no reduction of the droplet
 386 volume was obtained in the absence of pepsin in the gastric fluid. Indeed, pepsin hydrolyses
 387 the β -lactoglobulin protein initially coating the droplet surface, facilitating the adsorption and
 388 activity of lipase AN for triglyceride lipolysis.



389

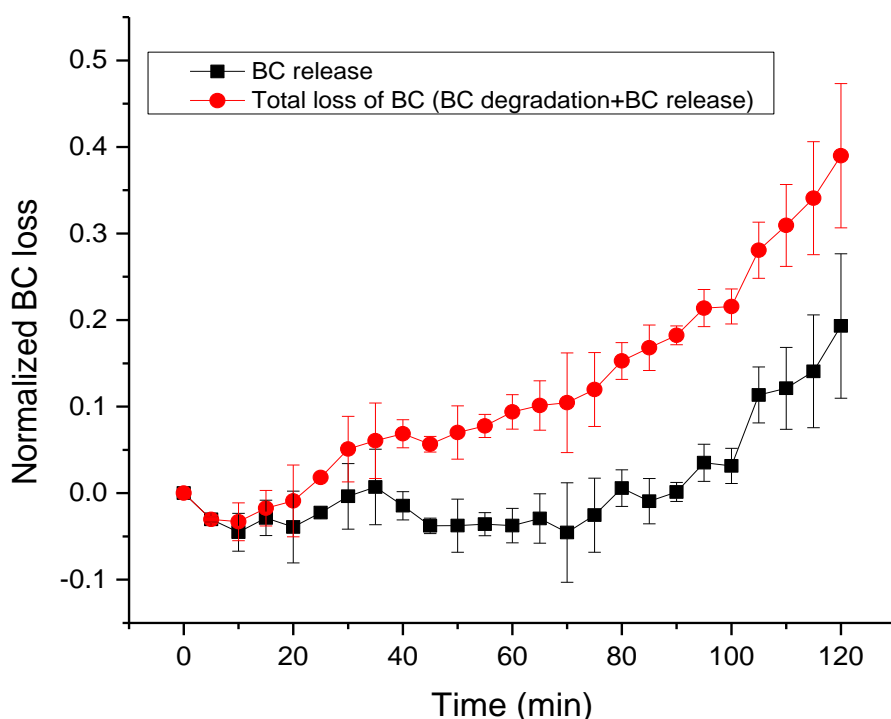
390 Fig. 5 a) Evolution of the normalized volume of TC droplets, and b) evolution of the
 391 normalized BC concentration inside TC droplets during gastric digestion with different gastric
 392 fluid compositions. In the cases of buffer with or without lipase AN, no change in droplet
 393 volume was observed.

394

395 Fig. 5b shows the evolution of BC concentration inside the TC droplets during the gastric
 396 phase. In the absence of pepsin (lipase AN or buffer only), as there was no change in droplet
 397 volume, the 20% decrease of BC concentration reveals a 20% degradation of BC. This result
 398 shows that this degradation is mostly due to the low pH condition (pH 3.0) during the gastric
 399 phase, as the addition of lipase AN has no significant effect on BC degradation. A 25%
 400 degradation of BC in emulsions during the gastric phase was reported by Kopec et al.
 401 (Kopec, Gleize, Borel, Desmarchelier, & Caris-Veyrat, 2017), regardless of the presence or
 402 absence of pepsin. In the case with lipase AN and pepsin, two different regions are
 403 observed, with a BC concentration decreasing trend during the first 40 min, followed by an
 404 increasing trend until the end of the gastric phase. During the first 40 min, there is almost no
 405 reduction of the droplet volume, thus the decrease of the BC concentration reveals the
 406 degradation of BC inside the oil droplet. The kinetics of BC degradation is similar than in the
 407 absence of pepsin, confirming the limited effect of pepsin on BC degradation. The region of
 408 increasing BC concentration is observed during the reduction of the droplet volume. In this
 409 region, it is likely that both BC degradation and BC release contribute to the kinetics.

410 In order to discriminate each contribution, we assumed that the degradation of BC in the
 411 presence of pepsin is similar to the case without pepsin (buffer+lipase AN). Thus, both BC
 412 degradation and BC total loss (degradation+release) could be calculated from the data
 413 presented in figs. 5a and 5b. Then, the BC release could be deduced. The evolutions of the
 414 normalized total loss and release of BC during the gastric phase are shown in fig. 6. This

415 result shows that BC release during the gastric phase is low (about 20%), occurring
416 significantly only after 100 min. This was not expected as BC is highly hydrophobic and
417 should only release in the presence of bile salt micelles. However, a similar result (up to 30%
418 BC release) was reported in the case of highly stable MCT emulsions using decaglycerol
419 monolaurate as the emulsifier (Liu, Hou, Lei, Chang, Gao, 2012). The absence of
420 coalescence might explain the efficiency of the release, although another factor is needed,
421 such as the possibility of tricaprylin lipolytic products (or decaglycerol monolaurate in Liu,
422 Hou, Lei, Chang, Gao, 2012) to form micelles able to solubilize BC.
423



424
425 Fig. 6 Normalized BC total loss (BC degradation+BC release) and BC release during gastric
426 digestion, in the case of buffer+ lipase AN+pepsin.
427

428 4. Conclusion

429 This work shows that the real-time kinetics of lipophilic bioactive molecules can be studied
430 using droplet microfluidics, as monitored by confocal fluorescence microscopy. The design
431 and fabrication of the setup were optimized to obtain a long hydrophilicity persistency of the
432 channel surface, and to facilitate the oil droplet generation and trapping. The development of
433 an open-close procedure of the outlets enabled the generation and trapping of oil droplets on

434 a single chip, and solved the issue of flow instabilities by compensating for the high oil
435 viscosity. These results show the potential of immobilized oil droplets to screen the reactivity
436 of lipophilic molecules. In the second article of this series and in subsequent works, the use
437 of this lab on a chip platform will be extended to different edible oils and fat-soluble
438 micronutrients. A comprehensive study of the degradation of antioxidant lipophilic molecules
439 during the gastric phase will be carried out as well. The whole approach can be generalized
440 to screen the reactivity of lipophilic molecules in the context of bioavailability studies which
441 are conducted in nutrition, pharmacology, and toxicology.

442

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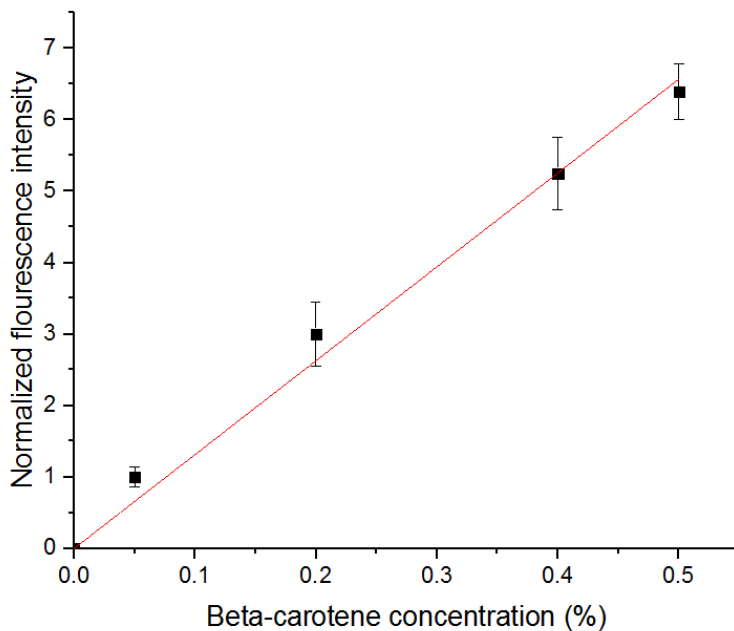
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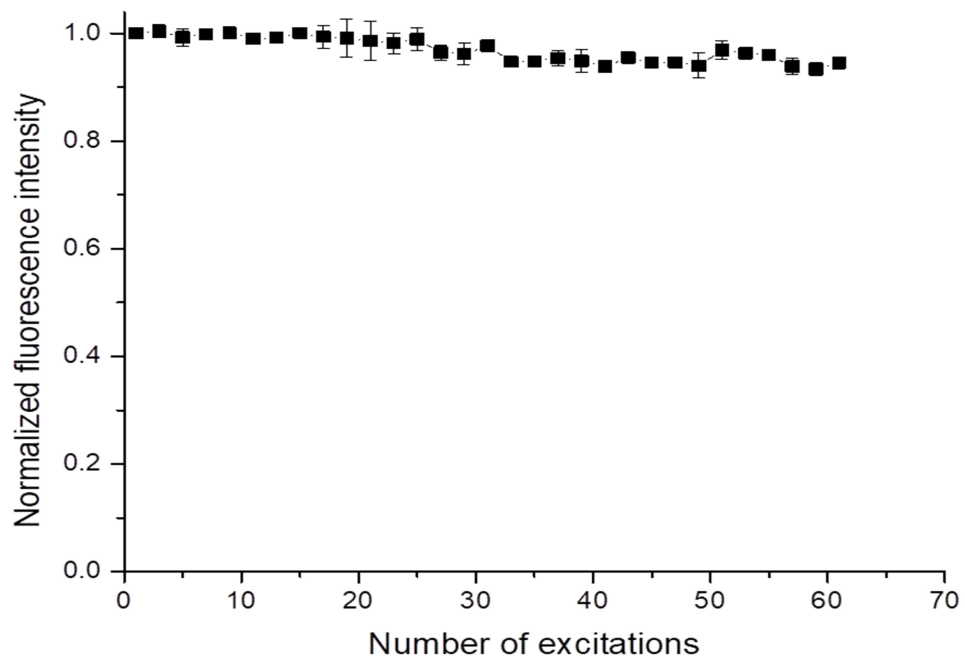
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Calibration curve for beta-carotene concentration and degradation due to the intestinal buffer at pH 7.0

A factor that may have an effect on the real-time measurement of fluorescence is BC degradation due to the repeated laser excitation at 488 nm. This figure shows the effect of a pulse excitation (500 ms pulse duration) repeated every 2 min on the fluorescence intensity of TC droplets with added 0.2 wt% BC. The measured fluorescence intensity decreased by 1% and 5% after 25 and 60 excitations (corresponding to 50 and 120 min), respectively. About 25 time points were used to cover the digestion kinetics, so by the end of the experiment, only 1% of the variation of the fluorescence intensity could be attributed to degradation, which was neglected.



Calibration curve of the average fluorescence intensity in the TC droplets as a function of BC concentration ($R^2=0.9902$).



Evolution of the average fluorescence intensity in the TC droplets exposed to the intestinal buffer at pH 7.0 as a function of the number of laser excitations.

Gastric coalescence effect on intestinal digestion

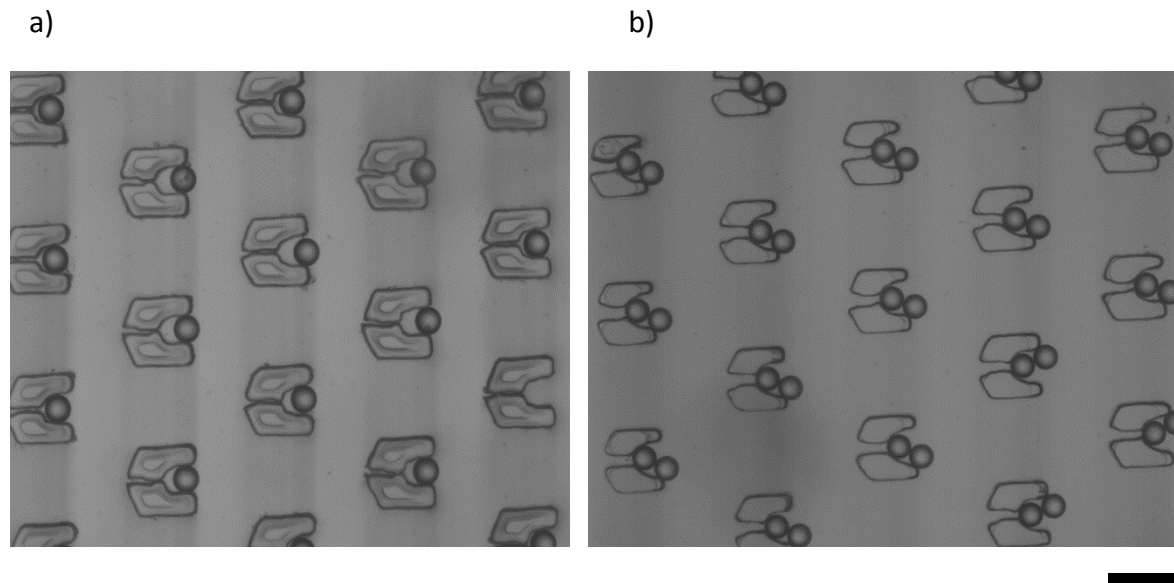


Fig. 1 Oil droplets trapped inside the chamber: a) Single droplet trapping, b) double droplet trapping. The scale bar represents 200 μm .

For this experiment, two droplets of 90 μm per trap were subjected to gastrointestinal digestion to test the effect of coalescence. A gastric phase (buffer+lipase AN+pepsin) of 60 min preceded the intestinal phase. Fig. 2a) shows the droplets at different times of gastrointestinal digestion. Coalescence occurred in some but not all traps near the end of the gastric phase. Thus intestinal digestion could be monitored simultaneously for both uncoalesced (diameter 90 μm) and coalesced (diameter 112 μm) droplets. Fig. 2b) shows the different kinetics of intestinal digestion in these two cases. The rate of volume decrease for the uncoalesced droplets is higher than that for the coalesced droplet (rate ratio of about 1.28). This is likely a surface area effect, as gastric coalescence caused the fusion of two droplets into a single larger droplet, reducing the surface area compared to separate droplets. The surface area is indeed reduced by a factor $2 \cdot (90/112)^2$, that is about 1.29, explaining the ratio between the rates of volume decrease.

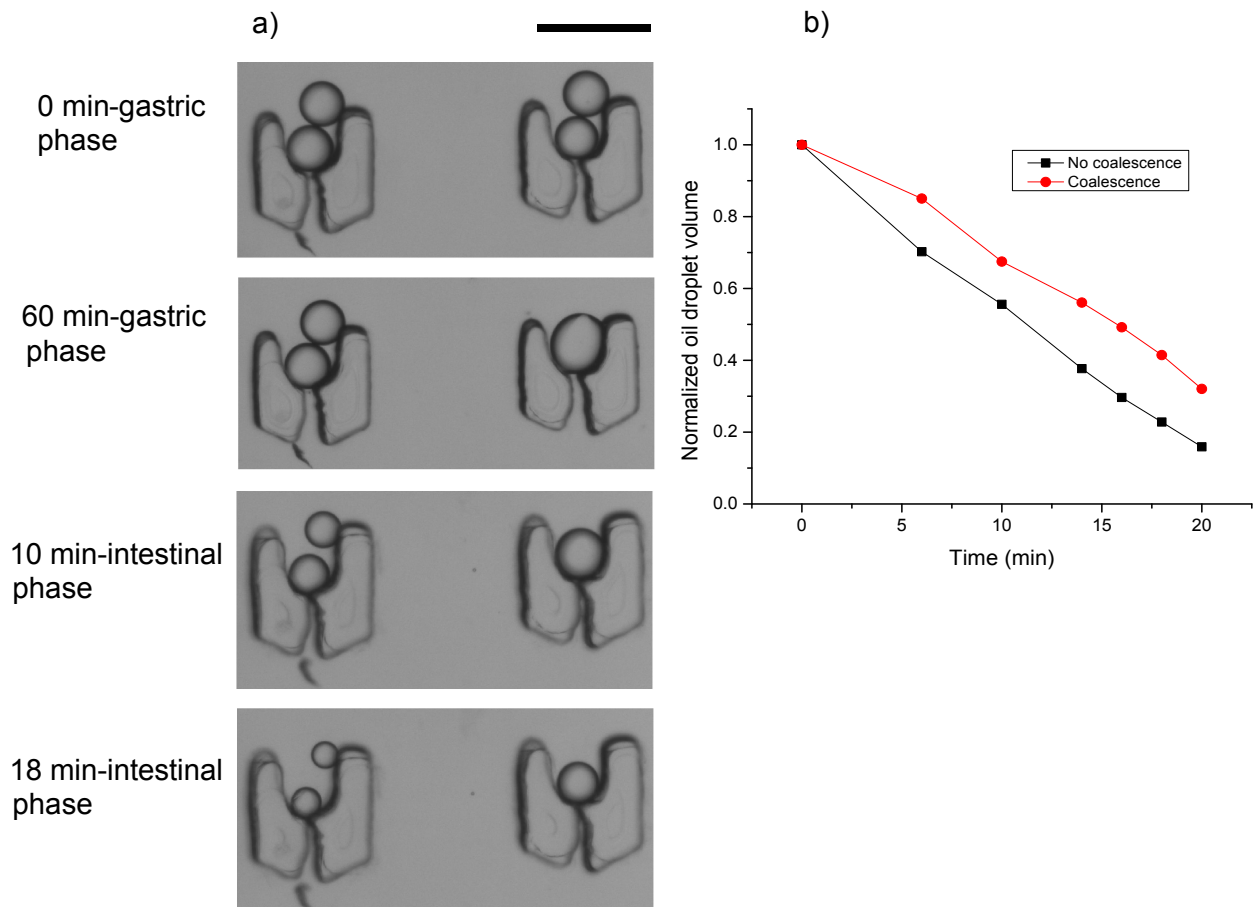
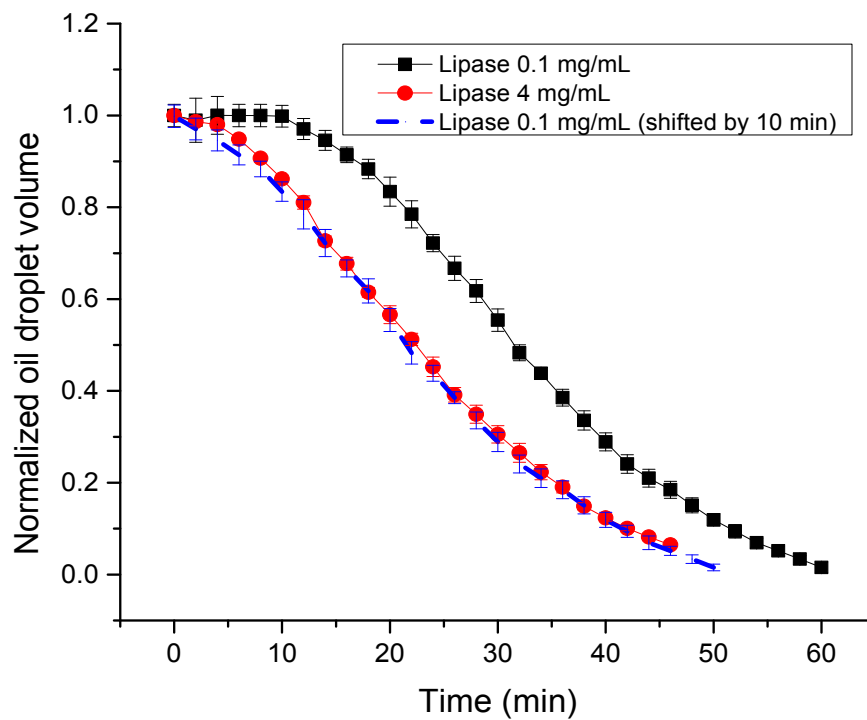


Fig. 2 a) Images of uncoalesced and coalesced droplets during gastrointestinal digestion, b) Evolution of the normalized volume of TC droplets during intestinal digestion (with or without coalescence during the gastric phase). The black scale bar is 200 μm .

Effect of lipase concentration on the intestinal digestion of oil droplets

Oil droplets were subjected to intestinal digestion with lower lipase concentrations (0.1 mg mL^{-1}) to compare with the normal case of lipase concentration 4 mg mL^{-1} . Tricaprylin digestion with the two lipase concentrations is shown below. In the case of the low lipase concentration (0.1 mg mL^{-1}), a longer lag phase is observed at the beginning of the digestion. This lag phase likely represents the time needed to saturate the oil droplet surface with lipase. Thus, a lower lipase concentration results in a longer lag phase. When the curve for the lower lipase concentration (0.1 mg mL^{-1}) is shifted by 10 min (lag phase), both curves superimpose (see figure). This result means that as long as lipase saturates the droplet surface, lipid digestion proceeds with the same kinetics regardless of the lipase concentration in the digestive fluid.



Effect of lipase concentration on intestinal digestion of TC oil droplets