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## Dynamic modeling highlights the major impact of droplet coalescence on the in vitro digestion kinetics of a whey protein stabilized submicron emulsion

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1     **Dynamic modeling highlights the major impact of droplet coalescence on**  
2           **the *in vitro* digestion kinetics of a whey protein stabilized submicron**  
3   **emulsion**

4  
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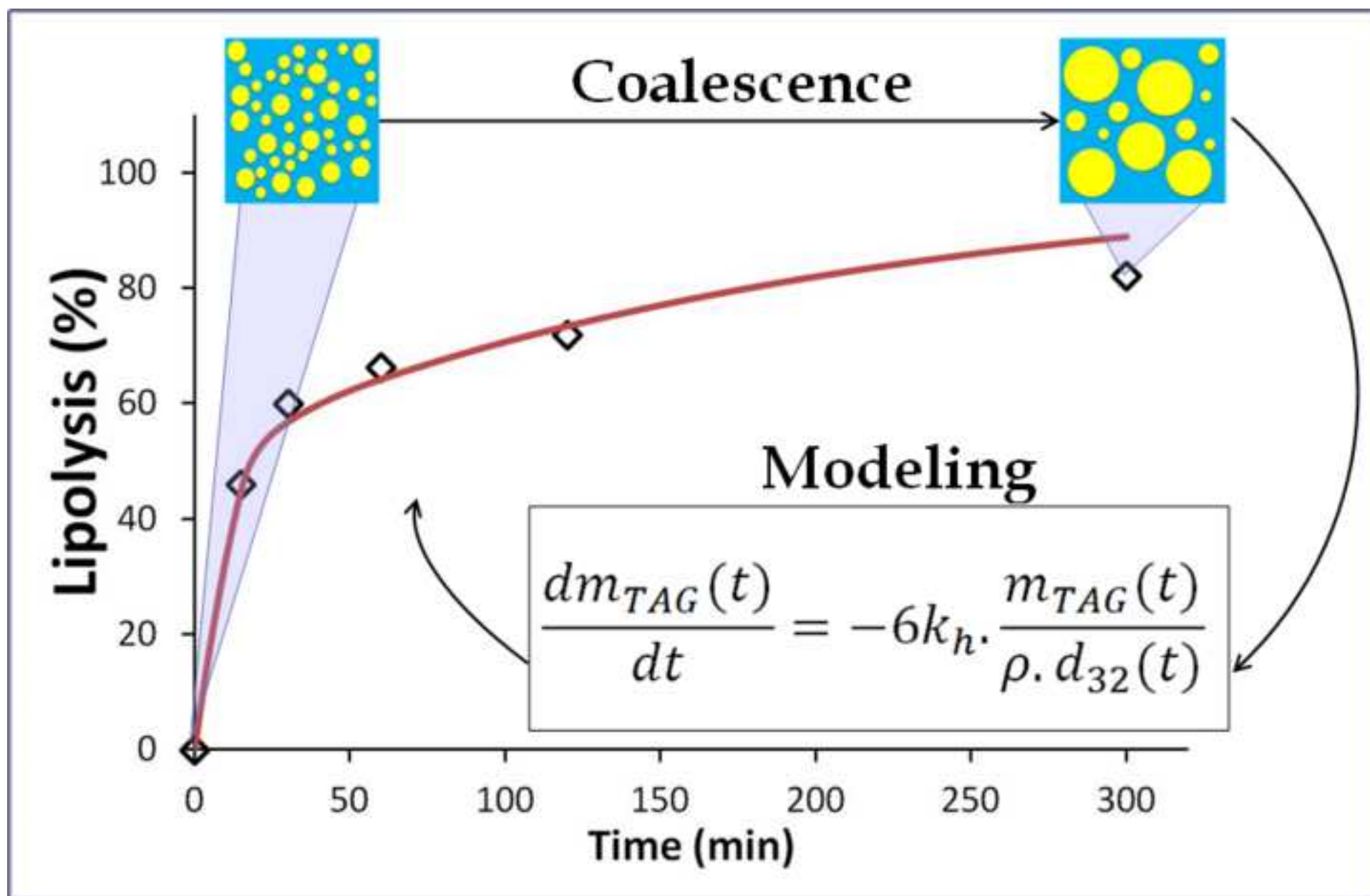
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**Highlights:**

- A whey protein stabilized emulsion was submitted to *in vitro* digestion.
- The kinetics of pancreatic lipolysis plateaued after ~ 30 min of intestinal digestion.
- A marked coalescence of the oil droplets occurred concurrently.
- A mathematical model was developed and successfully used to relate both sets of data.
- Droplet coalescence, and not enzyme inhibition, was the key mechanism explaining the rate of lipid digestion.

15 **Abstract**

16 Whey protein stabilized submicron oil-in-water emulsions have been reported to remain  
17 relatively stable in size during the gastric phase and to coalesce during the intestinal phase of  
18 *in vitro* digestion experiments. The aim of this study was to understand the impact of oil  
19 droplet coalescence on the intestinal lipolysis kinetics during an *in vitro* digestion of such  
20 emulsion, and to develop a mathematical model able to predict the experimental observations.  
21 A submicron whey protein stabilized emulsion made of a mixture of medium-chain (MCT)  
22 and long-chain triacylglycerols (LCT) was prepared and submitted to gastro-intestinal *in vitro*  
23 digestion. Triacylglycerol concentrations and droplet size distributions were measured before  
24 and after the gastric phase and during the intestinal phase using HPLC and laser granulometry,  
25 respectively. MCT were fully digested within 15 min of intestinal digestion, whereas LCT  
26 were still detected after 5 hours. Moreover, the intestinal lipolysis of LCT showed a two-stage  
27 behavior with an initial fast rate that markedly slowed down after about 30 min, a time at  
28 which a sudden rise in the droplet sizes, attributed to coalescence, was also observed. A  
29 mathematical model based on the experimentally measured droplet sizes and assuming a rate  
30 of lipolysis proportional to the interfacial area was developed and successfully used to  
31 reproduce the observed kinetics. Our results support the idea that droplet coalescence during  
32 the intestinal phase was the main reason for the marked slowdown of the kinetics of lipid  
33 digestion, hence suggesting that inhibition of the lipolysis reaction could be a secondary  
34 factor only.

35

36 **Keywords:** Pancreatic lipase, Lipid digestion, Droplet size, Polyunsaturated Fatty Acids,  
37 Simulation.

38       **1. Introduction**

39 Key parameters that govern food digestion *in vivo* are very hard to identify for practical  
40 reasons. *In vitro* methods, for which the conditions are strictly controlled, have therefore been,  
41 and are still, largely used. Concerning the digestion of emulsified lipids, both their  
42 compositional and structural properties can affect digestion [1]. It is known that short chain  
43 fatty acids are released faster and to greater extents than long-chain fatty acids [2-6], and that  
44 polyunsaturated fatty acids such as docosahexanoic acid (DHA) and eicosapentaenoic acid  
45 (EPA) are highly resistant to lipolysis [7-8]. The composition and the surface area of the  
46 interface surrounding lipids are also very important parameters because digestive lipases must  
47 adsorb on the droplet surface to reach their substrates. On the one hand, the kinetics of  
48 lipolysis increases with the interfacial area, and hence with decreasing droplet sizes [3, 5-6, 9-  
49 10]. On the other hand, the rate of the reaction depends on the lipase affinity for the interfacial  
50 layer and may therefore be modulated by the nature of the emulsifiers used to stabilize the  
51 emulsions [11-12].

52 Moreover, if lipid digestion is initiated in the stomach by the gastric lipase, most of the  
53 reaction (70-90%) is performed in the upper part of the intestine by the combined action of  
54 the pancreatic lipase, its colipase, and bile salts [1]. Beyond the properties of the native  
55 emulsions, the structural modifications they may undergo within the stomach will affect the  
56 intestinal phase, and hence the overall kinetics of the digestion [13]. Emulsions can remain  
57 stable, flocculate or coalesce during the gastric phase depending on several parameters such  
58 as the initial droplet size, the type of emulsifiers, and the composition of the surrounding  
59 medium [14-17]. The rate of lipid hydrolysis during the intestinal phase can thus be impacted  
60 by changes in the droplet surface area induced by the gastric structuring of the emulsions: the  
61 lower the interfacial area when emptied into the duodenum, the slower the digestion. The

62 stability of the oil droplets against flocculation and coalescence in the stomach is therefore  
63 essential to understand why two resembling emulsions may lead to different digestion kinetics.  
64 Several studies have also highlighted that some emulsions can remain relatively stable during  
65 the gastric phase but undergo important modifications during the intestinal phase of digestion.  
66 Whey protein stabilized submicron emulsions are good examples thereof. They have been  
67 reported to remain stable in size or flocculate in simulated gastric conditions [14, 18-19]. In  
68 either case, the initial rate of lipolysis during the subsequent intestinal phase was fast and  
69 comparable to other gastric stable emulsions [14], suggesting that flocculation during the  
70 gastric step did not alter much the intestinal lipolysis kinetics. In parallel, other studies have  
71 shown that whey protein stabilized emulsions are highly prone to coalescence during the  
72 intestinal phase of *in vitro* digestion [11, 13, 19-20]. The effect of such an intestinal  
73 coalescence of the oil droplets on the kinetics of pancreatic lipolysis have, however, not been  
74 investigated so far.

75 Therefore, our study aimed at studying and modeling the influence of droplet coalescence on  
76 the kinetics of pancreatic lipolysis during an *in vitro* gastrointestinal digestion of a whey  
77 protein stabilized emulsion. The emulsion was prepared from a blend of medium-chain  
78 triacylglycerols and a microalgae oil rich in docosahexaenoic acid (DHA), a long-chain  $\omega$ 3  
79 polyunsaturated fatty acid which daily recommended intake is 250 mg [21]. The evolution of  
80 both the oil droplet sizes and the lipolysis kinetics were monitored throughout gastro-  
81 intestinal *in vitro* digestion experiments. A mathematical model, in line with previously  
82 published ones [5, 22-23], was developed to quantitatively evaluate the relationships between  
83 the intestinal lipolysis kinetics and the evolution of the droplet surface area.

## 84      **2. Material and methods**

### 85      **2.1 Materials**

86      The oil containing medium-chain triacylglycerols (Miglyol 812S) (MCT): C8:0 (54%) and  
87      C10:0 (43%) was purchased from Sasol GmbH, Germany. The oil containing long-chain  
88      triacylglycerols (DHASco) (LCT): docosahexaenoic acid (DHA, C22:6 n-3, 40%), C12:0  
89      (4%), C14:0 (12%), C16:0 (12%) and C18:1 n-9 (24%) was obtained from Martek, via DSM  
90      Nutritional Products Ltd, Switzerland. Whey protein powder (Prolacta 95) was purchased  
91      from Lactalis Ingredients, France. Pepsin (P7012), mucin (M2378), pancreatin (P7545),  
92      pancreatic lipase (L3126) and bile extract (B8631) were from porcine origin and obtained  
93      from Sigma-Aldrich, France. Water was Milli-Q water. Solvents for liquid chromatography  
94      were chloroform for HPLC (Carlo Erba), methyl alcohol for HPLC (99.9%, Carlo Erba),  
95      ammonia solution (30%, Carlo Erba).

96

### 97      **2.2. Emulsion preparation**

98      An emulsion composed of 80% of aqueous phase and 20% of oil (w/w) was made. The  
99      aqueous phase was prepared by dissolving 4% (w/w) of whey protein powder, used as  
100     emulsifier, in a 0.1M sodium phosphate buffer at pH 7.0. The oil phase consisted of 62.5%  
101     LCT and 37.5% MCT (w/w) mixed together. A rotor-stator homogenizer (SilentCrusher M  
102     equipped with the 12F generator from Heidolph Instruments, Germany) was used in a pre-  
103     emulsification step (5 min, 20000 rpm). The coarse emulsion was successively homogenized  
104     for 5 min at 500 bars and for 10 min at 1000 bars under nitrogen flow with a high-pressure  
105     homogenizer (C3-EmulsiFlex, Sodexim SA, France) temperature-controlled at 4°C to produce  
106     the emulsion with droplet diameters below micron. A 50% (w/w) maltodextrin in water  
107     solution was then added as a 1:1 (v/v) ratio to the emulsion and the mixture was thereafter  
108     freeze-dried. The dried emulsion was then conditioned in oxygen hermetic bag under vacuum



109 and kept at  $-20^{\circ}\text{C}$  until uses. The dried emulsion was rehydrated in Milli-Q water to obtain a  
110 final oil concentration of 3.2% (w/w) on the day of the *in vitro* experiments.

111

### 112 **2.3 Emulsion digestion**

113 3 mL of the rehydrated emulsion, corresponding to an oil mass of about 96 mg, was placed  
114 into 22.4 mL headspace vials hermetically sealed with Teflon/silicon septa and aluminum  
115 caps. These vials were kept in a temperature controlled chamber at  $37^{\circ}\text{C}$  under magnetic  
116 stirring ( $400\text{ rpm}\cdot\text{min}^{-1}$ ) throughout the duration of the experiments. The gastric phase  
117 duration was 60 min and was launched by adding 2.12 mL of simulated gastric fluid (SGF)  
118 and  $40\ \mu\text{L}$  of 1M HCl to reach a final pH of 2.5 in the reaction vials. The SGF solution  
119 contained  $3.9\ \text{g}\cdot\text{L}^{-1}$  of pepsin,  $2.4\ \text{g}\cdot\text{L}^{-1}$  of mucin, 120 mM of NaCl, 2 mM of KCl and 6 mM  
120 of  $\text{CaCl}_2$ . The intestinal phase duration was then launched for 300 min maximum by adding  
121  $4.86\ \text{mL}$  of simulated intestinal fluid (SIF) into the vials and  $100\ \mu\text{L}$  of 1M  $\text{NaCO}_3$  to reach a  
122 final pH of 6.5. The simulated intestinal fluid (SIF) contained  $30.8\ \text{g}\cdot\text{L}^{-1}$  of bile extract  
123 powder,  $0.82\ \text{g}\cdot\text{L}^{-1}$  of pancreatin,  $0.41\ \text{g}\cdot\text{L}^{-1}$  of pancreatic lipase, and the same electrolyte  
124 concentrations as the SGF.

125 The native emulsion (NE), and samples taken at the end of the gastric phase (G60), at  $t = 0$   
126 min of the intestinal phase (I0) using a modified SIF that contained all constituents except  
127 pancreatin and lipase, and at 15, 30, 60, 120 and 300 min of intestinal digestion (I15 to I300)  
128 were analyzed. One vial was used for one sampling time and one type of measurement  
129 (quantification of LCT and MCT by HPLC or droplet size by laser granulometry) so that the  
130 contents of 16 vials were analyzed in total (2 methods times 8 sampling times) for one  
131 digestion. Three independent digestion experiments, further denoted replicates, were  
132 performed. Samples devoted to droplet size measurements were analyzed immediately,  
133 whereas samples devoted to HPLC measurements were kept at  $-80^{\circ}\text{C}$  until further analysis.

134

#### 135 **2.4 Quantification of LCT and MCT by HPLC**

136 HPLC paired with an evaporative light scattering detector (ELSD) was used to quantify the  
137 decrease of both LCT and MCT masses during the time course of the *in vitro* digestions. Total  
138 lipids were extracted from 1.5 mL of the native emulsions or of the stomach media and from 3  
139 mL of the intestinal media according to the Bligh and Dyer [24] procedure with minor  
140 modifications in the ratio  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$  1/2/1. Before the HPLC analyses, the lipid  
141 extract was dissolved in  $\text{CHCl}_3$  down to  $0.3 \text{ mg}\cdot\text{mL}^{-1}$  for native emulsion and stomach media  
142 and to  $0.7 \text{ mg}\cdot\text{mL}^{-1}$  for intestinal media. HPLC operating conditions were similar to those  
143 described in [25] using a Uptip-prep Strategy column ( $2.2 \mu\text{m SI}$ ,  $150\times 4.6 \text{ mm}$ , Interchim,  
144 Montluçon, France) and  $30 \mu\text{L}$  of injected lipid extract. As illustrated in **Fig. 1**, injected  
145 masses of pure MCT and LCT ranging from  $0.5$  to  $9 \mu\text{g}$  led to a power law calibration curve  
146 with no distinction of the TAG chain-length.

147 The triacylglycerol region of an HPLC chromatogram stemming from an undigested sample is  
148 presented in **Fig. 2**. The retention times of LCT ( $1.21 \text{ min}$ ) and MCT ( $1.32 \text{ min}$ ) were  
149 different but their signals partly overlapped. As illustrated in **Fig. 2**, signal deconvolution was  
150 therefore undertaken using a specifically developed algorithm running with the Matlab™  
151 software (The MathWorks Inc., Natick, MA) and the recovered LCT and MCT signals were  
152 converted into masses using the calibration curve (**Fig. 1**). Reliable results were obtained  
153 using this procedure as shown from the comparison of the mean LCT and MCT masses of  
154  $57.3 \pm 2.7$  and  $35.0 \pm 2.0 \text{ mg}$  (estimated over 15 undigested samples) with the  $59.7$  and  $35.7$   
155  $\text{mg}$  targeted masses in each digestion vial, respectively. LCT and MCT masses were finally  
156 converted into lipolysis percentages using **Eq. 1**:

$$\text{lipolysis}(t) = \frac{m_{TAG_0} - m_{TAG}(t)}{m_{TAG_0}} \times 100 \quad (1)$$

157 where  $m_{TAG_0}$  and  $m_{TAG}(t)$  are the masses (mg) of LCT or MCT initially present in the vials  
158 and measured by HPLC at time t, respectively.

159

## 160 **2.5 Droplets size measurement**

161 The volume-based distribution of oil droplet sizes was measured using a Mastersizer S  
162 (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 2 mW He-Ne laser of  $\lambda =$   
163 633 nm and the 300RF lens with detection limits of 0.05 and 900  $\mu\text{m}$ . The refractive index  $n_0$   
164 of the aqueous phase was 1.33 and the properties of the dispersed phase were 1.457 for the  
165 refractive index and 0.001 for the absorption. Samples were pre-diluted 100-fold with the  
166 desired solution (with or without sodium dodecyl sulfate, SDS, as deflocculating agent), and  
167 then diluted with distilled water to reach an oil volume concentration near 0.01% for the  
168 circulation in the measurement cell. All analyses were performed at room temperature as  
169 described previously [8, 25]. The surface-weighted mean diameter,  $d_{32}$ , corresponding to the  
170 droplet diameter having the same ratio of volume to surface area as the droplet distribution,  
171 was calculated according to:

$$d_{32} = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2}$$

172 where  $n_i$  is the number of droplets of diameter  $d_i$ .

173

## 174 **3. Mathematical modeling**

### 175 **3.1 Model assumptions and equations**

176 Lipolysis is mediated by the pancreatic colipase-lipase system which absorbs onto the droplet  
177 interface and splits the *sn*-1 and *sn*-3 ester bonds of triacylglycerols (TAG). The first step of  
178 the reaction generates one free fatty acid (FFA) and a diacylglycerol (DAG) which is further  
179 transforms into a second FFA and the *sn*-2-monoacylglycerol. Our model simulates the

180 kinetics of lipolysis as inferred from the disappearance of the TAG molecules so that, in  
 181 principle, it only characterizes the first step of the reaction. Nevertheless, the lack of detected  
 182 DAG in the course of the experiments strongly suggests that the second reaction step was not  
 183 rate-limiting in our experimental conditions, and hence that similar lipolysis kinetics would  
 184 have been recovered by monitoring the appearance of the final products of the reaction. The  
 185 main modeling assumptions were as follows:

186 A1: The rate of TAG hydrolysis was assumed proportional to the interfacial area of the  
 187 droplets [13-14, 22]. This means that the surface reaction rate was taken as constant in the  
 188 considered experimental conditions. Thus:

$$\frac{dm_{TAG}(t)}{dt} = -k_h \cdot A(t) \quad (2)$$

189 where  $k_h$  is the hydrolysis rate constant ( $\text{mg} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ) and  $A(t)$  is the interfacial area ( $\text{m}^2$ ) at  
 190 time  $t$  (min).

191 A2: The droplets were considered as spheres, and the interfacial area of the droplets was  
 192 assumed to be adequately described by the size distributions measured by laser granulometry  
 193 and their corresponding  $d_{32}$ . Hence, one can write that:

$$A(t) = 6 \frac{V_{TAG}(t)}{d_{32}(t)} \quad (3)$$

194 where  $d_{32}(t)$  is the surface-weighted mean diameter (nm) at time  $t$ , and  $V_{TAG}(t)$  is the  
 195 volume of TAG in the sample ( $\text{mm}^3$ ) at time  $t$ . **Eq. 3** can be further transformed into:

$$A(t) = 6 \frac{m_{TAG}(t)}{\rho \cdot d_{32}(t)} \quad (4)$$

196 where  $\rho$  is the mass density of TAG ( $\text{mg}/\text{mm}^3$ ) and  $m_{TAG}(t)$  is the mass of TAG (mg) at time  
 197  $t$ .

198 Combining **Eq. 1** and **4**, the evolution of the TAG mass is given by:

$$\frac{dm_{TAG}(t)}{dt} = -6k_h \cdot \frac{m_{TAG}(t)}{\rho \cdot d_{32}(t)} \quad (5)$$

199 To solve the differential equation **Eq. 5**, one also needs to know how the droplet sizes evolve  
200 as a function of time. In the present study, we resorted to two different assumptions on  $d_{32}(t)$ ,  
201 leading to two different versions of the model:

202 A3a: In one version, it was assumed that all droplets had the same diameter and that the total  
203 number of droplets remained constant, hypotheses that lead to the following equation [5]:

$$d_{32}(t) = d_0 \sqrt[3]{\frac{m_{TAG}(t)}{m_{TAG_0}}} \quad (6)$$

204 where  $m_{TAG_0}$  is the mass of TAG initially introduced in the reaction vial and  $d_0$  is the droplet  
205 diameter of the native emulsion.

206 A3b: In the other version,  $d_{32}(t)$  was estimated by linear interpolations of the experimental  
207 values recovered using laser granulometry.

208 For both model versions, the masses calculated by solving **Eq. 5** were finally converted into  
209 percentages of lipolysis using **Eq. 1** to enable the comparison of the model simulations with  
210 experimental data.

211

### 212 **3.2 Model fitting and parameter estimation**

213 The lipolysis of MCT was so fast that it was already finished at  $t = 15$  min, *i.e.* the first  
214 sampling time. Only the lipolysis of LCT was therefore considered to confront the model to  
215 the experimental data. The differential equation **Eq. 5** was numerically solved using a LCT  
216 mass density,  $\rho$ , of  $0.92 \text{ g.cm}^{-3}$ , and each of the previously described assumptions (A3a or b)  
217 for the droplet size evolution as a function of time,  $d_{32}(t)$ . The unknown hydrolysis rate,  $k_h$ ,  
218 was then estimated by fitting model predictions to the LCT lipolysis results determined by  
219 HPLC. The recovered value, expressed in mg of TAG per minute and square meter of  
220 interfacial area ( $\text{mg.m}^{-2}.\text{min}^{-1}$ ), was then converted into  $\mu\text{mol.m}^{-2}.\text{min}^{-1}$  for comparison

221 purposes with the literature using a LCT molar mass of  $900 \text{ g}\cdot\text{mol}^{-1}$ . Numeric calculations  
222 were performed using Matlab™ software (The MathWorks Inc., Natick, MA).

223

## 224 **4. Results and Discussion**

### 225 **4.1 Kinetics of lipolysis of LCT and MCT**

226 The evolution of HPLC chromatograms during the intestinal phase stemming from one *in*  
227 *vitro* digestion experiment is shown in **Fig. 3A**, and the mean percentages of lipolysis  
228 calculated over the three replicates are presented in **Fig. 3B**. Initially, 2 peaks were clearly  
229 visible in the chromatograms. The signal of the native emulsion and at  $t = 0$  min of the  
230 intestinal phase (modified SIF with no lipase) were similar since no gastric lipase was used in  
231 this study. At  $t = 15$  min of intestinal digestion, the signal arising from MCT entirely  
232 disappeared, indicating that MCT were fully hydrolyzed in only few minutes. In contrast,  
233 about 20% of the initial LCT mass was still detected after 300 min (*i.e.* 5h) of intestinal  
234 digestion, showing that lipolysis was much slower for LCT than for MCT (**Fig. 3B**). In fact,  
235 lipolysis of LCT was relatively fast during the first 30 minutes of digestion but was greatly  
236 slowed down afterwards, resulting in a two-stage curve typical of most *in vitro* lipolysis  
237 studies on submicron emulsions made of long-chain triacylglycerols [4-5, 14].

238 Higher rates of lipolysis of MCT compared to LCT have been reported in many studies using  
239 pure MCT and LCT emulsions or MCT/LCT mixed emulsions as in the present study [2-5].  
240 This is generally attributed to the higher water solubility of medium-chain FFAs than long-  
241 chain FFAs. Indeed, the low water solubility of long-chain FFAs would lead to their  
242 accumulation at the interface that would, in turn, inhibit the lipase activity by steric hindrance  
243 until they are removed by bile salts or by forming soap with calcium ions [26]. In contrast, the  
244 higher water solubility of medium-chain FFAs would facilitate their release from the interface,  
245 and hence promote further hydrolysis of triacylglycerols at the droplet surface. According to

246 the composition of our emulsion, other factors may also have contributed to the higher rate of  
247 MCT hydrolysis. Indeed, it has been reported that triacylglycerols containing  
248 docosahexaenoic acid are more resistant to pancreatic lipase [7], possibly because of an  
249 inhibitory effect induced by the presence of a double bond near the carboxyl group. For  
250 MCT/LCT mixed emulsions, it has also been shown that MCT hydrolysis can be promoted to  
251 the detriment of that of LCT [2] because of a preferential location, or turnover, of MCT at the  
252 droplet interface [2-3]. It is therefore likely that different mechanisms have contributed to the  
253 marked difference we observed in the lipolysis kinetics of MCT and LCT.

254

#### 255 **4.2 Evolution of the droplet size**

256 The evolution of the particle size distribution (measured without deflocculating agent)  
257 stemming from one *in vitro* digestion experiment is shown in **Fig. 4A**. The native emulsion  
258 (NE) presented a monomodal distribution with a mean  $d_{32}$  of 0.26  $\mu\text{m}$ . The mean size  
259 increased considerably during the gastric phase since the measured  $d_{32}$  was  $3.00 \pm 0.46 \mu\text{m}$   
260 after 60 min of contact with the SGF (G60). However, the size distribution and the measured  
261  $d_{32}$  returned close to their original values after dilution of the same sample in a 1% SDS  
262 solution (not shown) or after addition of a SIF with no pancreatin or lipase (I0). This  
263 demonstrates that the increase of the mean diameter during the gastric phase was caused by  
264 droplet flocculation, and that the subsequent addition of bile led to a deflocculation of these  
265 droplet aggregates. During the intestinal phase, the droplet size distribution remained similar  
266 during the first 15 min (I15). It was suddenly shifted toward considerably larger diameters at  
267 about  $t = 30$  min (mean volume diameter of about 9  $\mu\text{m}$ ) and remained relatively stable until  
268 the end of the experiment (I30 to I300), with a good repeatability of the surface weighted  
269 diameters over the three replicates (**Fig. 4B**).

270 According to the demonstrated tendency of bile to deflocculate the droplet aggregates formed  
271 during the gastric phase, the increase of the particle size during the intestinal phase most  
272 assuredly resulted from a coalescence of the oil droplets. This conclusion is moreover  
273 consistent with previous studies showing that whey protein stabilized submicron emulsions  
274 undergo coalescence during *in vitro* intestinal digestion [11, 13, 19-20]. We may even  
275 highlight that the evolution of the size distributions we measured is highly similar to previous  
276 results obtained during the digestion of a whey protein stabilized soya oil emulsion with an  
277 initial  $d_{32}$  of 0.37  $\mu\text{m}$ , and for which an intense coalescence was observed by confocal  
278 microscopy after 10 to 30 min of intestinal digestion [19]. Hence, even if flocculation cannot  
279 be totally excluded from our own set of data, we will only refer to the term of coalescence in  
280 the rest of this article.

281 We may also highlight the remarkable simultaneity of the increase of the droplet size (**Fig. 4B**)  
282 and of the decrease of the lipolysis rate (LCT in **Fig. 3B**). Although the authors did not point  
283 out this particular aspect, it seems that droplet coalescence during the intestinal phase was  
284 also concomitant with a decrease of the lipolysis rate in the recent study of Li and coworkers  
285 [19]. It is indeed well known that the rate of lipolysis decreases with decreasing surface area,  
286 and hence with increasing droplet size [3, 14, 16, 22, 27]. We can therefore wonder how  
287 much of the decrease of the LCT lipolysis kinetics at about  $t = 30$  min (**Fig. 3B**) was induced  
288 by droplet coalescence. This was further explored using a modeling approach.

289

### 290 **4.3 Modeling results**

291 We remind that MCT was fully hydrolyzed in less than 15 min so that only LCT lipolysis was  
292 considered for the modeling. The results obtained with three different mathematical models  
293 are presented in **Fig 5A**. First, the dotted line represents our model version that assumes a  
294 constant number of droplets of identical diameter (assumption A3a), and which decreases in



295 size upon hydrolysis of the TAG they contain (**Fig. 5B**). The fit was very bad because the  
296 assumed mechanisms could not reproduce the strong decrease of the reaction kinetics at about  
297 30 min.

298 Second, the dashed line represents the model proposed by Li and McClements [5]. This model  
299 is similar to the previous one but further assumes that a fraction of the TAG can remain  
300 undigested. It provided a very good fit of our experimental data ( $R^2 = 0.9888$ ) and led to a  
301 lipolysis extent of  $77.3 \pm 2.6 \%$  and a surface rate constant ( $k_h$ ) of  $9.4 \pm 1.5 \mu\text{mol.m}^{-2}.\text{min}^{-1}$ ,  
302 which is rather close to the  $13.8 \mu\text{mol.m}^{-2}.\text{min}^{-1}$  reported for a corn oil emulsion in [5]. As  
303 noticed by the authors, these rate constants should nevertheless be considered as upper values  
304 whenever droplet flocculation or coalescence takes place because, in such cases, the model  
305 would not adequately simulate the evolution of the available interfacial area. This is also why  
306 the mean diameter simulated with this model decreased from 350 to 213 nm (**Fig. 5B**), a trend  
307 that is not consistent with our experimental results (**Fig. 4**).

308 Finally, the solid line represents our model version that accounts for the experimentally  
309 measured  $d_{32}$  (**Fig. 5A and B**). This model also reproduced the experimental data very well  
310 ( $R^2 = 0.9882$ ). The underlying interpretation is however entirely different since the marked  
311 slowdown of the reaction kinetics around 30 min is here fully explained by the decrease of the  
312 interfacial area caused by droplet coalescence (*i.e.* no upper fraction of the digested lipids is  
313 assumed here). The only unknown parameter in this model is the surface reaction rate ( $k_h$ )  
314 that was estimated to be  $2.4 \pm 0.1 \mu\text{mol.m}^{-2}.\text{min}^{-1}$ .

315 To support the above considerations, the interfacial surface area was calculated for both our  
316 model accounting for coalescence and the model of Li and McClements (**Fig. 6**). According  
317 to the model of Li and McClements, the droplet size reduces upon TAG hydrolysis, leading to  
318 a corresponding decrease of the surface area. The lipolysis nevertheless plateaued (**Fig. 5A**)  
319 despite a large remaining droplet surface area. This suggests that the interface was no more

320 available for the enzymatic action, or in other words, that the reaction was inhibited by a  
321 mechanism such as an accumulation of the reaction products at the droplet surface. According  
322 to our model however, the slowdown of lipolysis was clearly, and solely, related to the sharp  
323 decrease of the interfacial area induced by droplet coalescence. At 300 min, the available  
324 interfacial area was low but nonzero, in agreement with the fact that lipolysis still proceeded  
325 slowly. Note also that the reaction rate constants estimated with both models reflect these  
326 differences of simulated surface area since they are expressed per unit of interfacial area. This  
327 is indeed why the value recovered using our model (assumption A3b) is about 4 times smaller  
328 than the value estimated with the model of Li and McClements ( $2.4$  and  $9.4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$ ,  
329 respectively).

330 Taking into account the evolution of the experimentally measured droplet sizes, the  
331 mathematical model allowed a good prediction of the intestinal lipolysis kinetics. The  
332 decrease of the interfacial area was thus the major reason for the slowdown of the reaction  
333 rate after about 30 min. We may even highlight that our model slightly overestimates the last  
334 experimental point of the kinetics at  $t = 300$  min (**Fig. 5A**). Thus, our results are still  
335 compatible with an inhibition of the enzymatic reaction at the interface, but as a second order  
336 factor.

337 On the one hand, our results confirm that the kinetics of lipolysis are essentially proportional  
338 to the interfacial area [22]. On the other hand, they may also offer a complementary  
339 explanation for the strong decreases of the lipolysis kinetics that are frequently observed after  
340 few minutes of intestinal *in vitro* digestions. Indeed, such slowdowns are often attributed to an  
341 inhibition induced by the reaction products that accumulate at the interface, with no or little  
342 attention given to a possible coalescence or flocculation of the emulsion droplets. This is most  
343 probably because such phenomena have not been expected to occur in the conditions  
344 encountered in the intestinal phase because of high concentrations of bile salts and no

345 macroscopic visual evidences. Nevertheless, several recent studies have shown that droplet  
346 flocculation and coalescence might in fact be encountered during the intestinal phase of *in*  
347 *vitro* experiments [11, 13, 17, 19-20], similarly to what has now been established for the  
348 gastric phase [14, 16-17].

349 The exact cause for the occurrence of coalescence still remains to be studied because the  
350 nature of the interfacial layer is continuously evolving, especially in the case of protein-  
351 stabilized emulsion. The TAG composition of the droplets is evolving in the course of the  
352 reaction [28] and a competitive adsorption process takes place at the interface between  
353 emulsifier molecules, enzymes, bile salts and the products of the lipolysis reactions. It has  
354 been reported that hydrolysis of the proteins adsorbed at the interface can weaken the droplet  
355 repulsion forces and favor droplet flocculation or coalescence [19, 25], possibly explaining  
356 why protein stabilized emulsions seem more sensitive to these phenomena [27]. Droplet  
357 coalescence during digestion has also been reported to be promoted by the accumulation of  
358 monoacylglycerols and fatty acids at the interface [16, 28], that is, by the same mechanism as  
359 that usually put forward to support an inhibition of the enzymatic reaction. More studies are  
360 therefore needed to determine the frequency of droplet coalescence during *in vitro* intestinal  
361 digestions and to better understand its consequences on the lipolysis kinetics.

362

## 363 **5. Conclusion**

364 Our study confirms previously reported results showing that the kinetics of lipolysis is much  
365 faster for MCT than for LCT when they are mixed together in the same emulsion. In  
366 agreement with recent studies, it also confirms that whey protein stabilized submicron  
367 emulsions are prone to coalescence during the intestinal phase of *in vitro* digestions.  
368 Moreover, by accounting for the experimentally measured droplet distributions in a modeling  
369 approach, we were able to adequately reproduce the two-stage lipolysis curve recovered for

370 LCT with an initial fast reaction rate that markedly slowed down after about 30 min. These  
371 modeling results demonstrate that droplet coalescence had a considerable impact on the  
372 lipolysis kinetics of the remaining LCT by causing a sharp reduction of the interfacial area  
373 available for the adsorption of pancreatic lipase-colipase. Contrarily to what is generally  
374 postulated for intestinal lipid digestion, our findings suggest that inhibition of the enzymatic  
375 reaction might not always be the key mechanism explaining why *in vitro* lipolysis kinetics of  
376 emulsified lipids often plateaus before the reaction is completed.

377

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384 properties of food by sharing our knowledge on the digestive process (INFOGEST)’,  
385 <http://www.cost-infogest.eu>

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462  
463



1 **Fig 1.** Calibration curve relating the HPLC signal to the injected mass of TAG. The light  
2 scattering detector responded the same way to both LCT (triangles) and MCT (diamonds).

3

4 **Fig 2.** HPLC chromatogram stemming from an undigested sample (circles) superimposed  
5 with the results of the deconvolution process: LCT signal (dashed line), MCT signal (dotted  
6 line) and their sum (solid line).

7

8 **Fig 3.** (A) Typical evolution of HPLC chromatograms during the intestinal phase of *in vitro*  
9 digestion: native emulsion (dashed line) and, from top to bottom, at  $t = 0, 15, 30, 60, 120$  and  
10  $300$  min after the SIF addition, respectively. (B) Extent of lipolysis for MCT (diamonds) and  
11 LCT (triangles) during the intestinal phase. Means and standard deviations (smaller than the  
12 symbol size) were calculated over 3 replicates.

13

14 **Fig 4.** (A) Typical evolution of the droplet size distributions measured without deflocculating  
15 agent during *in vitro* digestion. From back to front: Native emulsion (NE), samples taken at  
16 the end of the gastric phase (G60), and at  $t = 0, 15, 30, 60, 120$  and  $300$  min (I0 to I300) after  
17 the SIF addition, respectively. (B) Evolution of the  $d_{32}$  during the intestinal phase. Means and  
18 standard deviations (vertical bars) were calculated over 3 replicates.

19

20 **Fig 5.** (A) Extent of LCT lipolysis measured by HPLC (symbols) and the fits obtained with i)  
21 our model that does not account for the observed coalescence (dotted line, assumption A3a), ii)  
22 the model of Li and McClements [5] (dashed line), and iii) our model that accounts for  
23 coalescence using the measured  $d_{32}$  (solid line, assumption A3b). (B) Comparison of the  
24 measured (symbols) and simulated  $d_{32}$  for the different models (same line coding).

25

26 **Fig 6.** Evolution of the interfacial area during the intestinal phase according to our model that  
27 accounts for coalescence using the measured  $d_{32}$  (solid line, assumption A3b) and to the  
28 model of Li and McClements [5] (dashed line).

29

Figure1

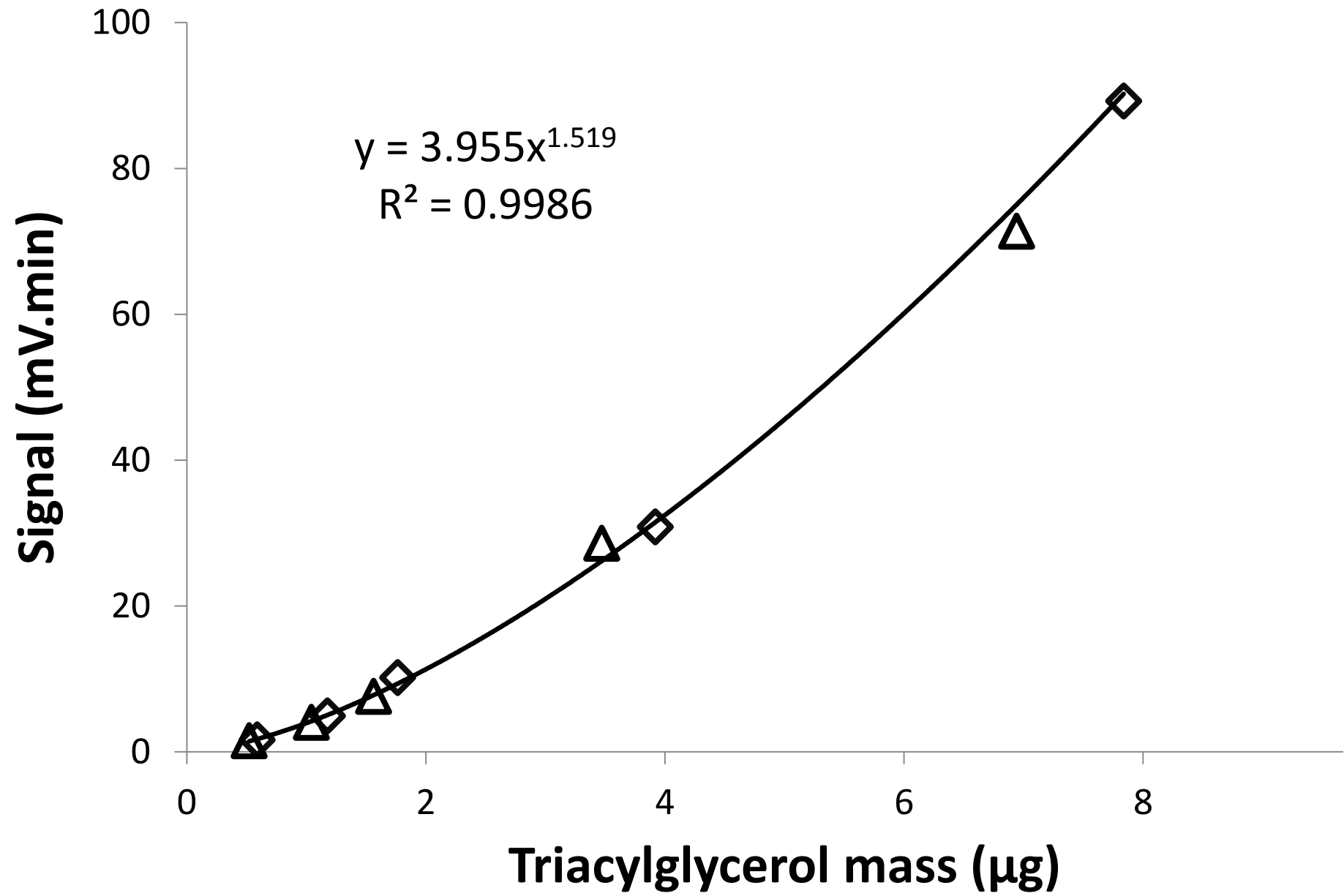


Figure2

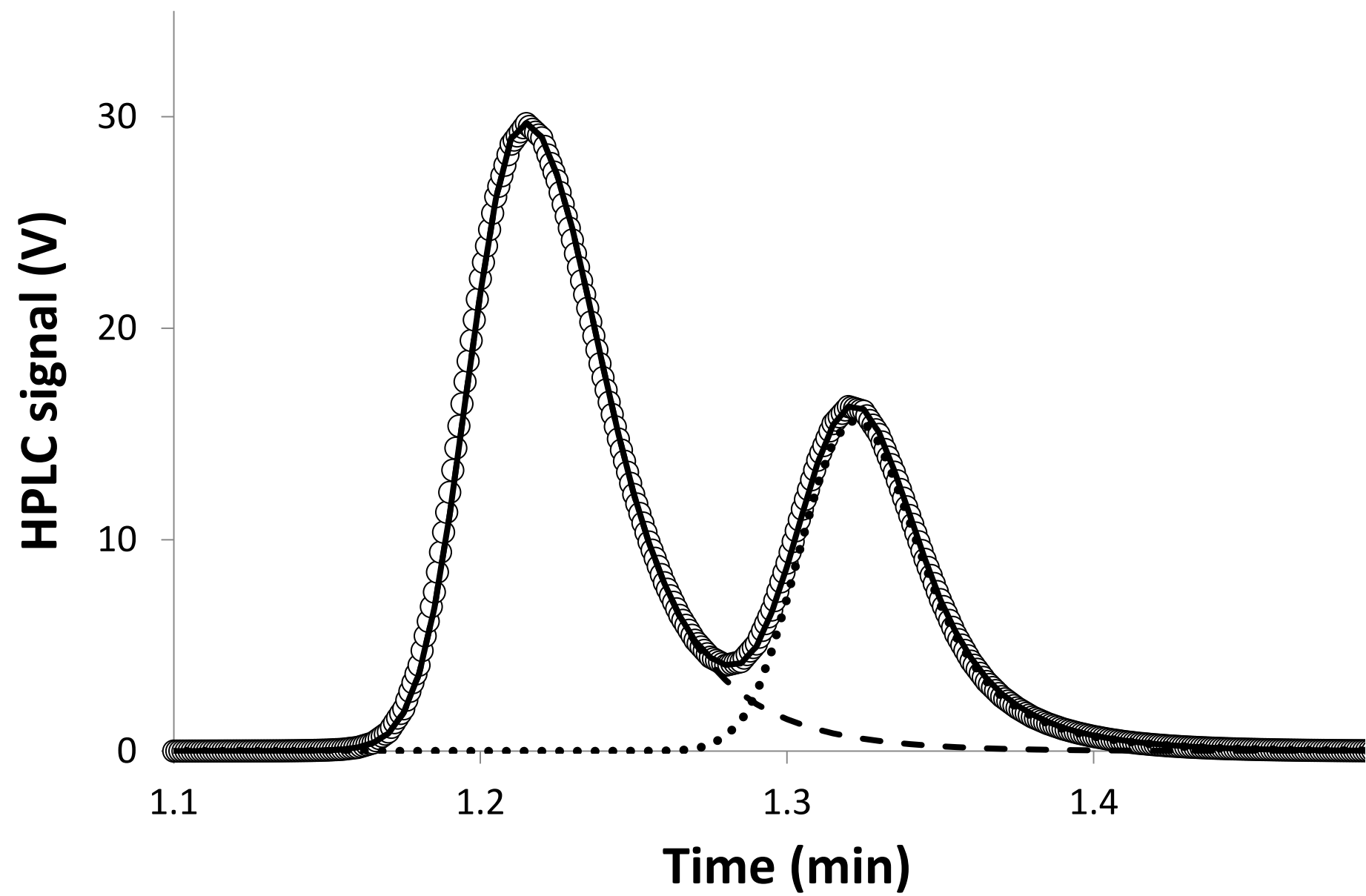


Figure3

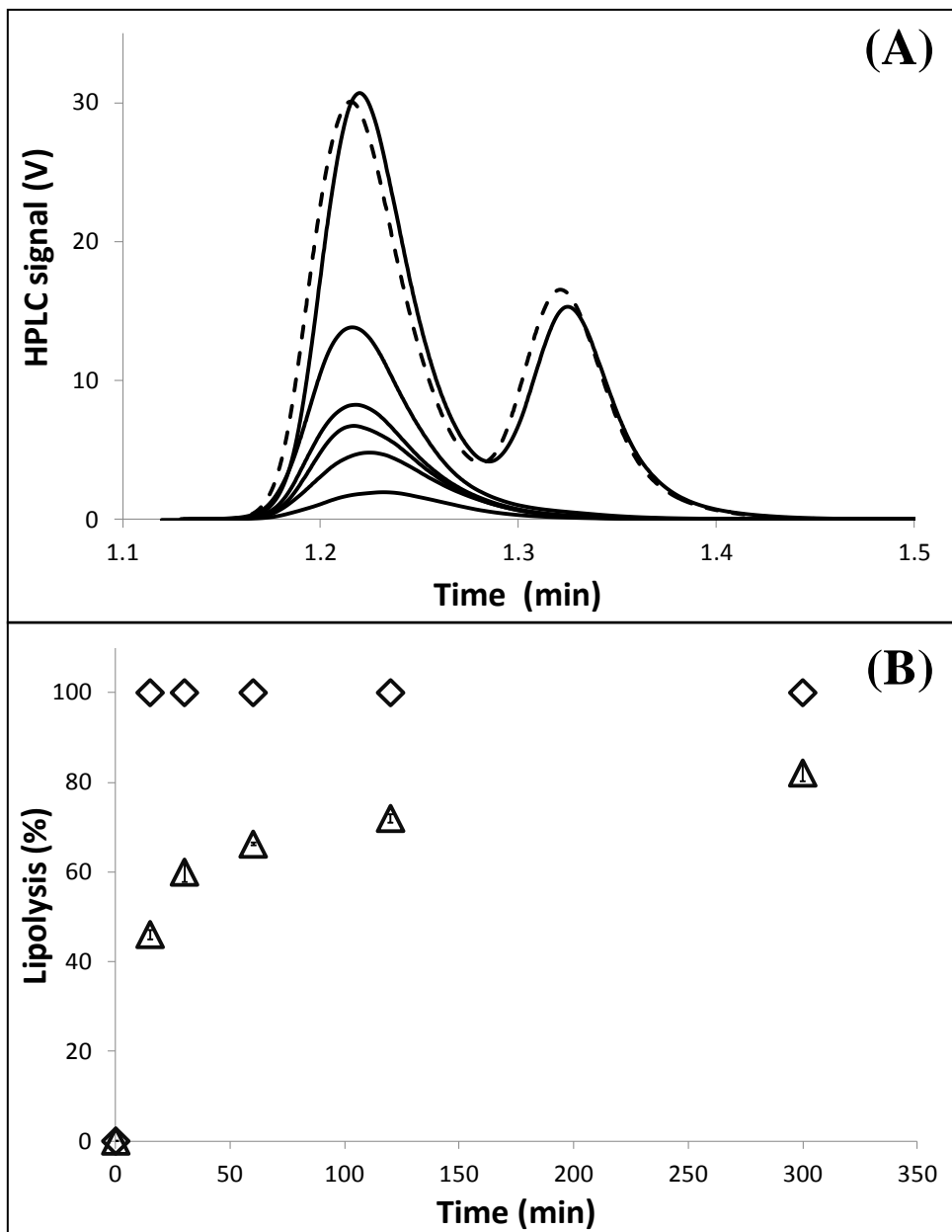


Figure4

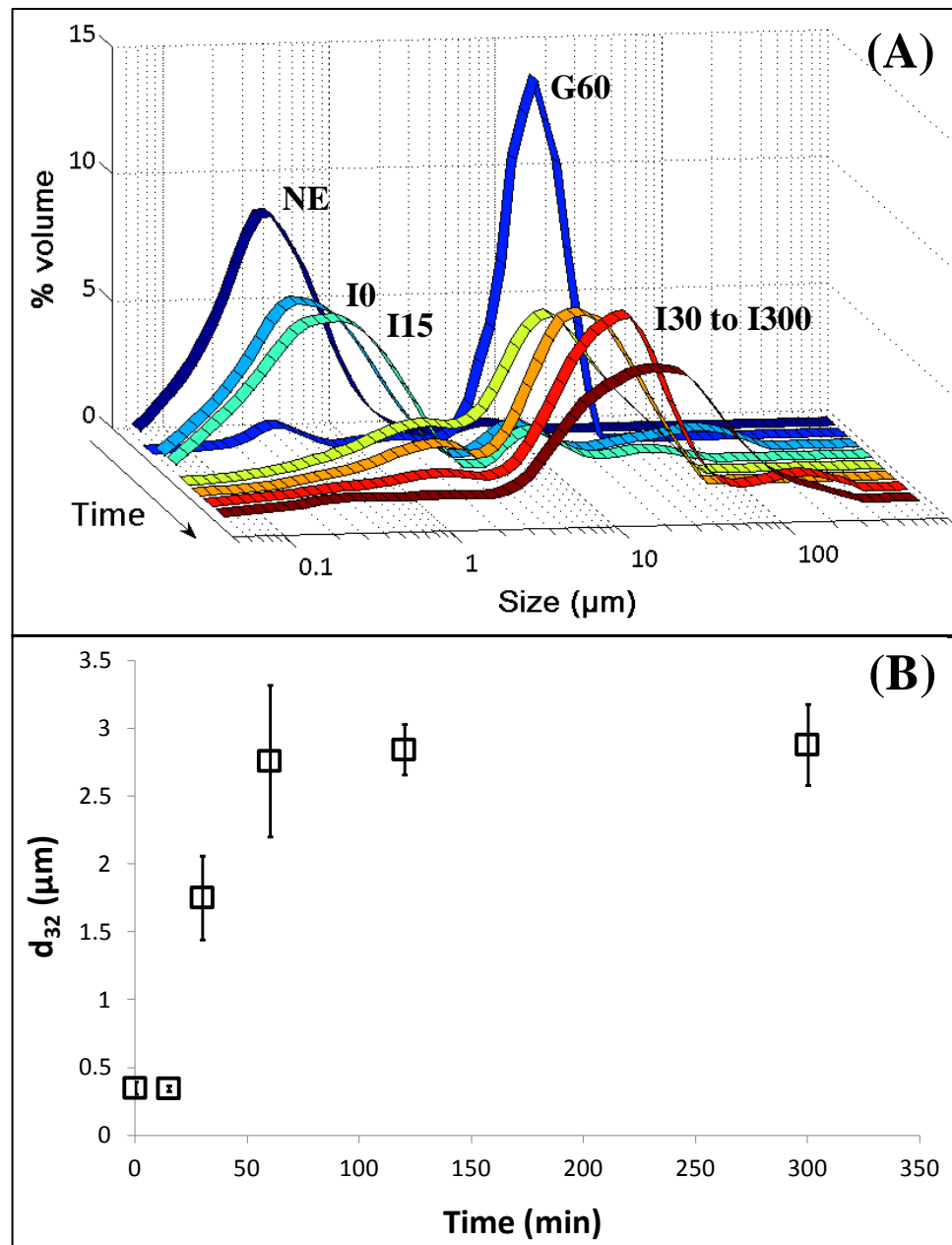


Figure5

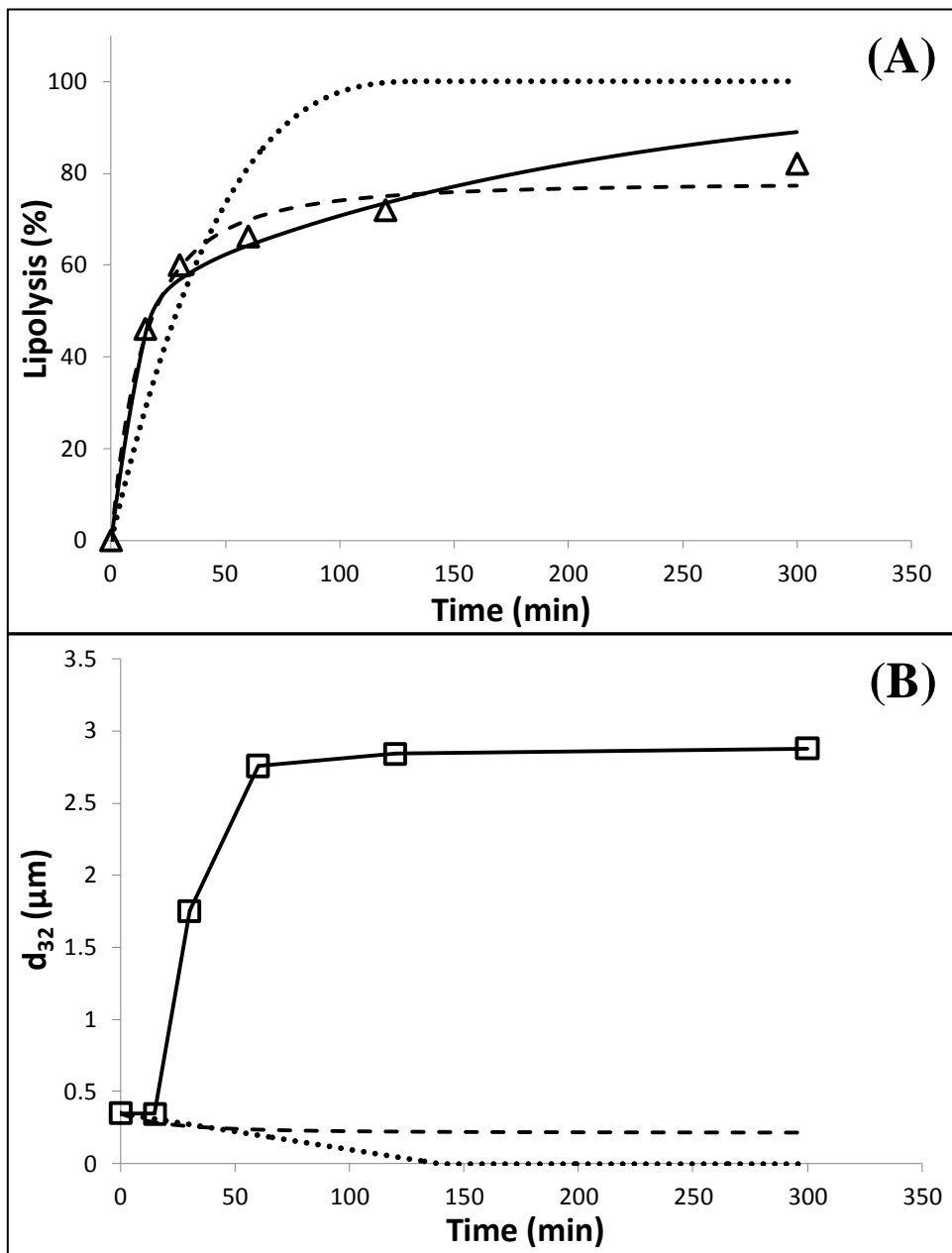


Figure6

