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## Mechanisms Governing the Transfer of Pure and Plant Matrix Carotenoids Toward Emulsified Triglycerides

Pascale Goupy, Claude Genot, Faiza Hammaz, Charlotte Halimi, Catherine Caris-Veyrat, Patrick Borel

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1 **MECHANISMS GOVERNING THE TRANSFER OF PURE AND PLANT MATRIX**  
2 **CAROTENOIDS TOWARD EMULSIFIED TRIGLYCERIDES.**

3  
4 Pascale GOUPY<sup>1</sup>, Claude GENOT<sup>2</sup>, Faiza HAMMAZ<sup>3</sup>, Charlotte HALIMI<sup>3</sup>, Catherine  
5 CARIS-VEYRAT<sup>1</sup>, Patrick BOREL<sup>3\*</sup>

6  
7 <sup>1</sup> UMR408 SQPOV « Sécurité et Qualité des Produits d'Origine Végétale », INRA, Avignon  
8 Université, F-84000 Avignon, France.

9 <sup>2</sup> UR1268 BIA « Biopolymères Interactions Assemblages », INRA, F-44316 Nantes, France.

10 <sup>3</sup> C2VN, INRAE, INSERM, Aix Marseille Univ, F-13005, Marseille, France.

11

12 **Keywords:**  $\beta$ -carotene, lutein, lycopene, phospholipids, serum albumin.

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14 \*Corresponding author:

15 [Patrick.Borel@univ-amu.fr](mailto:Patrick.Borel@univ-amu.fr)

16 UMR INRAE/INSERM/AMU "Center for CardioVascular and Nutrition Research of  
17 Marseille"

18 Faculté de Médecine

19 27, boulevard Jean Moulin

20 13005 Marseille, France

21 Phone: +33 (0)4 91 32 42 77

22

23 **Abbreviations:**  $\beta$ -carotene ( $\beta$ C), lycopene (LYC), lutein (LUT), phospholipids (PL), bovine  
24 serum albumin (BSA), triglycerides (TG).

25 **Abstract**

26

27 **Scope:** to assess the role of factors assumed to be involved in the transfer of carotenoids  
28 from plant matrices to dietary emulsions in the upper digestive tract. **Methods:** We first  
29 measured transfer as a function of time of pure  $\beta$ -carotene ( $\beta$ C), lutein (LUT) and lycopene  
30 (LYC) to triglyceride (TG) droplets dispersed in water. Then we measured the transfer to TG  
31 droplets stabilized with either bovine serum albumin (BSA), phospholipids (PL), or both.  
32 Finally, we measured transfer of tomato and spinach puree carotenoids to these emulsions.  
33 **Main results:** the maximal transfer efficiency of the pure carotenoids to uncoated emulsions  
34 was very efficient, ranging from 59 to 77%. However, it was dramatically impaired, ranging  
35 from 0.5 to 31% ( $p < 0.05$ ), when emulsions were stabilized by the emulsifiers. Conversely,  
36 when LUT, and to a less extent  $\beta$ C, but not LYC, was provided by the vegetable purees, its  
37 maximal transfer efficiency was significantly higher for the coated emulsions than for the  
38 uncoated one. **Conclusions:** Emulsifiers can dramatically impair the transfer of pure  
39 carotenoids to emulsion TG while they can facilitate the transfer of carotenoids from plant  
40 matrices. This suggests that specific interactions between plant matrix compounds and  
41 emulsifiers can enhance the transfer efficiency of carotenoids.

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## 50 **1) Introduction**

51

52 Carotenoids are hydrophobic plant pigments that belong to the lipid family. Although  
53 all their biological effects on the human body are far from being elucidated, some effects are  
54 acknowledged, e.g. the provitamin A activity of carotenoids that possess at least one  $\beta$ -ionone  
55 ring in their chemical formulae, and other are more and more assumed, e.g. anti-inflammatory  
56 properties<sup>[1-4]</sup> and role of lutein (LUT) and zeaxanthin in visual function.<sup>[5-7]</sup>

57 Carotenoid bioavailability is low and modulated by numerous factors.<sup>[8-10]</sup> It is assumed  
58 that this is mostly because they are both very hydrophobic and embedded in plant cells. It is  
59 also assumed that carotenoids can only migrate out of plant cells when these cells are broken,  
60 either through food processing, cooking or chewing. Indeed, carotenoids are deposited in  
61 different forms within plant cells, e.g. chloroplasts, chromoplasts, lipid-dissolved forms,<sup>[11]</sup> and  
62 it has been suggested that cells walls and chromoplast substructure represent important barriers  
63 for their bioaccessibility.<sup>[12, 13]</sup> Thus, carotenoids hardly integrate in mixed micelles, which are  
64 involved in their transport to their site of absorption.<sup>[14, 15]</sup> In fact, although during digestion it  
65 has been shown that a fraction of carotenoids directly transfers from plant matrices into  
66 micelles,<sup>[16]</sup> because of their high hydrophobicity most of the carotenoids present in plant food  
67 matrices transfer first to the triglyceride (TG) phase of the food bolus,<sup>[17]</sup> which is more or less  
68 dispersed as a so-called 'dietary emulsion'.<sup>[18, 19]</sup> Furthermore, because of their high  
69 hydrophobicity, non-oxygenated carotenoids, i.e. carotenes, are mostly solubilized in the core  
70 of the emulsion lipid droplets, i.e. in the TG phase,<sup>[20, 21]</sup> while oxygenated carotenoids, i.e.  
71 xanthophylls, are solubilized both in the core of the lipid droplets and in the phospholipid  
72 monolayer located at the droplet surface.<sup>[22]</sup> Indeed, dietary lipid droplets are coated by surface-  
73 active molecules, namely emulsifiers, naturally present in foods or secreted in the intestinal  
74 lumen during digestion.<sup>[23, 24]</sup> The main dietary emulsifiers are phospholipids (PL) and the

75 proteins that possess hydrophilic and hydrophobic sequence(s) of amino acids that allow them  
76 to adsorb at and stabilize the lipid droplet interface.<sup>[25]</sup>

77 Although the transfer of carotenoids from plant matrices to dietary emulsions has been  
78 suggested decade ago, and although several studies have studied the transfer of carotenoid to  
79 oil in vitro,<sup>[12, 26-30]</sup> in vivo experiments dedicated to measure the carotenoid transfer from plant  
80 matrices to dietary emulsions are scarce. In fact, to our knowledge there is only one clinical  
81 study dedicated to this topic.<sup>[31]</sup> In that study it has been shown that the three main dietary  
82 carotenoids, i.e.  $\beta$ -Carotene ( $\beta$ C), lycopene (LYC) and LUT, can be transferred from plant  
83 matrices to dietary emulsions in the human stomach during digestion. More precisely, up to  
84 about 60% carrot puree  $\beta$ -Carotene ( $\beta$ C) was transferred in the fat phase of the meal after 3 h  
85 digestion. A second interesting observation of this study was that the transfer efficiency of  
86 carotenoids can be very different depending on carotenoid species and on the nature of the food  
87 matrix. For instance, the maximal transfer efficiency of tomato puree LYC was less than 10%  
88 while that of carrot puree  $\beta$ C was about 60%.

89 Yet, factors that are involved in these very different transfer efficiencies have not been  
90 completely understood so far and it is for example not known whether the differences between  
91 the maximal transfer efficiency of LYC and  $\beta$ C were due to the different physicochemical  
92 properties of the carotenoids, as observed for the transfer of carotenoids between TG and mixed  
93 micelles,<sup>[32]</sup> or to the effect of the food matrix,<sup>[9, 11, 33-36]</sup> which can result both from physical  
94 barriers or from interactions with molecules that are associated with carotenoids in plant  
95 matrices.<sup>[11]</sup> Furthermore, concerning the dietary emulsions, the effects of emulsifier(s) that  
96 coat the lipid droplets, and which can also be present as isolated or as molecular assemblies in  
97 the aqueous medium, on the transfer efficiency of carotenoids has never been investigated,  
98 although a recent study has suggested a role of PL on the transfer efficiency of tomato LYC to  
99 oil.<sup>[29]</sup> Yet, we hypothesize that these compounds might impair the transfer of carotenoids to oil

100 droplets by creating a barrier hindering the direct diffusion of carotenoid to this hydrophobic  
101 phase.<sup>[37]</sup>

102         This project aimed at assessing the role of the three main factors that we assume to  
103 modulate the transfer of carotenoids to dietary emulsions, i.e. carotenoid species, presence and  
104 species of emulsifiers, and presence and nature of the plant matrix. To that aim, we first  
105 compared the transfer of three pure solid carotenoids, with contrasted physicochemical  
106 characteristics,<sup>[10, 32]</sup> to uncoated sunflower oil droplets dispersed in a slightly acid aqueous  
107 medium (pH 4.0), considered as representative of the physicochemical conditions found in the  
108 first step of digestion, that is during bolus formation in the stomach. Then, we studied the effect  
109 of the presence and the type of emulsifiers that coat these droplets by measuring the transfer of  
110 the pure carotenoids to the TG droplets stabilized with either a model protein: bovine serum  
111 albumin (BSA), well-known for its emulsifying properties, a mixture of PL, which was mainly  
112 composed of L- $\alpha$ -phosphatidylcholine, or a mixture of the two emulsifiers, which best mimic  
113 what happens in vivo. We finally assessed the role of the environment of carotenoids in plant  
114 matrix by measuring the transfer of carotenoids from tomato and spinach purees to either the  
115 uncoated TG droplets or to the TG droplets coated with the emulsifiers. The results obtained  
116 have allowed us to suggest fundamental mechanisms that govern this first step of carotenoid  
117 bioavailability.

## 118 2) Materials and methods

119

### 120 Chemicals and vegetable sources of carotenoids

121 Acetone, dichloromethane, methanol, *n*-hexane, ethyl-acetate and 2-propanol were  
122 purchased from Fisher-Scientific (Illkirch, France). Sodium acetate, sodium chloride and formic  
123 acid were purchased from VWR (Fontenay-sous-Bois, France). Ammonium formate, (all-*E*)- $\beta$ -  
124 carotene ( $\beta$ C) ( $\geq 95\%$ ), bovine serum albumin (BSA) (fraction V A-9647, MW ca. 66500 g  
125 mol<sup>-1</sup>), and trans- $\beta$ -Apo-8'-carotenal ( $> 95\%$ ) were purchased from Sigma-Aldrich (St  
126 Quentin-Fallavier, France). L- $\alpha$ -phosphatidylcholine from dried egg yolk ( $\geq 50\%$  TLC) was  
127 from Sigma-Aldrich (Buchs, Switzerland). Certificate of analysis of the lot used in this study  
128 indicated that it contained 76% L- $\alpha$ -phosphatidylcholine. A previous analysis of another lot of  
129 the same product by our lab (data not shown) showed that this product also contained some  
130 triglycerides (TG) and a mixture of phosphatidylethanolamine, sphingomyelin,  
131 lysophosphatidylcholine and phosphatidylinositol. It is therefore called the "PL mixture"  
132 thereafter in the manuscript. (all-*E*)-Lycopene (LYC) ( $>90\%$ ) was purified in the lab as  
133 previously described<sup>[38]</sup> from a tomato oleoresin provided by Conesa (Badajoz, Spain). (all-*E*)-  
134 Lutein (LUT) ( $>99\%$ ) was also purified in the lab,<sup>[39]</sup> but from a Naturex (Montfavet, France)  
135 oleoresin. Sunflower oil (Lesieur, France) was bought in a local supermarket. We chose this  
136 food oil because it is a commonly consumed oil. However, we acknowledge that other results  
137 could have been obtained with other oils. Indeed, it has been shown that the solubility of  
138 carotenoids increased when the chain-length of the triglycerides' fatty acids decreased.<sup>[22]</sup>

139 Two common dietary sources of carotenoids were used in this study: tomato, which is  
140 rich in LYC and  $\beta$ C and also contains LUT, and spinach, which is rich in LUT and also  
141 contains  $\beta$ C. Cold break tomato puree (cultivar Terradou®) was prepared according to previous  
142 work<sup>[40]</sup>. We chose this source of tomato because it was very well characterized and it allowed

143 us to compare data obtained in this study with data obtained with the same tomato matrix in a  
144 previous study by our lab on the diffusivity of lycopene from tomato puree to oil.<sup>[29]</sup> Canned  
145 spinach leaves were bought in a local supermarket (Casino brand). We chose canned spinach  
146 leaves in order to compare data obtained in this study with data obtained with the same spinach  
147 matrix in a previous study by our lab on the effect of spinach matrix on lutein bioaccessibility  
148 and bioavailability.<sup>[33]</sup> To have a homogeneous matrix, spinach leaves were diluted four times  
149 with deionized water, i.e. 100 g drained spinach leaves were dispersed in a total volume of 400  
150 mL water, and transformed in puree using a rotor stator homogenizer Silentcrusher (Heidolph,  
151 Grosseron, France) set at 16000 rpm for 2 min under dim light at room temperature.

152

### 153 Phospholipid purification

154 In order to measure the association of carotenoids with pure PL we eliminated residual  
155 neutral lipids (mainly triglycerides) from the egg yolk product rich in L- $\alpha$ -phosphatidylcholine  
156 according to the method modified from Gladkowski et al.<sup>[41]</sup> In summary, absolute ethanol (10  
157 mL) was added to 2 g of the egg yolk product in a 50 ml flask placed on a magnetic stirrer.  
158 After solubilization, the solution was placed at -20°C for 4 h and then centrifuged at 3000 x g at  
159 4°C for 5 min. The supernatant was recovered, placed again at -20°C for 12 h and centrifuged  
160 at 3000 xg at 4°C for 5 min. The new supernatant was recovered and evaporated under vacuum.  
161 Cold acetone (20 mL) was finally added to solubilize contaminating TG and the mixture was  
162 shake in ice. PL, which are insoluble in acetone, were precipitated, collected and washed with  
163 cold acetone to remove remaining TG still present. Purified PL were stored at -20 ° C until  
164 used.



165

166 Standard solutions of carotenoids

167 Standard solutions of carotenoids ( $\sim 50 \mu\text{mol.L}^{-1}$ ) were prepared by dissolving the dry  
168 standards in dichloromethane. Apo-8'-carotenal was dissolved in absolute ethanol. The  
169 integrity of the carotenoid standards and their concentrations in the stock solutions were  
170 checked and calculated by using a Specord S-600 diode-array UV-vis spectrophotometer  
171 (Analytical Jena, Saint-Aubin, France). The following molar absorption coefficients were used:  
172  $128000 \text{ L.mol}^{-1}.\text{cm}^{-1}$  at 460 nm for  $\beta\text{C}$  in dichloromethane,  $178000 \text{ L.mol}^{-1}.\text{cm}^{-1}$  at 482 nm  
173 for LYC in dichloromethane,  $140516 \text{ L.mol}^{-1}.\text{cm}^{-1}$  at 444 nm for LUT in methanol containing  
174 1% dichloromethane,  $108900 \text{ L.mol}^{-1}.\text{cm}^{-1}$  at 457 nm for apo-8'-carotenal in absolute ethanol.

175

176 CAR extraction from the vegetable sources of carotenoids

177 Tomato and spinach puree carotenoids were extracted using a method described by  
178 Serino et al.<sup>[42]</sup> with few modifications. More precisely, 500 mg of spinach puree or 250 mg of  
179 tomato puree were put in 2 ml Eppendorf tubes containing around 100 mg of 0.1 mm Zirconia/  
180 Silica Beads (Biospec products, Bartlesville, USA) and 50  $\mu\text{L}$  of a stock solution of apo-8'-  
181 carotenal (33  $\mu\text{M}$  in ethanol) used as internal standard. Then, 100  $\mu\text{L}$  of saturated aqueous  
182 NaCl solution, 100  $\mu\text{L}$  of n-hexane, 200  $\mu\text{L}$  of dichloromethane and 800  $\mu\text{L}$  of ethyl-acetate  
183 were successively added, each time followed by a 60 s full-speed high-energy shaking, using a  
184 FastPrep<sup>®</sup> homogenizer (Thermo scientific, Waltham, USA). Then, the Eppendorf tubes were  
185 centrifugated (5 min at  $10,000 \times g$  at  $4^\circ\text{C}$ ) and the upper phase was collected. Then, the pellet  
186 was re-extracted with the same successive solvents and the second upper phase was pooled  
187 with the first one and evaporated under argon to obtain the extracted lipids containing the  
188 carotenoids. Carotenoids were quantified by HPLC as described in the dedicated paragraph.  
189 Tomato puree contained  $7.88 \pm 0.53 \text{ mg}/100 \text{ g}$  fresh weight LYC,  $0.26 \pm 0.015 \text{ mg}/100 \text{ g}$   $\beta\text{C}$ , and

190 0.05±0.01 mg/100 g LUT. Spinach puree contained 2.34±0.02 mg/100 g fresh weight LUT and  
191 0.36±0.02 mg/100 g βC.

192

193

194

195 Preparation of sunflower oil-in-water emulsions

196 Four different emulsions were prepared: sunflower oil droplets dispersed in an aqueous  
197 medium (uncoated emulsion), oil droplets stabilized by BSA (BSA-emulsion), oil droplets  
198 stabilized by PL (PL-emulsion) or oil droplets stabilized by both emulsifiers (BSA/PL-  
199 emulsion). We studied the effect of the combination of the two emulsifiers because in most  
200 meals the two types of emulsifiers are simultaneously present and partition at the droplet  
201 interface according to their own interfacial properties. All systems were adjusted to pH 4.0  
202 because we aimed to study the transfer of carotenoids from vegetable food matrices to dietary  
203 emulsions that occurs in the human stomach,<sup>[31]</sup> and pH 4.0 is the mean pH observed in the  
204 human stomach after ingestion of vegetable-rich meals that contained tomato and spinach  
205 purees.<sup>[31]</sup> The proportions of the different component families, i.e. TG, PL and proteins, were  
206 representative of those occurring in the human gastrointestinal tract during digestion of a  
207 typical western meal.<sup>[31]</sup> Sunflower oil was chosen as the source of long chain TG because it is  
208 one of the most consumed one in Western countries. For the preparation of the uncoated-  
209 emulsions, 4 g of sunflower oil were added to 32 mL 10 mM acetate buffer at pH 4.0 in a 60  
210 mL bottle. Then, the mixture was vigorously agitated by using a magnetic barrel stirrer for 1  
211 min at room temperature. Obviously, this mixture was unstable, and the TG droplets coalesced  
212 very quickly. Thus, it was used extemporaneously in the transfer experiments described in the  
213 dedicated paragraph. The PL, BSA and BSA/PL-emulsions were prepared as follows: the  
214 emulsifiers were first dispersed in 32 mL 10 mM pH 4.0 acetate buffer (BSA: 4.44 g/L; PL-rich

215 mixture: 3.13 g/L; BSA 4.44 g/L + PL-rich mixture 3.13 g/L). Then the emulsifiers were  
216 dispersed using a rotor stator homogenizer (Heidolph Silent Crusher M) at 24,000 rpm for 2  
217 min at room temperature. Then, 4 g of sunflower oil was added to the emulsifier dispersions.  
218 The mixtures were then sonicated (Qsonica sonicator and a 3/4" probe) to obtain normal  
219 distributions with mean particle sizes ranging between 2 and 4  $\mu\text{m}$ , which was close to the size  
220 of lipid droplets observed in the human gastrointestinal tract during digestion.<sup>[19]</sup> The  
221 sonication conditions were empirically adjusted to obtain emulsions that were stable during the  
222 transfer experiments. Concerning the BSA and BSA/PL-emulsions the power was set at 20-  
223 25W, the amplitude at 40, and the sonication was performed in ice for 4 periods of 30 s with  
224 rest intervals of 30 s. Concerning the PL-emulsions, the power was set at 40 W, the amplitude  
225 at 80, and the sonication was performed in ice for 4 periods of 60 s with rest intervals of 30 s.  
226 The final pH of the mixtures was adjusted to 4.0 with either HCl or NaOH depending on the  
227 initial pH.

228

#### 229 Measurement of the size distribution of the emulsion lipid droplets

230 The size distribution of the stabilized emulsions was measured using a Mastersizer 2000  
231 laser diffraction instrument (Malvern Panalytical SARL, Orsay, France). Each emulsion was  
232 prepared 3 times independently as described above and 2 measures were performed on each  
233 sample. Thus, means were obtained from 6 values. The parameters modes: D [4,3], D [3,2],  
234 Dv10, Dv50 and Dv90 were calculated using the instrument's software. Calculations were  
235 performed using the Mie theory with the following refractive indexes: 1.475 for oil, 1.333 for  
236 the dispersant, and an absorbance value of 0.01 for the emulsion particles. The specific surface  
237 area ( $\text{m}^2/\text{g}$ ) was calculated by the software (total area of the particles divided by the total  
238 weight of oil). This calculation was based on the assumption that the particles were both  
239 spherical and non-porous.<sup>[43]</sup>

240

241 Measurement of the zeta potential of the emulsion lipid droplets and of the tomato and spinach  
242 particles

243 The zeta potential was measured on stabilized emulsions or vegetable puree particles  
244 dispersed and diluted with 10 mM acetate buffer at pH 4.0 (dilution ratio between 50 and  
245 5000). Measurements were performed in triplicate at 25°C using the Wallis zeta potential  
246 analyser (Cordouan Technologies, Pessac, France). Ten sequential runs were averaged to obtain  
247 one value. Zeta potentials were calculated using Henry's equation <sup>[44]</sup> and the Smoluchowski  
248 approximation. A mean zeta-potential measurement was obtained from three independent  
249 samples. Main settled parameters were: laser power 70%, low resolution (5 Hz), dielectric  
250 constant (relative permittivity) 78.06, applied field 21.47 mV, solvent (water) viscosity  
251 0.888cP, carrier frequency 8223 Hz.

252

253 Determination of the apparent solubility of carotenoids in aqueous solutions containing the  
254 emulsifiers

255 We deposited about 50 µg of each pure carotenoid solubilized in dichloromethane in 8  
256 mL glass tubes that possessed plugs and we dry evaporated them at room temperature under  
257 argon and gentle agitation (Vibrax Orbital movement shaker model VXR basic set at 500 rpm).  
258 The gentle agitation was performed to obtain a homogeneous film of carotenoids glued at the  
259 bottom of to the glass tube. Then, we added 2 mL 0.1 mM pH 4.0 acetate buffer containing  
260 either BSA, PL-rich mixture, purified PL, or a 50/50 mixture of BSA and PL-rich mixture, or  
261 purified PL. Emulsifier concentrations were the same than those used to make the emulsions,  
262 i.e. 4.44 g/L for BSA and 3.125 g/L for PL-rich mixture and purified PL. After 4 h incubation  
263 at 37°C under gentle agitation (same shaker and setting than above), the solutions were  
264 centrifugated (1000 x g for 5 min at 4 °C) to discard potential carotenoid crystals remaining in

265 suspension. Carotenoids remaining in the aqueous medium were theoretically associated with  
266 the emulsifiers because of their very low solubility in water, were quantified by UPLC.

267

268 Measurement of the transfer of carotenoids from either pure carotenoids or vegetable puree  
269 carotenoids to either uncoated or coated emulsions

270 To study the transfer of pure carotenoids to the TG phase of the different emulsions we  
271 dry evaporated about 50 µg of each carotenoid in 8 mL glass tubes as described above. Then,  
272 we added 2 mL of the different emulsions. Note that in the case of uncoated emulsions we first  
273 added 2 mL 10 mM pH 4.0 acetate buffer then we added 0.25 mL sunflower oil in order to  
274 avoid direct contact of the oil with the carotenoid film. We then closed the tubes with their  
275 plugs, and we incubated them at 37°C under constant stirring for 240 min (same shaker and  
276 setting than above). After 0.5, 15, 30, 60, 90, 120, 150 and 240 min, 1.5 mL of the mixtures  
277 were sampled (we used one glass tube per incubation time), and TG, which was theoretically  
278 rid of emulsifiers,<sup>[20, 21]</sup> was recovered as described below.

279 To study the transfer of vegetable puree carotenoids to the TG phase of the different  
280 emulsions we first diluted the vegetable purees with ultrapure H<sub>2</sub>O (MilliQ system) to obtain  
281 liquid mixtures that could be readily blended with the emulsions and that provided a quantity of  
282 carotenoids nearly equivalent to that of pure carotenoids. Indeed, these dilutions provided  
283 around 20 and 50 µg of total carotenoids per incubation condition for spinach and tomato  
284 purees, respectively. Two mL of the emulsions were then added, and the mixtures were  
285 incubated at 37°C under constant stirring (same apparatus and speed than above) for 240 min.  
286 Samples of the mixtures were collected at the same interval times than above. The TG phase,  
287 free of emulsifiers, was recovered as described below.

288 To break the emulsions and recover the TG phase free of emulsifiers and of vegetable  
289 puree particles, samples that came from the transfer experiments were centrifuged for 15 min at

290 5,000 x g, frozen overnight at -20°C, thawed at room temperature and centrifuged again for 30  
291 min at 5,000 x g. This allowed to obtain a floating layer of pure TG at the top of the tubes.  
292 Furthermore, to avoid any contamination of the TG samples with emulsifiers that are located at  
293 the interface between the TG and the aqueous phase,<sup>[20, 21]</sup> only a fraction of the TG layer  
294 floating at the top of the centrifuge tube, about 0.1 g, was collected. Each experiment was  
295 performed in triplicate.

296

### 297 CAR quantification by UPLC

298 Carotenoids extracted from the vegetable puree matrices or incorporated in the emulsion  
299 TG phases recovered after the transfer experiments were analyzed by HPLC as described  
300 thereafter. The dried extracts of carotenoids obtained after tomato and spinach puree extraction  
301 were first dissolved in 1 mL of dichloromethane/methanol (50/50; v/v) that contained 3.3 µM  
302 apo-8'-carotenal as internal standard. The recovered emulsion TG phases containing the  
303 transferred carotenoids were weighed and dissolved in 200 µL of 2-propanol and 800 µL of  
304 dichloromethane/methanol (50/50; v/v), which also contained 3.3 µM apo-8'-carotenal as  
305 internal standard. The analyses were performed using an ACQUITY UPLC® system (Waters  
306 Corp., Milford, USA) linked to a diode array detector 190–800 nm. Empower™ 2 software  
307 (Waters Corp.) was used for instrument control and chromatogram integrations. The separation  
308 was carried out using an ACQUITY UPLC® HSS T3 C18 column (150 mm × 2.1 mm, internal  
309 diameter 1.8 µm; Waters Corp.), which temperature was set at 50 °C. Solvents used were  
310 methanol/water (80:20, v/v) (solvent A) and ethyl acetate (solvent B) containing 0.2% formic  
311 acid. Gradient conditions were: A, 100%, 0–2 min; A, 100–30%, 2–20 min; A, 30–20%, 20–22  
312 min; A, 20–100%, 22–23 min; and returned to the initial conditions (solvent A, 100%) for a 4  
313 min re-equilibration period<sup>[45]</sup>. The system operating pressure was 800 bars at initial gradient  
314 conditions and injection volume was 7.5 µL with partial-loop with needle overfill injection.

315 The peaks were detected at 440 and 470 nm for LUT and the carotenes (LYC and  $\beta$ C),  
316 respectively. The limit of quantification was 2 ng for LYC, 3 ng for  $\beta$ C and 0.2 ng for LUT.

317

### 318 Calculations and statistics

319 The transfer efficiency was defined as the percentage of carotenoids recovered in the  
320 TG phase of the emulsions (measured mg CAR/g TG x 0.2 g TG, which was the total amount  
321 of TG provided by the 2 mL emulsion used in each experiment) relative to the amount of  
322 carotenoids, either pure or provided by the vegetable purees, that was incubated with the  
323 emulsions. The maximal transfer efficiency was the highest measured transfer efficiency that  
324 was mostly obtained after 240 min. incubation.

325 Results were expressed as means  $\pm$  SEM. Differences between means were assessed  
326 using ANOVA followed by *post hoc* Tukey-Kramer tests. All *p* values under 0.05 were  
327 considered significant. Statistical comparisons were performed using StatView software,  
328 version 5.0 (SAS Institute Inc., Cary, NC).

329

### 330 Dosage information / Dosage regimen

331 The doses of pure carotenoids used in this study were non  
332 supraphysiological/pharmacological. Indeed, we incubated 50  $\mu$ g pure carotenoids per tube  
333 while the average intake of each of these phytochemicals is higher than 1 mg/person/d.<sup>[46]</sup>

334 **3) Results**

335

336 *Main characteristics of the different emulsions*

337           Because we hypothesized that differences in transfer efficiency, and/or transfer rate, of  
338 carotenoids between the different emulsions could be due to different characteristics of the  
339 emulsions we measured both their lipid droplet size distribution and their electric charge.  
340 Indeed, for a given amount of oil, the lower the size of the droplets the higher the emulsion  
341 specific surface area ( $\text{m}^2/\text{g}$  oil) and thus the surface of the lipid droplets that can get in contact  
342 with the carotenoids. Note that the characteristics of the uncoated emulsion were not measured  
343 because, as stated in the material and method section, it was composed of coarse non-  
344 homogeneous TG droplets, apparently in the mm range, which coalesced very quickly. As  
345 expected, all coated emulsions exhibited monomodal particle size distributions with droplet  
346 sizes in the range 0.5-4  $\mu\text{m}$  (supporting information Fig. S4). Several parameters characterizing  
347 the lipid droplet size distributions of the different coated emulsions are shown in table 1. All of  
348 them, except the D3,2, were significantly ( $p < 0.05$ ) different between the 3 emulsions. The  
349 specific surface area of the PL-emulsion was about 60% higher than that of the BSA-emulsion  
350 and about two times higher than that of the BSA/PL-emulsion. We calculated, from the mass  
351 charge of BSA at the interface (theoretical surface load) given by the literature and the  
352 emulsion's specific surface area, that in the BSA-emulsion about 25% of the BSA was  
353 adsorbed at the oil-water interface, meaning that around 75% of the BSA remained in the  
354 aqueous phase.<sup>[47]</sup> Concerning the PL-emulsion, 65 % of PC were adsorbed at the interface.  
355 Finally, concerning the BSA/PL-emulsion, about 30 % PL present in the system were enough  
356 to cover the TG droplets and we assumed that all BSA remained in the aqueous phase. Indeed,  
357 it is acknowledged that there is a preferential adsorption of low-molecular weight emulsifiers as  
358 compared to high molecular weight emulsifiers such as proteins.<sup>[47-49]</sup>



359 Concerning the zeta potential, as for the lipid droplet size distribution it was not  
360 possible to measure it for the uncoated emulsion because this emulsion was too unstable. The  
361 zeta potential of the BSA-emulsion was  $24.6 \pm 0.4$  mV, that of the PL-emulsion was  $-9.3 \pm 0.4$   
362 mV, while that of the BSA/PL-emulsion was intermediate, i.e.  $8.3 \pm 0.6$  mV. These zeta  
363 potentials were significantly different ( $p < 0.05$ ). Concerning the vegetable puree particles, they  
364 had close negative charges, i.e.  $-11.5 \pm 0.2$  and  $-10.2 \pm 0.2$  mV for the tomato and spinach  
365 puree particles, respectively.

366

### 367 *Stability of carotenoids in the emulsions*

368 To accurately measure the transfer efficiency of carotenoids toward the TG phase of the  
369 studied emulsions, and because it has been shown that Z-isomerization of LYC can enhance the  
370 transfer efficiency,<sup>[30, 50]</sup> we checked whether carotenoids were stable in these emulsions during  
371 the experiment. To that purpose we quantified carotenoid concentrations as well as possible  
372 formation of carotenoid (Z)- isomers, at regular time intervals from up to 240 min, in the TG  
373 phase of BSA/PL-emulsions (data not shown). Neither the total amount of each carotenoid  
374 incorporated in TG nor their (Z) to (all-E)-isomer ratios significantly changed ( $p > 0.05$ ) over  
375 time, i.e. all along the 240 min. This shows that the studied carotenoids were relatively stable in  
376 the studied emulsions, at least in the conditions of our experiments, i.e. 240 min at pH 4.0 at  
377 37°C, which agrees with recent results showing that  $\beta$ C is not significantly degraded in the  
378 upper human gastrointestinal tract lumen during digestion.<sup>[51]</sup>

379

### 380 *Apparent solubility of carotenoids in emulsifiers dispersed in aqueous medium*

381 Results reported in table 2 show that the apparent solubility of carotenoids in the  
382 emulsifiers dispersed in an aqueous medium depended both on carotenoid and emulsifier  
383 species. For example, while about 11% of LUT was recovered in PL-rich mixture dispersion,

384 only about 0.5% of  $\beta$ C was. Concerning LYC it was not detected in the aqueous medium  
385 whatever the emulsifier(s) used. PL were better than BSA to solubilize  $\beta$ C, 0.52 vs 0.03%  
386 ( $p < 0.05$ ), while it was the opposite for LUT, 19.54 vs 11.12% ( $p < 0.05$ ). The apparent solubility  
387 of both carotenoids were higher when BSA and PL were mixed than when they were used  
388 alone ( $p < 0.05$ ). Finally, when purified PL were used instead of the PL mixture, which was a  
389 mixture of PL, lysophosphatidylcholine, TG and sphingomyelin (see material and methods), the  
390 apparent solubility of  $\beta$ C was non significantly different, 0.77 vs 0.52%, while that of LUT was  
391 significantly decreased, 5.82 vs 11.12%. Also, the mixture of BSA plus purified PL led to a  
392 lower apparent solubility of LUT than the mixture of BSA plus PL mixture.

393

#### 394 *Transfer of carotenoids toward uncoated sunflower oil droplets*

395 Figure 1 shows the time-dependence of carotenoid incorporation (wt % of initial  
396 amount of each carotenoid added in the system) in uncoated sunflower oil dispersed in acetate  
397 buffer at pH 4.0. As shown in Fig. 1A the transfer of pure solid carotenoids, which initially  
398 glued to the wall of the glass tubes, to the uncoated TG droplets, increased as a function of time  
399 to nearly reach a plateau. The maximal transfer efficiency was about 59% for LUT and about  
400 72 and 77% for LYC and  $\beta$ C, respectively (Fig. 5). When carotenoids were provided in the  
401 tomato puree matrix the curves of transfer showed very different shapes (Fig. 1B) and the  
402 maximal transfer efficiencies of all the carotenoids were significantly ( $p < 0.05$ ) lower than that  
403 of the pure carotenoids (supporting information Fig. S1A, S2A and S3A). The maximal transfer  
404 efficiency for LYC was about 12% and that for  $\beta$ C and LUT were about 27 and 28%,  
405 respectively (supporting information Fig. S3A, S2A and S1A). Finally, when carotenoids were  
406 in spinach puree (Fig. 1C) the maximal transfer efficiencies of carotenoids to uncoated TG  
407 droplets were the lowest with values of about 8% for LUT and about 5% for  $\beta$ C (supporting

408 information Fig. S1A and S2A). Note that there is no data on the transfer of LYC from spinach  
409 puree because this carotenoid is not significantly present in this vegetable.

410

#### 411 *Transfer of carotenoids toward sunflower oil droplets emulsified with BSA*

412 The only difference between Fig. 2 and Fig. 1 is that the carotenoids were incubated  
413 with TG droplets stabilized with BSA instead of uncoated TG droplets. Fig. 2A shows that the  
414 presence of BSA dramatically impaired the transfer of pure carotenoids to TG located in the  
415 core of these droplets. Indeed, the maximal transfer efficiencies of the solid pure carotenoids  
416 ranged between 0.5 and 4.4% as compared to values higher than 59% for pure solid carotenoids  
417 to the uncoated emulsion (Fig. 5). In contrast, when the carotenoids were provided by tomato  
418 puree (Fig. 2B), their maximal transfer efficiencies for the BSA-emulsion (7.6% for LYC,  
419 26.8% for  $\beta$ C, and 51.6% for LUT; Fig. 6B) were higher than those observed when they were  
420 provided as pure solids (0.5% for LYC, 0.7% for  $\beta$ C, and 4.4% for LUT; Fig. 5). The same  
421 observation could be made for the maximal transfer efficiencies of carotenoids provided in  
422 spinach puree. They were higher for the BSA-emulsion than for the uncoated emulsion.

423

#### 424 *Transfer of carotenoids toward sunflower oil droplets emulsified with PL*

425 Fig. 3A and 5 show that the maximal transfer efficiencies of the pure carotenoids to  
426 these emulsions ranged between 1.5% for LYC to 30.9% for LUT. When the carotenoids were  
427 provided by tomato puree (Fig. 3B), the maximal transfer efficiency dramatically increased for  
428 LUT, reaching 72.5% (Fig 6A). The maximal transfer efficiency of LYC also increased, from  
429 1.5 to 8.5% (Fig. 5C and 6C). Finally, when the carotenoids were provided by the spinach  
430 puree (Fig. 3C), their maximal transfer efficiencies were about 41% and 10% for LUT and  $\beta$ C  
431 (Fig. 7A and 7B), respectively.

432

433 *Transfer of carotenoids toward sunflower oil droplets stabilized with both BSA and PL*

434 Fig. 4A shows that the maximal transfer efficiency of pure carotenoids to TG located in  
435 BSA/PL-emulsions was very low. Indeed, it ranged between 0.9% for LYC to 8% for  $\beta$ C (Fig.  
436 5C, A and B). As observed for the two other emulsions, when LUT was provided in tomato  
437 puree its maximal transfer efficiency to the TG of the BSA/PL-emulsion dramatically increased  
438 ( $p < 0.05$ ) reaching about 81% (Fig. 6A) as compared to pure LUT (7.0%, Fig. 5A). The  
439 maximal transfer efficiencies of the two other carotenoids also significantly ( $p < 0.05$ ) increased  
440 reaching about 10 and 32% for tomato puree LYC and  $\beta$ C (Fig. 6C and B) as compared to pure  
441 carotenoids, 0.9 and 8.0%, respectively (Fig. 5C and B). The curves of transfer of spinach  
442 carotenoids to the BSA/PL-emulsion are shown in Fig. 4C and the corresponding maximal  
443 transfer efficiencies are shown in Fig. 7. In summary, the spinach- $\beta$ C maximal transfer  
444 efficiency was about 14% and that of spinach-LUT was about 50%.

445

446 *Comparison of the maximal transfer efficiencies of pure carotenoids toward sunflower oil when*  
447 *this oil was either uncoated or emulsified with PL, BSA or both*

448 Fig. 5 to 7 give a summary of the maximal transfer efficiencies measured in the transfer  
449 experiments shown in Fig. 1 to 4. They allow us to compare the maximal transfer efficiency  
450 measured for each CAR, either pure or incorporated in the two different vegetable purees, with  
451 each different species of emulsion.

452 Concerning the transfer of pure carotenoids, two general observations can be made.  
453 First, the transfer was dramatically impaired when the TG were coated with the emulsifiers  
454 (Fig. 5). Second, the lowest maximal transfer efficiencies were obtained when BSA was used  
455 as the only emulsifier. Regarding the effect of carotenoid species, it is noteworthy that LUT  
456 had the lowest maximal transfer efficiency to uncoated TG, i.e. about 59%, while LYC and  $\beta$ C  
457 exhibited close maximal transfer efficiencies for this emulsion, i.e. about 72 and 77%

458 respectively. This ranking markedly changed in the presence of emulsifiers with LYC having  
459 the lowest maximal transfer efficiency, regardless the emulsifiers.

460

461 *Comparison of the maximal transfer efficiencies of tomato puree carotenoids toward sunflower*  
462 *oil when this oil was either uncoated or emulsified with PL, BSA or both*

463 The maximal transfer efficiencies of tomato puree LUT to the different emulsion  
464 species were always higher than those of the two other tomato carotenoids (Fig. 6). The  
465 maximal transfer efficiencies of the three tomato carotenoids were always higher when PL and  
466 BSA were both present, i.e. in the BSA/PL-emulsions, than when they were used alone.

467

468 *Comparison of the maximal transfer efficiencies of spinach puree carotenoids toward*  
469 *sunflower oil when this oil was either uncoated or emulsified with PL, BSA or both*

470 The first observation that arises from Fig. 7 is that, conversely to what was observed  
471 with pure carotenoids (Fig. 5), the lowest maximal transfer efficiencies of both spinach  
472 carotenoids were observed with the uncoated emulsion. Moreover, the effect of the emulsifiers  
473 on the maximal transfer efficiencies of the spinach carotenoids depended on carotenoid species.  
474 Indeed, concerning spinach LUT, PL led to a significantly ( $p < 0.05$ ) higher maximal transfer  
475 efficiency than BSA while it was the opposite for  $\beta$ C.

476

477 *Comparison of the maximal transfer efficiency of LUT toward the different emulsions when*  
478 *LUT was provided either as a pure solid or incorporated in tomato or spinach puree*

479 Results presented in supporting information Fig. S1 are not commented because they  
480 are presented, in another way, in Fig. 5 to 7. However, we believe that showing these results  
481 this way can help the reader to better see another interesting result of this study, that is the  
482 effect of the food matrix on the transfer of LUT to the different emulsion species.

483

484 *Comparison of the maximal transfer efficiency of  $\beta$ C toward the different emulsions when  $\beta$ C*  
485 *was provided either as a pure solid or incorporated in tomato or spinach puree*

486 Same comment for supporting information Fig. S2 than for supporting information Fig.  
487 S1, but concerning  $\beta$ C.

488

489 *Comparison of the maximal transfer efficiency of LYC toward the different emulsions when*  
490 *LYC was provided either as a pure solid or incorporated in tomato puree*

491 Same comment for supporting information Fig. S3 than for supporting information Fig.  
492 S1 and S2 but concerning LYC. Obviously, there is no data for spinach LYC because this  
493 carotenoid is not present in this vegetable.

#### 494 **4) Discussion**

495

496 Before discussing the results in details it should be mentioned that the transfer values  
497 that we observed for the vegetable puree carotenoids to the BSA/PL-emulsion, which is the  
498 emulsion assumed to best mimic the dietary emulsions that exist *in vivo*, showed similar trends  
499 than what was observed *in vivo*.<sup>[31]</sup> Indeed, we measured about 10% maximal transfer  
500 efficiency for tomato LYC that can be compared to about 6% recovered in the fat phase of a  
501 meal observed *in vivo* after 3 h digestion,<sup>[31]</sup> and about 50% for spinach LUT as compared to  
502 about 30% measured *in vivo*.<sup>[31]</sup>

503 In the first part of this study we aimed to obtain basic data on the transfer efficiency of  
504 carotenoids to TG. We thus measured the transfer efficiency of pure carotenoids toward  
505 uncoated TG droplets dispersed in an aqueous medium. Results showed that this transfer was  
506 very efficient. Indeed, the maximal transfer efficiency ranged between 59 and 77% depending  
507 on carotenoid species. To explain this observation, we hypothesize that the transfer efficiency  
508 depends both on the collisions between the agitated TG droplets and the film of carotenoids  
509 stuck to the glass tubes, and on the hydrophobic attraction between carotenoids and TG  
510 molecules when TG droplets enter in contact with the solid pure carotenoid film. Indeed, the  
511 transfer of nonpolar molecules from a polar environment to a nonpolar environment is  
512 driven both by the free energy gain<sup>[52]</sup> and the hydrophobic force.<sup>[53]</sup> The involvement of the  
513 hydrophobic force is further supported by previous finding<sup>[28]</sup> and by the fact that the maximal  
514 transfer efficiency of LUT was lower than that of  $\beta$ C and LYC, i.e. 59 vs 77 and 72%  
515 respectively, which can be related to their respective hydrophobicity schematically reflected by  
516 their Log *P*, i.e. 11.78 vs 15.51 and 15.19, respectively (www.chemspider.com). Nevertheless,  
517 the use of Log *P*, which measures the partition of a molecule between octanol and water,  
518 appears not to be accurate to predict the solubility of carotenoids in oil because (Z)-isomers of

519 lycopene, which have the same Log  $P$  than the (all-E)-isomer, are more soluble in oil than the  
520 (all-E)-isomer.<sup>[30, 50]</sup> Nevertheless, because carotenoid hydrophobicity has also been correlated  
521 with transfer of carotenoids from tomato and carrot based matrices to oil,<sup>[28]</sup> as well as with  
522 their transfer efficiency from lipid droplets toward mixed micelles,<sup>[32, 54]</sup> these results suggest  
523 that the hydrophobic force is a key player in carotenoid bioaccessibility.

524 Because dietary fats are coated with amphiphilic dietary molecules in the upper  
525 gastrointestinal tract,<sup>[18]</sup> the second part of this study was dedicated to assessing the effect of  
526 the main dietary emulsifiers, i.e. phospholipids and proteins, on the transfer of pure solid  
527 carotenoids to the TG located in the core of lipid droplets.<sup>[20-22]</sup> When TG droplets were  
528 emulsified in the presence of the emulsifiers, the maximal transfer efficiencies of pure  
529 carotenoids were dramatically impaired (Fig. 5). Furthermore, the ranking of carotenoids  
530 regarding their maximal transfer efficiency markedly changed with pure LYC having the  
531 lowest maximal transfer efficiency, regardless the emulsifier, meaning that the inhibitory effect  
532 of the emulsifiers on the transfer of carotenoids was higher for LYC than for  $\beta$ C and LUT. This  
533 inhibition was *a priori* surprising because, as expected, when the TG were stabilized by the  
534 emulsifiers the size of the emulsion lipid droplets decreased and the specific surface area, and  
535 thus the available surface of exchange between carotenoids and TG, dramatically increased  
536 (table 1). Thus, the mechanism(s) that lead(s) to the inhibitory effect of the emulsifiers on the  
537 transfer of carotenoids far outweigh(s) the hypothesized potential of the emulsifiers in  
538 increasing transfer by increasing the surface of transfer. Several inhibitory mechanisms can be  
539 proposed. First, it can be hypothesized that, when the emulsions stabilized with the emulsifiers  
540 were incubated with the solid films of pure carotenoids, a fraction of the carotenoids could have  
541 been incorporated/associated to the fraction of the emulsifiers that remained dispersed in the  
542 aqueous medium,<sup>[55, 56]</sup> becoming not available for the transfer to the lipid droplets. Indeed, the  
543 results obtained in this study on the apparent solubility of carotenoids in the emulsifiers



544 confirm that these compounds can associate with PL <sup>[52, 57, 58]</sup> and BSA. <sup>[59-61]</sup> They further show  
545 that the apparent solubility in the emulsifiers is mainly determined by carotenoid species, the  
546 carotenoid with two polar groups, LUT, being better associated with the emulsifiers than the  
547 two other carotenoids, likely because it associates more easily with amphiphilic molecules.  
548 Nevertheless, such a mechanism is not sufficient to explain the inhibitory effect of the  
549 emulsifiers on the transfer of carotenoids because the transfer of LUT, which was by far the  
550 carotenoid that showed the greatest apparent solubility in PL and BSA (table 2), was less  
551 affected by the presence of the emulsifiers than LYC, which was not significantly associated  
552 with the emulsifiers (Fig. 5). We thus hypothesize that the emulsifiers adsorbed at the droplet  
553 interface could also create a physical barrier (or an energy barrier) that impairs the contact of  
554 carotenoids with TG and thus impairs their transfer into the lipid droplets. Furthermore, we  
555 hypothesize that this barrier is most important for the carotenoids that are not soluble in the  
556 emulsifiers than for those who are. Indeed, we assume that carotenoids solubilized in the  
557 emulsifier located at the droplet interface <sup>[22]</sup>, and thus in contact with the TG core of the  
558 droplets, can easily transfer to the TG phase, while carotenoids not solubilized in the interface  
559 emulsifiers can hardly transfer. This hypothesis explains why the transfer of pure LYC, which  
560 was not soluble in the emulsifiers, was more impaired by the emulsifiers than that of the two  
561 other pure carotenoids (Fig. 5). Thus, when TG are coated with these emulsifiers, the transfer  
562 efficiency of pure carotenoids to TG results from opposite effects: negative effects due to both  
563 the quenching of the hydrophobic force by the emulsifier barrier between carotenoids and TG  
564 and to the trapping of a fraction of carotenoids by the fraction of emulsifiers that remain in the  
565 aqueous phase, <sup>[22, 55, 62]</sup> and positive effects due to both the facilitated transfer of carotenoids  
566 that are incorporated in the fraction of emulsifiers located at the lipid droplet interface, and to  
567 the higher surface of exchange induced by the emulsifiers. Obviously, these hypotheses should  
568 be verified by dedicated experiments and we cannot extrapolate these observations to the effect

569 of other dietary emulsifiers on the transfer of other carotenoid species. Nevertheless, our results  
570 and their interpretations allow us to hypothesize that the transfer efficiency of any carotenoid  
571 species to the TG phase of an emulsion stabilized with amphiphilic molecules will mainly  
572 depend on the ability of these carotenoids to associate with the amphiphilic molecules located  
573 at the interface and to those solubilized in the aqueous phase.

574         The third part of this study aimed at assessing whether plant matrices, which contain  
575 physical barriers that can impair carotenoid diffusion,<sup>[13, 26]</sup> and which also contain carotenoids  
576 that can be in different physical states and that can be associated with different matrix  
577 molecules,<sup>[11]</sup> can significantly affect the transfer efficiency. To that aim we chose two  
578 vegetables that are usual dietary sources of carotenoids, i.e. tomato<sup>[63]</sup> and spinach,<sup>[33]</sup> and we  
579 turned them into puree to mimic the size of their particles in the stomach during digestion.  
580 After having studied the transfer of carotenoids in the same conditions than those used for the  
581 pure carotenoids, we first observed that the transfer efficiency of the carotenoids incorporated  
582 in these matrices to the uncoated TG droplets was markedly lower than that of the pure  
583 carotenoids (supporting information Fig. S1A, S2A and S3A). To explain this observation, we  
584 first hypothesize that this is because carotenoids in plant cells have to cross different barriers  
585 that impair their release from the cells.<sup>[12, 13]</sup> Nevertheless, this hypothesis disagrees with the  
586 observation that the vegetable puree carotenoids were, in most cases, better transferred to the  
587 coated emulsions than the pure carotenoids (supporting information Fig. S1 to S3). Therefore,  
588 we suggest that some compounds in the vegetable purees are able to improve the transfer by a  
589 mechanism that far outweighs the inhibitory effect of the cell barriers. A second hypothesis to  
590 explain the lower transfer of vegetable puree carotenoids to uncoated TG droplets, as compared  
591 to pure carotenoids, is that plant cells contain amphiphilic molecules, e.g. proteins and PL,<sup>[64]</sup>  
592 that adsorb at the uncoated TG droplet interface and that create a barrier that impairs the  
593 transfer. A second, and very intriguing, observation was that the transfer of both spinach and

594 tomato puree LUT was significantly improved by the presence of the emulsifiers (supporting  
595 information Fig. S1B, S1C and S1D vs supporting information Fig. S1A). This phenomenon  
596 was not observed for tomato  $\beta$ C and LYC (supporting information Fig. S2 and supporting  
597 information Fig. S3) and it was of less amplitude for spinach  $\beta$ C (supporting information Fig.  
598 S2). To explain this phenomenon, we suggest that the complexes of molecules that were  
599 associated with LUT in spinach and tomato purees and with  $\beta$ C in spinach puree, or which had  
600 been associated with these carotenoids after the transformation of these matrices in purees,  
601 associate very efficiently to the TG droplets coated with emulsifiers. The electric charges of the  
602 coated or uncoated TG droplets and of the vegetable purees (Table 2) cannot explain why the  
603 plant complexes of molecules apparently readily associate with coated TG droplets, while they  
604 do not to uncoated TG droplets. Thus, we hypothesize that spinach and tomato LUT, as well as  
605 spinach  $\beta$ C, were associated with molecules that facilitate anchoring of these complexes to  
606 interfaces coated with emulsifiers. Furthermore, the fact that the transfer efficiency of tomato  
607 LYC was not enhanced by the presence of emulsifiers (supporting information Fig. S3) was  
608 perhaps because LYC in tomatoes is present as crystals in chromoplasts<sup>[11]</sup> and these crystals  
609 are not coated by the molecules that, we hypothesized to, have a high affinity for interfaces  
610 coated with emulsifiers. A third observation on the transfer efficiency of the vegetable puree  
611 carotenoids to emulsion TG was that this transfer was always more efficient for tomato LUT  
612 and  $\beta$ C than for spinach LUT and  $\beta$ C (supporting information Fig. S1 and supporting  
613 information Fig. S2). We hypothesis, but this should be verified in a dedicated study, that  
614 carotenoids located in these two different plant matrices could have to cross different biological  
615 barriers,<sup>[12, 13]</sup> to go out of these matrices. Another possibility is that the size of the cells or the  
616 percentage of remaining intact cells was different in the two purees. Whatever the mechanism,  
617 the fact that the transfer is different in the two different plant matrices is in agreement with the  
618 very variable effect of different plant matrices on carotenoid bioaccessibility.<sup>[13, 36]</sup>

619 In summary, this study has allowed us to suggest fundamental mechanisms governing  
620 the transfer of carotenoids to the TG phase of dietary emulsions. We have first suggested that  
621 this transfer is basically governed by the hydrophobic force between carotenoids and TG. Then  
622 we have shown that amphiphilic molecules that coat TG droplets strongly impair the transfer of  
623 pure carotenoids, likely by creating a barrier between them and TG. Furthermore, the transfer  
624 of carotenoids that are significantly solubilized in the amphiphilic molecules is less affected.  
625 We then found that plant carotenoids hardly transfer to uncoated TG droplets, and we have  
626 suggested that this is because they have to cross structural barriers but it is also likely that  
627 amphiphilic molecules present in plant matrices coat the interface and hinder the transfer.  
628 Conversely, we found that spinach and tomato puree LUT, and to a less extent spinach  $\beta$ C,  
629 were better transferred to TG droplets coated with emulsifiers than to uncoated TG droplets and  
630 we hypothesized that this is because lipid/protein complexes that are present with these  
631 carotenoids in the studied vegetable matrices facilitate anchoring of these complexes to coated  
632 lipid droplets. Further experiments are needed to complete this pioneering study. Indeed, it is  
633 likely that digestive enzymes that hydrolyze lipids and proteins in the gastric and intestinal  
634 lumen, as well as bile salts that adsorb at the lipid droplet interface during digestion, modify the  
635 transfer rate and/or the transfer efficiency of carotenoids. It is also likely that different results  
636 would have been obtained with other types of food oil. Indeed, carotenoid solubility in TG  
637 depends on the type of TG fatty acids.<sup>[22]</sup> Nevertheless, we believe that a full understanding of  
638 the mechanisms that govern the transfer of plant carotenoids to food emulsions will allow  
639 researchers to propose new strategies to improve the bioavailability of these phytochemicals.

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644

645 **Author contributions:**

646 The authors' responsibilities were as follows---PB and CC-V: designed the research and have  
647 primary responsibility for final manuscript content. PG and FH: conducted research. PB and  
648 CG: analysed data and interpreted them. PB: wrote the paper; and all authors: read and  
649 approved the final manuscript.

650

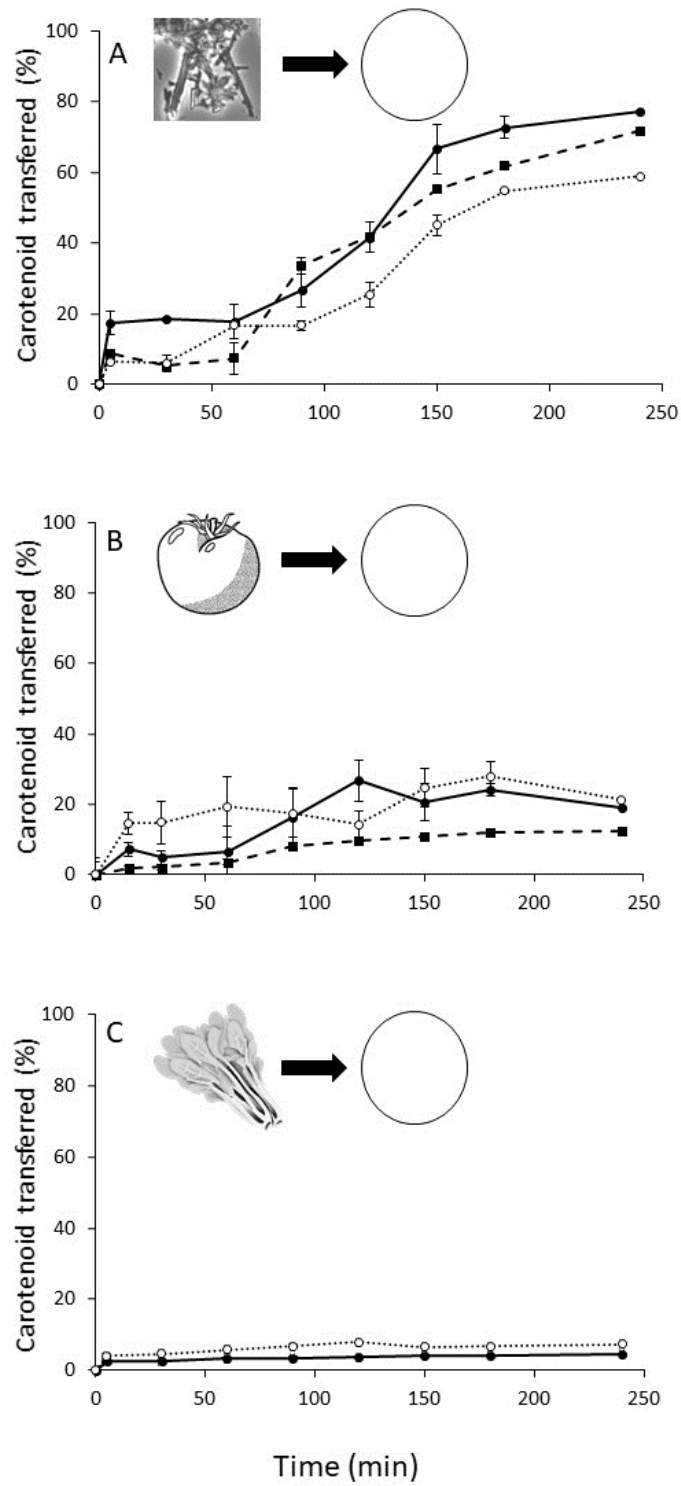
651 **Conflicts of interest:**

652 None of the authors reported a potential conflict of interest.

653

654 **Funding:**

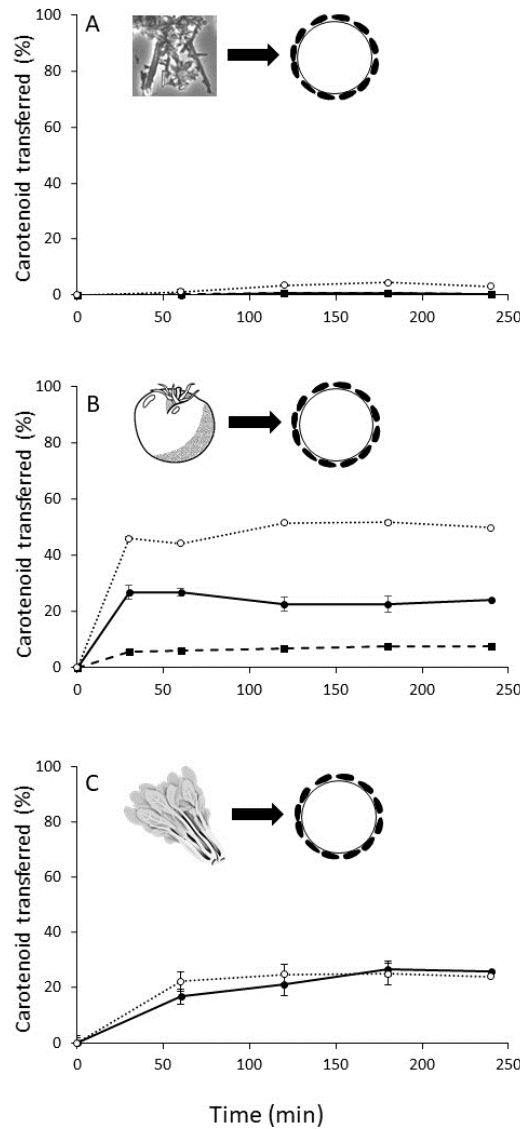
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656 Agricultural Research (INRA): AIC Cepia "Factors limiting the bioaccessibility of carotenoids  
657 during digestion" and Action Prioritaire AlimH "Bioavailability of micronutrients".



659

660 **Figure 1.** Transfer of carotenoids to uncoated sunflower TG.

661 The graph shows the percent of carotenoid transferred as a function of incubation time (min). **A:** the photography  
 662 of lycopene crystals (magnification 100x) and the circle helps to see that this is the transfer of pure solid  
 663 carotenoids to uncoated TG droplets in aqueous medium. **B:** the drawing of tomato helps to see that this is the  
 664 transfer of tomato puree carotenoids, i.e. lycopene (LYC),  $\beta$ -carotene ( $\beta$ C) and lutein (LUT), to uncoated TG  
 665 droplets. **C:** the drawing of spinach leaves helps to see that this is the transfer of spinach puree carotenoids, i.e.  $\beta$ -

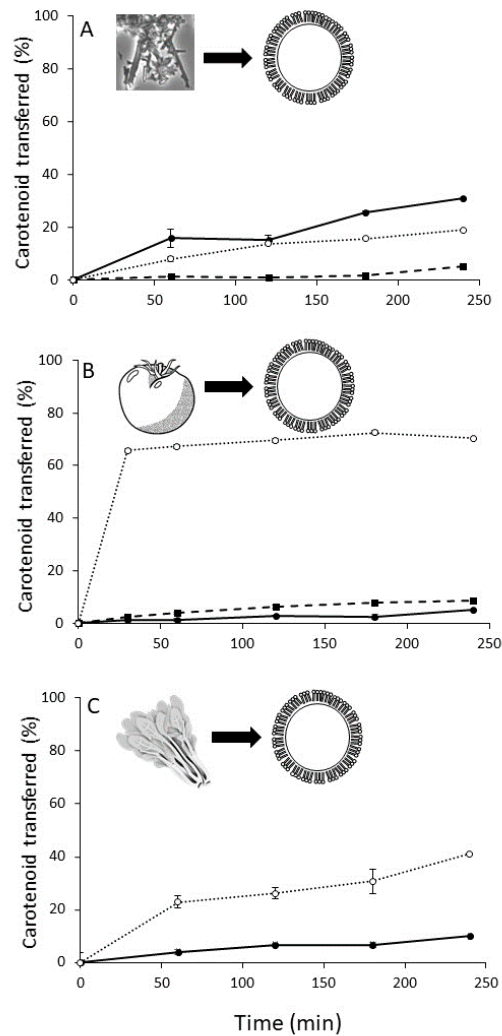


666 carotene ( $\beta$ C) and lutein (LUT), to uncoated TG droplets. ■ LYC, ●  $\beta$ C, ○ LUT. Dots represent mean  $\pm$  SEM of 4  
 667 independent experiments.

668

669 **Figure 2.** Transfer of carotenoids to sunflower TG located in emulsions stabilised by BSA.

670 The graph shows the percent of carotenoid transferred as a function of incubation time (min). **A:** the photography  
 671 of lycopene crystals (magnification 100x) and the circle coated with black forms helps to see that this shows the  
 672 transfer of pure solid carotenoids to BSA-emulsions. **B:** the drawing of tomato helps to see that this is the transfer  
 673 of tomato puree carotenoids, i.e. lycopene (LYC),  $\beta$ -carotene ( $\beta$ C) and lutein (LUT), to BSA-emulsions. **C:** the  
 674 drawing of spinach leaves helps to see that this is the transfer of spinach puree carotenoids, i.e.  $\beta$ -carotene ( $\beta$ C)



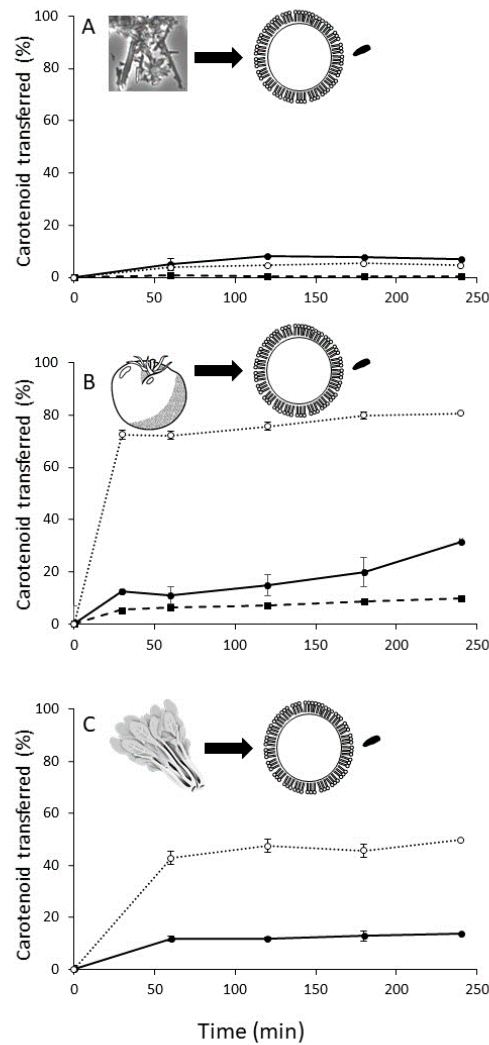
675 and lutein (LUT), to BSA-emulsions. ■ LYC, ●  $\beta$ C, ○ LUT. Dots represent mean  $\pm$  SEM of 4 independent  
 676 experiments.

677

678 **Figure 3.** Transfer of carotenoids to sunflower TG located in emulsions stabilised by PL-rich mixture.



679 The graph shows the percent of carotenoid transferred as a function of incubation time (min). **A:** the photography  
 680 of lycopene crystals (magnification 100x) and the circle coated with two arm pins helps to see that this shows the  
 681 transfer of pure solid carotenoids to PL-emulsions. **B:** the drawing of tomato helps to see that this is the transfer of  
 682 tomato puree carotenoids, i.e. lycopene (LYC),  $\beta$ -carotene ( $\beta$ C) and lutein (LUT), to PL-emulsions. **C:** the  
 683 drawing of spinach leaves helps to see that this is the transfer of spinach puree carotenoids, i.e.  $\beta$ -carotene ( $\beta$ C)

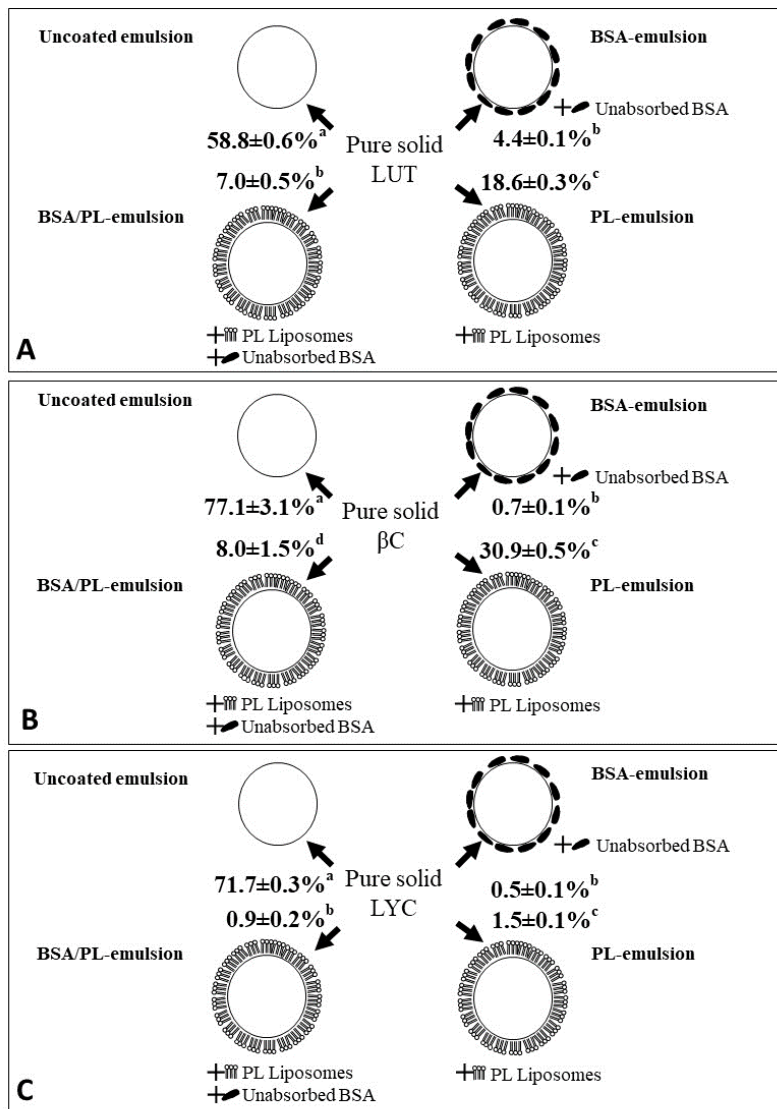


684 and lutein (LUT), to PL-emulsions. ■ LYC, ●  $\beta$ C, ○ LUT. Dots represent mean  $\pm$  SEM of 4 independent  
 685 experiments.

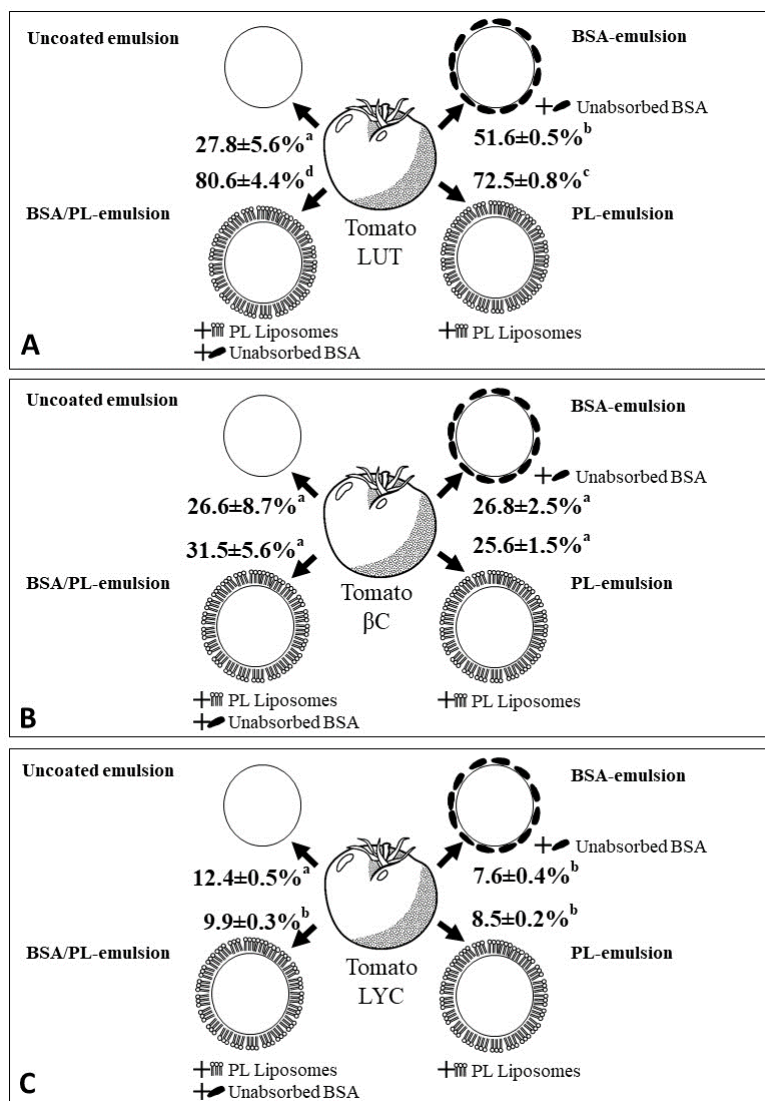
686

687 **Figure 4.** Transfer of carotenoids to sunflower TG located in emulsions stabilised by a mixture of BSA and PL as  
 688 emulsifiers.

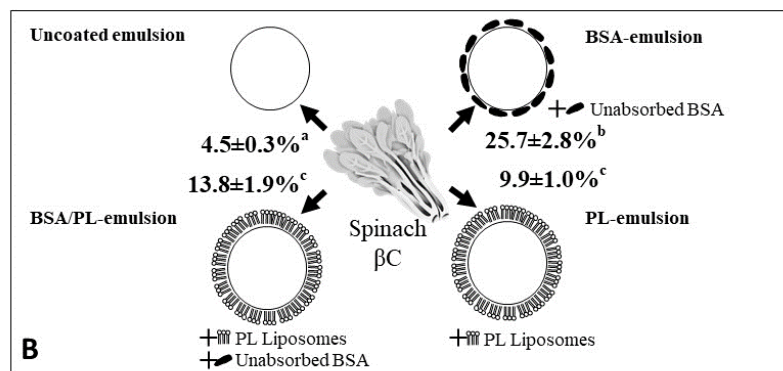
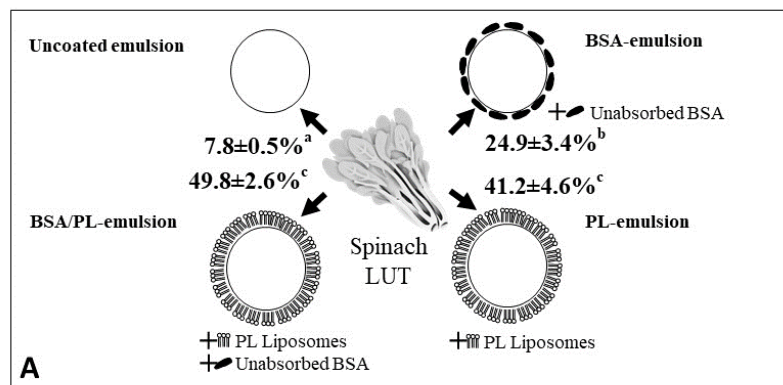
689 The graph shows the percent of carotenoid transferred as a function of incubation time (min). **A:** the photography  
 690 of lycopene crystals (magnification 100x) and the circle coated with two arm pins, together with a black form  
 691 close to it, helps to see that this shows the transfer of pure solid carotenoids to BSA/PL-emulsions. **B:** the drawing of tomato  
 692 helps to see that this is the transfer of tomato puree carotenoids, i.e. lycopene (LYC),  $\beta$ -carotene ( $\beta$ C)  
 693 and lutein (LUT), to BSA/PL-emulsions. **C:** the drawing of spinach leaves helps to see that this is the transfer of  
 694 spinach puree carotenoids, i.e.  $\beta$ -carotene ( $\beta$ C) and lutein (LUT), to BSA/PL-emulsions. ■ LYC, ●  $\beta$ C, ○ LUT.  
 695 Dots represent mean  $\pm$  SEM of 4 independent experiments.



696 **Figure 5.** Summary of the maximal transfer efficiencies measured for each pure solid carotenoid in each type of  
 697 emulsion. The maximal transfer efficiencies were obtained from the curves presented in figures 1 to 4. A:  
 698 Maximal transfer efficiency of pure lutein (LUT) to the different emulsions, B: Maximal transfer efficiency of  
 699 pure  $\beta$ -carotene ( $\beta$ C), C: Maximal transfer efficiency of pure lycopene (LYC). The theoretical localisation of the  
 700 emulsifiers is presented. For example, it has been calculated (see result section) that 65% of PL were located at the  
 701 lipid droplet interface and that the remaining fraction was dispersed in the aqueous phase. Values represent mean  $\pm$   
 702 SEM of 4 independent experiments. In each figure, means that bear different superscript letters are significantly  
 703 different ( $p < 0.05$ ), ANOVA followed by Tukey-Kramer post-hoc test.



704 **Figure 6.** Summary of the maximal transfer efficiencies measured for each tomato puree carotenoid in each type  
 705 of emulsion. The maximal transfer efficiencies were obtained from the curves presented in figures 1 to 4. A:  
 706 Maximal transfer efficiency of pure lutein (LUT) to the different emulsions, B: Maximal transfer efficiency of  
 707 pure  $\beta$ -carotene ( $\beta$ C), C: Maximal transfer efficiency of pure lycopene (LYC). The theoretical localisation of the  
 708 emulsifiers is presented. For example, it has been calculated (see result section) that 65% of PL were located at the  
 709 lipid droplet interface and that the remaining fraction was dispersed in the aqueous phase. Values represent mean  $\pm$   
 710 SEM of 4 independent experiments. In each figure, means that bear different superscript letters are significantly  
 711 different ( $p < 0.05$ ), ANOVA followed by Tukey-Kramer post-hoc test.



712

713 **Figure 7.** Summary of the maximal transfer efficiencies measured for each spinach puree carotenoid in each type  
714 of emulsion.

715 The maximal transfer efficiencies were obtained from the curves presented in figures 1 to 4. A: Maximal transfer  
716 efficiency of pure lutein (LUT) to the different emulsions, B: Maximal transfer efficiency of pure  $\beta$ -carotene ( $\beta$ C).  
717 The theoretical localisation of the emulsifiers is presented. For example, it has been calculated (see result section)  
718 that 65% of PL were located at the lipid droplet interface and that the remaining fraction was dispersed in the  
719 aqueous phase. Values represent mean  $\pm$  SEM of 4 independent experiments. In each figure, means that bear  
720 different superscript letters are significantly different ( $p < 0.05$ ), ANOVA followed by Tukey-Kramer post-hoc test.

721 **Table 1**

722

723 Main characteristics of the emulsions stabilised by BSA, PL mixture or both\*.

	BSA-emulsions	PL-emulsions	BSA/PL-emulsions
Dv10 ( $\mu\text{m}$ )	$0.81 \pm 0.05^a$	$0.43 \pm 0.02^b$	$1.55 \pm 0.06^c$
Dv50 ( $\mu\text{m}$ )	$1.81 \pm 0.12^a$	$1.42 \pm 0.09^b$	$2.30 \pm 0.05^c$
Dv90 ( $\mu\text{m}$ )	$3.84 \pm 0.25^a$	$2.75 \pm 0.13^b$	$4.60 \pm 0.20^c$
D3,2 ( $\mu\text{m}$ )	$1.52 \pm 0.15^a$	$1.48 \pm 0.04^a$	$2.46 \pm 0.11^b$
D4,3 ( $\mu\text{m}$ )	$2.11 \pm 0.19^a$	$0.95 \pm 0.04^b$	$1.18 \pm 0.12^c$
Specific Surface area ( $\text{m}^2/\text{g oil}$ )	$3.99 \pm 0.17^a$	$6.50 \pm 0.12^b$	$3.04 \pm 0.05^c$
Non-adsorbed emulsifier**			
g/L in aqueous phase	3.4	0.9	1.7 (PL); 4.44 (BSA)
% in aqueous phase	77.6	35.1	69.7 (PL); 100*** (BSA)
Zeta potential (mV)	$24.6 \pm 0.4^a$	$-9.3 \pm 0.4^b$	$8.3 \pm 0.6^c$

724 Values are means  $\pm$  SEM of 6 measurements (3 different emulsions measured 2 times each).

725 Dv10 is the droplet diameter below which 10% of the total lipid volume of the sample is

726 contained. Dv50 is the median size. Dv90 is the droplet diameter below which 90% of the total

727 lipid volume of the sample is contained. D3,2 is the surface mean diameter. D4,3 is the volume

728 mean diameter. In each line, means bearing different letters are significantly different (ANOVA

729 followed by Tukey-Kramer post-hoc test,  $p < 0.05$ ).

730 \*: it was not possible to measure these characteristics for the uncoated emulsion, i.e. pure TG

731 suspended in aqueous medium, because the TG droplets were very unstable and coalesced very

732 quickly. \*\*: amounts of emulsifiers not absorbed at the droplet interface have been estimated

733 using 2 mg/mL as theoretical surface load for both BSA and PL,<sup>[47]</sup> \*\*\*: in the presence of PL  
734 in excess, BSA is estimated not to be adsorbed at the interface.

735 **Table 2**

736

737 Apparent solubility of pure carotenoids in aqueous dispersions of BSA, PL mixture, Purified

738 PL or mixtures of the emulsifiers.

	BSA	PL mixture	Purified PL	BSA+PL mixture	BSA+Purified PL
$\beta$ C	$0.03 \pm 0.02^{*a}$	$0.52 \pm 0.11^b$	$0.77 \pm 0.11^b$	$1.36 \pm 0.21^c$	$0.65 \pm 0.02^b$
LUT	$19.54 \pm 0.61^c$	$11.12 \pm 1.46^b$	$5.82 \pm 3.44^a$	$43.19 \pm 5.91^d$	$11.42 \pm 1.53^b$
LYC	ND	ND	ND	ND	ND

739 Pure carotenoids were incubated in aqueous solutions of either BSA, PL mixture, purified PL,

740 or a 50/50 mixture of both emulsifiers, and carotenoids recovered in the aqueous dispersions of

741 emulsifiers were quantified by UPLC. \*Maximal percentage of carotenoid recovered in

742 dispersions of emulsifiers. Values are mean  $\pm$  SEM of 3 to 9 measurements. ND: not detected,743 i.e.  $< 0.01\%$ . In each line, means bearing different letters were significantly different (ANOVA744 followed by Tukey-Kramer post-hoc test,  $p < 0.05$ ).



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