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1                   **Phytosterol vehicles used in a functional product modify carotenoid/cholesterol**  
2                   **bioaccessibility and uptake by Caco-2 cells**

3  
4                   **Magali Gies<sup>a,b</sup>, Adrien Servent<sup>a,b</sup>, Patrick Borel<sup>c</sup>, Claudie Dhuique-Mayer<sup>a,b\*</sup>**

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13  
14  
15                   **Abstract**

16                   Functional foods containing dispersible phytosterols (DPs) are recommended to reduce  
17                   cholesterol absorption in humans. However, only a few studies have been conducted on the effect  
18                   of DP vehicles on carotenoid bioaccessibility and uptake by intestinal cells. On a previously  
19                   developed fermented maize product containing both DPs and carotenoids, we aim to evaluate the  
20                   effect of DPs encapsulated by maltodextrin on the carotenoid/phytosterol/cholesterol interactions  
21                   during gastro-duodenal digestion. Thanks to maltodextrin properties,  $\beta$ -carotene and lycopene  
22                   bioaccessibilities were significantly improved by  $40.2 \pm 1.4\%$  while that of  $\beta$ -cryptoxanthin  
23                   decreased by  $14 \pm 0.1\%$ . Additionally, the presence of DPs reduced the cholesterol micellarization  
24                   by  $51.8 \pm 4.2\%$ . Despite a slight decrease in carotenoid uptake caused by the DP vehicle, related  
25                   to micelle size, an inhibition of cholesterol accumulation by Caco-2 cells was observed. These  
26                   results highlighted the key role of maltodextrin as a vehicle of DPs on carotenoid and cholesterol  
27                   absorption.

28  
29                   **Keywords**

30                   Dispersible phytosterols; maltodextrin; carotenes; xanthophylls; bioaccessibility; Caco-2 cells

## 31 1. Introduction

32 Phytosterols are frequently used in the functional food industry because they are known to be effective  
33 in lowering the LDL-cholesterol level (Gylling & Simonen 2015). Several dairy products, such as  
34 yogurts or margerines, are enriched in phytosterols and have been on the market for several years  
35 (Descalzo *et al.*, 2018). The daily recommended intake of phytosterols to obtain a cholesterol-lowering  
36 effect is 1.5 to 3 g. It allows reducing the cholesterol absorption from the gut by 30-40% leading to 7-  
37 12% decrease of plasma LDL-cholesterol (Chen, McClements, & Decker, 2013; EFSA Panel on  
38 Dietetic Products, Nutrition and Allergies (NDA), 2012; Marangoni & Poli, 2010; Shahzad *et al.*, 2017).  
39 Recently, it was explained that functional foods with plant sterols may be considered in  
40 hypercholesterolemic patients with intermediate and low global cardiovascular risk (Gylling *et al.*,  
41 2014).

42 Phytosterols are natural constituents of plant cell walls. Among the 250 identified phytosterol  
43 molecules,  $\beta$ -sitosterol is the most abundant phytosterol along with campesterol and stigmasterol.  
44 Because of the structural similarities between sitosterol and cholesterol (an additional ethyl group at  
45 position C-24 in sitosterol), it is known that sitosterol can reduce cholesterol absorption. The two main  
46 mechanisms involved in the cholesterol-lowering effect of phytosterols are : their competition with  
47 cholesterol molecules during the incorporation into mixed micelles in the digestive tract ; and the co-  
48 cristallization with cholesterol, leading to an increase in the fecal excretion of cholesterol (Marangoni &  
49 Poli 2010). Another mechanism described the competition between cholesterol and phytosterols for  
50 uptake by intestinal transporters (NPC1L1 and SR-BI) and a biliary secretion due to an apical efflux of  
51 phytosterols from the enterocytes via ABCG5/G8 (Nakano, Inoue, & Murakoshi, 2019). Moreover, the  
52 *Trans-Intestinal Cholesterol Excretion* (TICE) was described as a complementary mechanism of the  
53 biliary secretion, inversely correlated with the development of atherosclerosis (Blanchard, Moreau,  
54 Cariou, & Le May, 2014).

55 Data suggest that the type of phytosterols (free or esterified) and the food matrix can influence the  
56 interaction between phytosterols and cholesterol (Gleize *et al.*, 2016). Solubilized sterols are more  
57 often used in low-fat dairy products than sterol esters. They also induced a similar LDL-cholesterol  
58 lowering effect (Thomsen *et al.* 2004; Shaghaghi, Harding, & Jones 2014). Bohn *et al.* (2007) showed

59 that fat-free phytosterol-containing products were able to significantly decrease cholesterol  
60 micellarization and Caco-2 cell absorption, depending on the formulation of products. Moreover, while  
61 micro-encapsulation is known to enhance the stability of compounds and their bioaccessibility (Kopeck  
62 & Failla, 2018), Spilburg *et al.* (2003) demonstrated that powdered soy stanol-lecithin used as  
63 emulsifier decreased cholesterol absorption and LDL cholesterol when consumed in fat-free foods.

64 However, a major concern is the interaction between phytosterols and fat-soluble bioactive  
65 compounds, like carotenoids or tocopherols, during digestion (Noakes *et al.*, 2002; Richelle *et al.*,  
66 2004; Rudkowska, AbuMweis, Nicolle, & Jones, 2008). Indeed, recent meta-analyses reported the  
67 decrease of carotenoid concentrations in plasma after plant sterol consumption (Baumgartner *et al.*  
68 2017; Fardet *et al.* 2017).

69 Consequently, it is recommended to increase the consumption of fruits and vegetables rich in  
70 carotenoids together with the consumption of functional sterol products (Fardet *et al.* 2017).  
71 Carotenoids are known to be a natural antioxidant (such as lycopene (Lyc)), but also enhancers of the  
72 immune system, decreasing the risk of cardiovascular and eye diseases (Jomova & Valko, 2013).  
73 Some of them are indeed provitamin A carotenoids ( $\beta$ -carotene (Bc),  $\alpha$ -carotene,  $\beta$ -cryptoxanthin  
74 (Bcx)) and have many vital systemic functions after bioconversion into vitamin A in the organism  
75 (Fernández-García *et al.*, 2012). The lipophilic nature of carotenoids could explain the decrease in  
76 their absorption when phytosterols are present. Apolar Bc is solubilized within mixed micelles whereas  
77 Bcx, like xanthophyll, is probably located on the surface of lipid droplets (Borel *et al.*, 1996). Therefore,  
78 phytosterols can replace not only cholesterol in the core of mixed micelles but also other fat-soluble  
79 compounds, such as carotenoids (Baumgartner *et al.*, 2017). However, both esterified or free  
80 phytosterols are known to compete with carotenoids for absorption during gastro-duodenal digestion,  
81 in a dose-dependent manner, even if it is less with free phytosterols (Richelle *et al.*, 2004). That is why  
82 it is necessary to supplement a functional product containing phytosterols with carotenoids, to reach at  
83 least 2 mg per 125 g serving portion (Noakes *et al.*, 2002).

84 In this context, our laboratory developed a new probiotic functional food based on fermented maize,  
85 containing dispersible phytosterols (DPs) and enriched with natural carotenoids from papaya and  
86 melon. This yogurt like-product was designed to be an alternative to current functional dairy products  
87 (Gies, Descalzo, Servent, & Dhuique-Mayer, 2019). Due to possible interactions between phytosterols

88 and fat-soluble compounds for micellarization, the bioaccessibility of carotenoids and dietary  
89 cholesterol of this new functional food had to be assessed, taking into account the role of the vehicle  
90 of DPs. Indeed, to the best of our knowledge, there is no study on the impact of maltodextrin as  
91 vehicle of DPs on carotenoid bioaccessibility. Specifically, our aims were 1) to evaluate the  
92 bioaccessibility of carotenoids with and without added DPs micro-encapsulated with maltodextrin and  
93 2) to understand if these DPs decrease cholesterol micellarization during *in vitro* digestion of the  
94 fermented yogurt-like product with added cholesterol, in order to validate the cholesterol-lowering  
95 effect of these specific DPs. In the second part, fat-soluble cellular uptake was measured using *in vitro*  
96 digestion coupled with the Caco-2 cell culture model.

97

## 98 **2. Material and methods**

### 99 ***2.1. Fermented maize yogurt-like product***

100 This functional product was previously developed by our laboratory to obtain a probiotic yogurt-like  
101 food, based on fermented maize, designed to be functional. Indeed, it was enriched with carotenoids  
102 and DPs to confer a cholesterol-lowering effect and provide a high intake of provitamin A. The material  
103 contains 2.05 mg of carotenoids/portion of 125 g, which is nearly half of the estimated daily carotenoid  
104 consumption in several countries of 1.3 - 5.84 mg (Gies, Descalzo, Servent, & Dhuique-Mayer, 2019;  
105 Toti *et al.*, 2018). The manufacturing process included maize soaking, crushing, sieving, pasteurization  
106 and lactic fermentation. DPs and freeze-dried fruits (papaya and melon) were incorporated before the  
107 pasteurization step (Gies, Descalzo, Servent, & Dhuique-Mayer, 2019). Commercial DPs, S-80 WDP  
108 90% non-GMO with 80%  $\beta$ -sitosterol were provided by Vitaesterol® (Vitae Naturals, Spain). This type  
109 of DP is spray-dried with 10% maltodextrin (16.5-19.5 dextrose equivalent) and 3% sucroesters, in  
110 order to ensure good encapsulation and therefore their hydrophilic solubility.

111 The product was formulated either with or without DPs, and with maltodextrin with the same dextrose  
112 equivalent, or with maltodextrin and sucroesters. Interaction mechanisms during digestion were  
113 studied thanks to products with added standards of  $\beta$ -sitosterol (Supelco/Sigma-Aldrich, USA) and  
114 cholesterol (Sigma-Aldrich, France). The final products were kept frozen and in darkness at -20 °C  
115 until analysis.

116

117

## 118 **2.2. Fat-soluble compound analysis**

119 The carotenoids, phytosterols and tocopherols of the yogurt-like product were analyzed by a single  
120 analysis method UPLC-DAD-FLD with an UPLC –1290 System Infinity II (Agilent, USA) according to  
121 Gies, Descalzo, Servent, & Dhuique-Mayer (2019). Briefly, 1 g of product was saponified and  
122 extracted twice with n-hexane. Hexanic phases were evaporated under nitrogen and dissolved in 1 mL  
123 of a MTBE/methanol solution (4:1, v: v) before injection into the UPLC system.

124 Carotenoid extraction from digested samples was performed as previously described by Dhuique-  
125 Mayer *et al.* (2018). An aliquot of 10 mL of the micellar phase from a digested sample was extracted 3  
126 times with 10 mL of hexane and 5 mL of ethanol containing 150  $\mu$ L of  $\beta$ -apo-8'-carotenal as an internal  
127 standard. The pooled hexane extracts were evaporated and dissolved in 500  $\mu$ L of MTBE/methanol  
128 (4:1; v:v). Samples were injected according to the UPLC conditions described below.

129 The column used was a C30 YMC (150 x 4.6 mm; 3  $\mu$ m) (YMC Europe GMBH, Germany). Mobile  
130 phases were methanol as eluant A, water as eluant B and MTBE as eluant C, set at 1.5 and 1 mL/min  
131 flow rate for carotenoids/phytosterols/tocopherols and cholesterol respectively. The gradient used to  
132 separate carotenoids, phytosterols and tocopherols was the following: 0-1.5 min [60% A, 40% B]; 1.5-  
133 3 min [80% A, 20% B]; 3-12.5 min [80% A, 5% B, 15% C]; 12.5-15 min [15% A, 85% C]; 15-17 min  
134 [100% A] and back to the initial conditions for re-equilibration. The other gradient used for cholesterol  
135 was: 0-1 min [60% A, 40% B]; 1-1.5 min [80% A, 20% B]; 1.5-3 min [80% A, 5% B, 15% C]; 3-25 min  
136 [65% A, 5% B, 30% C]; 25-27.7 min [15% A, 85% C]; 27.7-30 min [100% A] and back to the initial  
137 conditions for re-equilibration. The column temperature was 20 °C and the injection volume was set  
138 between 10  $\mu$ L and 40  $\mu$ L. Detection was set at 210 nm (DAD) for phytosterols and cholesterol, 450  
139 and 470 nm (DAD) for carotenoids. Fluorescence detection (FLD) for tocopherols was set at 296 nm  
140 (excitation) and 330 nm (emission). Quantification was achieved using the external calibration curve of  
141 Bc,  $\beta$ -cryptoxanthin, lycopene,  $\alpha/\gamma$ -tocopherol standards (Extrasynthese, France) and  $\beta$ -sitosterol  
142 standard (Supelco, USA).

143

144 **2.3. *In vitro* digestion model**

145 The *in vitro* digestion model was previously developed by Reboul *et al.* (2006). It had been validated  
146 against human studies and was considered to be a reliable model for carotenoid behavior during *in*  
147 *vitro* digestion (Etcheverry, Grusak, & Fleige, 2012). 15 g of product samples were mixed in 32 mL of  
148 saline solution (NaCl 0.9%) and submitted to the *in vitro* digestion model according to Dhuique-Mayer  
149 *et al.* (2016). To mimic the gastric digestion phase, the pH was adjusted to 4.0 and 2 mL of pepsin  
150 were added before incubating the mixture at 37 °C for 30 min. To mimic the duodenal phase, the pH of  
151 the gastric mixture was raised to 6.0. Then, 9 mL of a solution containing porcine pancreatin (2  
152 mg/mL) and porcine bile extract (12 mg/mL) in 100 mmol/L trisodium citrate were added, as well as 4  
153 mL of porcine bile extract (0.1 g/mL) and 1 mL of cholesterol esterase (10 mg 32 U/mL in 6 mL of  
154 distilled water). Samples were subsequently incubated in a shaking water bath at 37 °C for 30 min to  
155 finish the digestion process. Micelles were separated by centrifugation at 48 000 g for 4 h at 10 °C  
156 using an Aventi JE rotor JA-20 (Beckman-coulter, USA), and the aqueous fraction was collected and  
157 filtered through a 0.20 µm filter (Whatman, U.K.). Aliquots were stored at -20 °C until analysis.

158 The maltodextrin and the sucroesters (as used in the commercial DPs with 10% maltodextrin and 3%  
159 sucroesters) were added to 15 g of the product before starting the *in vitro* digestion process.  
160 Maltodextrin (dextrose equivalent 16.5-19.5) was purchased from Sigma-Aldrich (France), while  
161 sucroesters (E473) were purchased from Louis François (France). β-sitosterol or cholesterol  
162 standards (Sigma-Aldrich, France) were solubilized in tetrahydrofuran (THF) at 0.25 g/mL and 0.05  
163 g/mL respectively. These solutions were also added into the 15 g of product before *in vitro* digestion,  
164 according to each experiment, to reach a concentration of β-sitosterol necessary to obtain a  
165 cholesterol-lowering effect, and to simulate the digestion of the cholesterol content of 5 g of fat food  
166 (based on pork rillettes in this study).

167

168 **2.4. *Mixed micelle size***

169 The size of mixed micelles from the micellar phase was measured at 25 °C using the photon  
170 correlation spectroscopy analysis (Zetasizer Nano-ZS, Malvern Instruments, UK).

171

172 **2.5. *Measurement of fat-soluble compound uptake by intestinal cells***

173 Caco-2 clone TC7 cells were a gift from Dr. M. Rousset (U178 INSERM, Villejuif, France). Culture cell  
174 conditions were performed according to Gence *et al.* (2018) with minor modifications. Briefly, cells  
175 were maintained in DMEM supplemented with 20% heat-inactivated fetal bovine serum, 1% non-  
176 essential amino acid, 1% streptomycin, and 2% L-glutamine. For each experiment, cells were sowed  
177 at a density of  $5 \times 10^5$  cells/25 cm<sup>2</sup> flask (Becton Dickinson, le Pont-de Chaix, France) for 21 days to  
178 obtain confluent differentiated cell monolayers. Carotenoid-rich micelles derived from the *in vitro*  
179 digestion were diluted at 1:4. At the beginning of each experiment, cell monolayers were washed with  
180 2 mL of phosphate buffered saline (PBS). Cell monolayers were incubated with 7 mL of diluted  
181 micelles for 2h at 37 °C. Media and cells were collected after the incubation period. Cell monolayers  
182 were collected in 2 mL of PBS. Note that for cholesterol measurement, a control without any  
183 incubation of micelles was used as a reference. All samples were stored at -80 °C under nitrogen  
184 before carotenoid extraction. HPLC analysis was carried out as reported by Gence *et al.* (2018).  
185 Cholesterol and  $\beta$ -sitosterol were detected at 210 nm and identified by comparaison with pure  
186 standards.

## 187 **2.6. Statistical analyses**

188 Data were analyzed statistically using one-way analysis of variance (ANOVA) in order to determine  
189 significant differences ( $p < 0.05$ ). Tukey's multiple comparison method was used as a post-hoc test to  
190 further compare means together.

191

## 192 **3. Results and discussion**

### 193 **3.1. Fat-soluble compound content of the fermented maize yogurt-like product**

194 Carotenoid, tocopherol and  $\beta$ -sitosterol contents of the fermented yogurt-like product are reported in  
195 Table 1. The three main carotenoids, which were Bcx, Bc and Lyc, came from papaya and melon  
196 extracts. They represented a total carotenoid content of  $26.3 \pm 0.8$  mg/kg, Bc being the major  
197 provitamin A carotenoid.  $\alpha$ -tocopherol and  $\gamma$ -tocopherol (provided by maize and fruits) content were of  
198  $3.8 \pm 0.1$  mg/kg. This is equivalent to 4% of the Recommended Daily Allowance, which is low, but  
199 valuable for their antioxidant role, both *in vitro* for the product stability and *in vivo* for the organism. The  
200 added DPs, represented 1.68 g/100 g available portion, the same as the minimum recommended



201 intake of 1.6 g/day to display a cholesterol-lowering effect (Chen, McClements, & Decker, 2013;  
202 Marangoni & Poli, 2010; Shahzad *et al.*, 2017). *In fine*, this product provided a functional balance  
203 between carotenoids and phytosterols, necessary to induce a cholesterol-lowering effect without a  
204 detrimental effect on carotenoids (Noakes *et al.*, 2002)

### 205 **3.2. Bioaccessibility of fat-soluble compounds in the fermented maize** 206 **yogurt-like product**

207 The bioaccessibility of  $\beta$ -cryptoxanthin,  $\beta$ -carotene, lycopene,  $\beta$ -sitosterol was studied in this product  
208 (Figure 1). Carotenoid bioaccessibilities were ranked as follows:  $29.9 \pm 3.0\%$  (Bcx),  $28.0 \pm 0.5\%$  (Bc)  
209 and  $5.5 \pm 0.5\%$  (Lyc), according to this order  $Bcx \geq Bc > Lyc$  in agreement with the literature (Kopeck &  
210 Failla, 2018). Indeed, it is known that efficiency of carotenoid micellarization is positively correlated  
211 with their hydrophilicity. Because of their presence in the food matrix, tocopherol bioaccessibility was  
212 also evaluated. Since their bioaccessibility was high (94-100%), interactions with other fat-soluble  
213 compounds were unlikely. Although tocopherol bioaccessibility could be variable among dietary  
214 sources and processes, the present study was in agreement with previous studies (Reboul *et al.*  
215 2006; Granado-Lorencio *et al.* 2007; Cilla *et al.* 2012).

216 It is interesting to underline that the  $\beta$ -sitosterol bioaccessibility was very low, with only  $1.6 \pm 0.3\%$ .  
217 This result suggested that a very slight fraction of  $\beta$ -sitosterol added in the formulation was  
218 micellarized. This result was confirmed by measuring the  $\beta$ -sitosterol content in the micellar phase, the  
219 aqueous phase and the pellets after *in vitro* digestion. It represented 0.22 mg/mL; 2.56 mg/mL and  
220 31.4 mg/mL respectively in these fractions.

221 It was supposed that a high amount of  $\beta$ -sitosterol was not micellarized because of the vehicle of DPs  
222 (mainly maltodextrin). Anyway, in this case, this low  $\beta$ -sitosterol micellarization (1.6%) represented  
223 approximately 34 mg of bioaccessible  $\beta$ -sitosterol/125 g serving portion, sufficient to obtain a  
224 cholesterol-lowering effect. Alemany *et al.* (2013) and Garcia-Llatas, Cilla, Alegría, & Lagarda (2015)  
225 also used microencapsulated phytosterols and observed low micellarization (from 2.9 to 6.4%) of the  
226 added phytosterols in milk-based fruit beverages. These authors reported that the presence of Bcx  
227 seemed to decrease the sitosterol bioaccessibility in milk-based fruit beverages, supposing an  
228 interaction between carotenoids and phytosterols for incorporation into mixed micelles. Moreover,  
229 Alvarez-Sala *et al.* (2016) underlined that the bioaccessibility of phytosterols was better for a product

230 containing whey proteins with milk fat globule membrane or soy lecithin than for a product containing  
231 extra olive oil and soy lecithin. They finally highlighted that the bioaccessibility of phytosterols was  
232 influenced by the type and the quantity of fat and emulsifiers used in the formulation. Blanco-Morales  
233 *et al.* (2018) showed that the addition of 5.0 g of galactooligosaccharides did not affect the phytosterol  
234 bioaccessibility, but could slightly increase the bioaccessibility of cholesterol. Differences between  
235 micro or nano-encapsulation and types of carriers of bioactive encapsulated compounds could have  
236 an influence on fat-soluble compound bioaccessibility (Soukoulis & Bohn, 2018).

237

### 238 **3.3. Effect of DPs and their vehicles on carotenoid bioaccessibility**

239 In order to better understand how these DPs influence carotenoid bioaccessibility, four products were  
240 compared: two products, with and without DPs, and two products with the vehicles of encapsulation  
241 mainly represented by maltodextrin, either with maltodextrin alone at 10% or maltodextrin associated  
242 with sucroesters at 10%-3% (Figure 2A and 2B).

243 Surprisingly, the bioaccessibility of both Bc and Lyc significantly ( $p < 0.05$ ) increased by  $40.2 \pm 1.4\%$  in  
244 the presence of added DPs as represented on Figure 2. Similarly, carotene bioaccessibility also  
245 increased in the presence of maltodextrin alone. Maltodextrin is a polysaccharide produced from  
246 partial hydrolyzed starch and currently used as an encapsulating agent of fat-soluble compounds  
247 (Medeiros *et al.*, 2019). It is used during the spray-drying of high carotenoid content extracts, and it is  
248 known to have properties such as low viscosity and coating capacity, which can also stabilize  
249 emulsions (Parikh, Agarwal, & Raut, 2014). Some authors reported that bioaccessibilities of spray-  
250 dried carotenoids encapsulated with maltodextrin increased significantly (Kyriakoudi & Tsimidou, 2018;  
251 Montero, Calvo, Gómez-Guillén, & Gómez-Estaca, 2016). Therefore, in our study, maltodextrin, added  
252 with DPs, significantly increased the bioaccessibility of carotenes.

253 Conversely, the bioaccessibility of Bcx, of which the concentration was low so it represented a minor  
254 provitamin A carotenoid in this product, decreased by 19 and 24% when DPs or maltodextrin  
255 respectively were added. This different behavior between carotene and xanthophyll (oxygenated  
256 forms) bioaccessibility was probably due to xanthophyll hydrophilicity. It is thought that xanthophylls  
257 (as Bcx) are located on the surface of the lipid droplets whereas apolar carotenes (such as Bc or Lyc)

258 are solubilized in the core of lipid droplets (Borel *et al.*, 1996). Maltodextrin and its stabilizing  
259 properties appeared to have a lower effect on Bcx bioaccessibility than on Bc, which is less polar.  
260 However, the bioaccessibility of carotenoids in the product with the maltodextrin alone were not  
261 significantly different ( $p > 0.05$ ) from the bioaccessibility of carotenoids in the product containing DPs.

262 The same experiment on the product without DPs but containing maltodextrin associated with  
263 sucroesters, reduced the vehicle effect for Bcx but maintained it for carotenes. The addition of  
264 sucroesters seemed to reduce the difference between carotenoid bioaccessibility from the product with  
265 DPs and that from the product with vehicles (Figure 2B). Therefore, it seemed that sucroesters, which  
266 are known to decrease carotenoid bioaccessibility, counteracted the maltodextrin effect (Grune *et al.*,  
267 2010). This second experiment didn't reveal any significant difference ( $p > 0.05$ ) between  
268 bioaccessibility of carotenoids in the product containing DPs and the product with the encapsulation  
269 excipients. Together, these results suggested that the bioaccessibility of carotenes in the product with  
270 DPs was enhanced by the vehicles of DPs used for encapsulation (mainly maltodextrin). Indeed, the  
271 competition for absorption during digestion between carotenes and phytosterols seemed to be avoided  
272 in these conditions.

273 Maltodextrin is known to be a fat-replacer in the food industry and can be used as a fat-like gel  
274 (Hofman, van Buul, & Brouns, 2016). It was thus possible that maltodextrin could enhance the  
275 micellarization of the most apolar carotenoids. After digestion of the product, the average diameter of  
276 particle size in the aqueous phase was of 51 nm while the mean diameter of the product without DPs  
277 measured 189 nm. These results suggested that maltodextrin could contribute to the formation of  
278 nanoemulsion systems with oil-in-water interfaces, efficient in enhancing carotenoid solubility or  
279 bioaccessibility (Liang *et al.*, 2013).

280 To better understand the role of the vehicles and their interaction between DPs and carotenoids, *in*  
281 *vitro* digestion of the product with added free  $\beta$ -sitosterol, without encapsulation, was investigated.  
282 Figure 3 shows that the bioaccessibility of Bcx and Bc decreased by 32 and 23% respectively when  
283 the product contained free  $\beta$ -sitosterol. Therefore, free  $\beta$ -sitosterol affected the micellarization of Bcx  
284 and Bc, while Lyc micellarization was not impacted. Interestingly, the bioaccessibility of free  $\beta$ -  
285 sitosterol was 11.8% compared to the dispersible  $\beta$ -sitosterol (1.6%). This last result showed that  $\beta$ -  
286 sitosterol without an excipient was more easily micellarized, which induced a detrimental effect on all  
287 types of carotenoid bioaccessibility. This is in agreement with several human studies reporting that

288 free or esterified (emulsion form, associated with lipids) phytosterols decreased plasma concentrations  
289 of carotenoids and/or tocopherols (Noakes *et al.*, 2002; Richelle *et al.*, 2004).

#### 290 **3.4. Impact of DPs on cholesterol micellarization during *in vitro* digestion**

291 In order to assess the effect of DPs on cholesterol micellarization, the yogurt was submitted to *in vitro*  
292 digestion with added cholesterol (Figure 4). The reduction in the micellarization of cholesterol was  
293 more than 50%. DPs used in our formulation had therefore a potential cholesterol-lowering effect.  
294 Beside this effect, carotene micellarization was not affected by DPs whereas the bioaccessibility of Bcx  
295 decreased from 28% to 18%. Although Bcx bioaccessibility was also affected by DPs in presence of  
296 cholesterol, it represented only 7% of the total carotenoid content of our product and was half  
297 provitaminic A. Nevertheless, using *in vitro* and *in vivo* studies, Granado-Lorencio *et al.* (2011) showed  
298 that Bcx bioavailability was not affected by the presence of water-dispersible microencapsulated  
299 phytosterols, but the vehicle of encapsulation was not mentioned. Conversely, Hernández-Alvarez *et*  
300 *al.* (2016) observed that the bioaccessibility of Bcx decreased while phytosterol bioaccessibility  
301 increased after the *in vitro* digestion of a dairy beverage enriched in milk fat globule membrane. They  
302 highlighted the competitive effect between Bcx and phytosterols for incorporation into micelles.  
303 However, they did not find a similar trend in human serum. Additionally, in a recent human study, Bcx  
304 seemed to enhance the cholesterol-lowering effect of DPs and to improve the lipid profile (Granado-  
305 Lorencio *et al.* 2014).

306

307 A recent meta-analysis study concluded that plant sterol intake decreased plasma concentrations of  
308 carotenoids and affected oxygenated carotenoids in different ways (Baumgartner *et al.*, 2017). In this  
309 review based on 41 trials, Bcx content in plasma was impacted less than the carotene ones,  
310 underlining their different behaviors.

311 In our study, we observed the opposite behavior for Bcx, because this carotenoid was more affected  
312 by the presence of DPs. However, in these meta-analyses data, the vehicles of the DPs were not  
313 considered or discussed, and it could be a critical point influencing carotenoid absorption.  
314 Nevertheless, Shaghghi, Harding, & Jones (2014) highlighted that DPs did not affect fat-soluble  
315 vitamins or carotenoids in plasma of moderately hypercholesterolemic subjects; the vehicle used was  
316 a polysorbate emulsifier and the carotenoids tested were carotenes and lutein but not Bcx. Note that *in*

317 *vivo* studies reflect carotenoid serum levels, while *in vitro* studies analyze carotenoid bioaccessibility  
318 by evaluating the available fraction for enterocytes. Thus, our results underlined the essential role of  
319 the phytosterol vehicles incorporated in a functional food. Therefore, the cholesterol-lowering efficacy  
320 of DPs in this product was demonstrated, without a detrimental effect on carotenes.

321

### 322 **3.5. Carotenoids and $\beta$ -sitosterol/cholesterol uptake by Caco-2 cells exposed to micelles** 323 **generated from *in vitro* digestion**

324 To investigate any potential effect of DPs on intestinal carotenoid uptake, differentiated Caco-2 cells  
325 were exposed to micelles generated from *in vitro* digestion of the products with and without DPs. The  
326 provitamin A carotenoids, Bcx and Bc, were preferentially absorbed with an average of 16% vs 7.2%  
327 respectively in comparison to Lyc (Table 2). Moreover, carotenoid uptake was significantly higher ( $p <$   
328 0.05) with micelles issued from *in vitro* digestion of the product without DPs. These results can be  
329 explained by the relationship between uptake and micelle size. Indeed, the size of mixed micelles  
330 formed during the *in vitro* digestion experiments was measured (Table 3). The presence of the  
331 vehicles (maltodextrin/sucroesters) with or without phytosterols significantly ( $p < 0.05$ ) modified the  
332 size of the micelles. Thus, micelles without phytosterols presented a mean radius (8.33 nm) smaller  
333 than those with the vehicles (9.41 nm for P and 10.61 nm for P without DP). The smaller-sized  
334 micelles seemed to be taken up better by intestinal cells. Moreover, the higher uptake of carotenoids  
335 obtained from digestion of the product without phytosterols could be explained by a mechanism  
336 involved in the regulation of intestinal transporters. Indeed, the uptake of carotenoids and phytosterols  
337 by enterocyte could be facilitated by the same transporter such as NPC1L1 or SR-BI and  
338 consequently, carotenoids could compete with phytosterols for binding to these transporters (Gleize *et*  
339 *al.*, 2016; Nakano, Inoue, & Murakoshi, 2019; Reboul, 2013). We performed the same experiments  
340 with the addition of cholesterol in order to observe the ability of DPs to reduce cholesterol  
341 accumulation by intestinal Caco-2 cells. A slight decrease of cholesterol uptake was observed when  
342 DPs were present in mixed micelles (~2%). However, in our study, the ratio of cholesterol to plant  
343 sterol was 1:2.5 while the ratio used in human studies is near to 1:5 corresponding to a dietary  
344 cholesterol intake of 300-400 mg/day (Bohn *et al.*, 2007). The supplementation of 1.5-2g of

345 phytosterols reduced by 7% the cholesterol intake (300 mg) in human studies. Finally, a decrease was  
346 observed for the uptake of  $\beta$ -sitosterol when cholesterol was present, from 7.9% to 5.8%.

347

#### 348 **4. Conclusion**

349 This study resulted in a better understanding of the role of DP vehicles on carotenoid bioaccessibility  
350 and uptake by Caco-2 cells of a functional cereal-fermented food. Our results showed that carotene  
351 bioaccessibility -or solubilization in aqueous phase during digestion- was improved by the vehicle of  
352 DPs, i.e. maltodextrin associated with sucroesters. Moreover, these DPs, incorporated in the  
353 functional fermented food, were able to induce a potential cholesterol-lowering effect by decreasing  
354 cholesterol micellarization without having a detrimental effect on carotene bioaccessibility. Despite a  
355 slight decrease in carotenoid uptake caused by the DP vehicle related to micelle size, an inhibition of  
356 cholesterol accumulation by Caco-2 cells was observed. Together, our findings underlined the key role  
357 of the vehicle of the phytosterols on carotenoid micellarization and Caco-2 cell uptake. Our results  
358 supported the use of DPs encapsulated in maltodextrin associated with sucroesters to design  
359 functional foods containing natural carotenoids. However, further studies are needed to clarify the  
360 specific behavior of Bcx in the interaction with phytosterols in different food matrices. Finally, dietary  
361 intervention studies are needed to validate the efficiency of this functional fermented product in  
362 humans, mainly the *in vivo* cholesterol-lowering effect without having a detrimental effect on  
363 provitamin A carotenoid bioavailability.

#### 364 **Conflict of interest**

365 The authors declare no conflict of interest.

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## 512 **Figure captions**

513 **Figure 1:** Bioaccessibility of fat-soluble compounds in the functional product

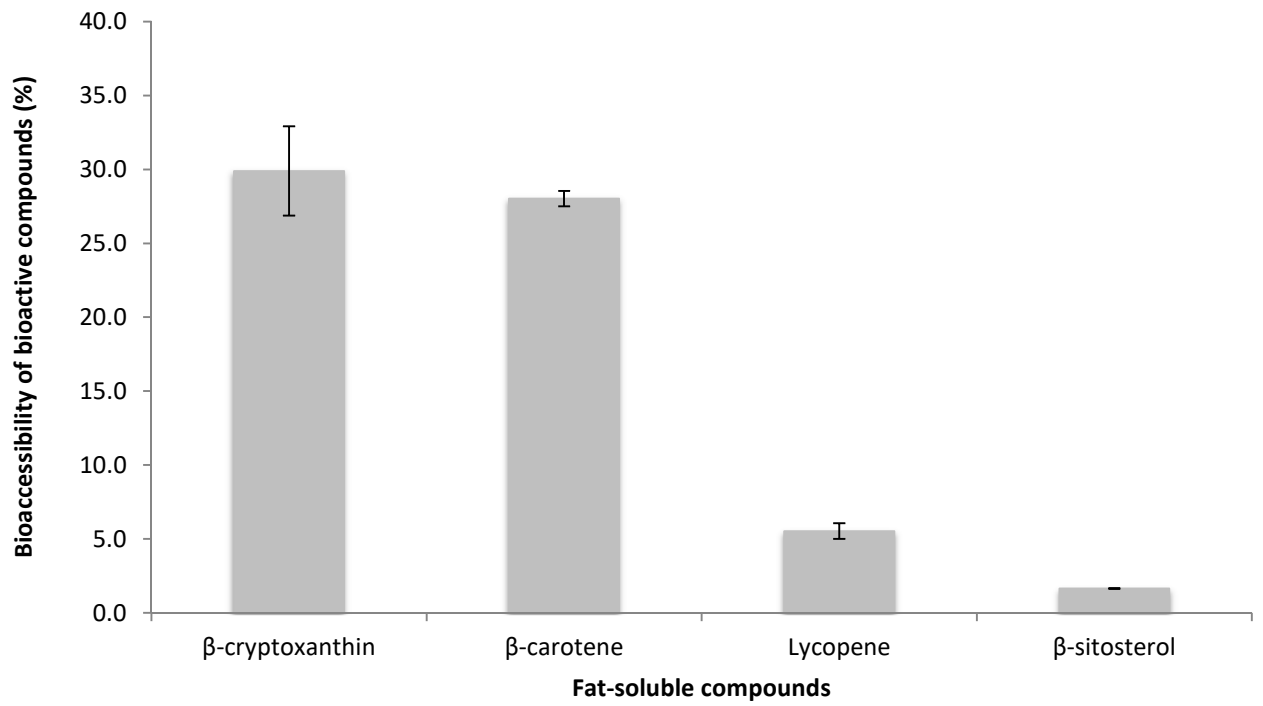
514 **Figure 2:** A) Carotenoid bioaccessibility of the functional Product (P) with and without Dispersible  
515 Phytosterols (+/-DP) and with maltodextrin vehicle (+m) B) Carotenoid bioaccessibility of the  
516 functional Product (P) with and without dispersible phytosterols (+/-DP) and with maltodextrin and  
517 sucroester vehicle (+m+s)

518 **Figure 3:** Carotenoid bioaccessibility of the functional Product (P) without Dispersible  
519 Phytosterols containing standard  $\beta$ -sitosterol; ■ P-DP; ■ P-DP +Bsito

520 **Figure 4:** Carotenoid bioaccessibility of the functional Product (P) with or without Dispersible  
521 Phytosterols in presence of standard cholesterol; ■ P-DP +chol; ■ P+DP +chol

522

523 **Figure 1**



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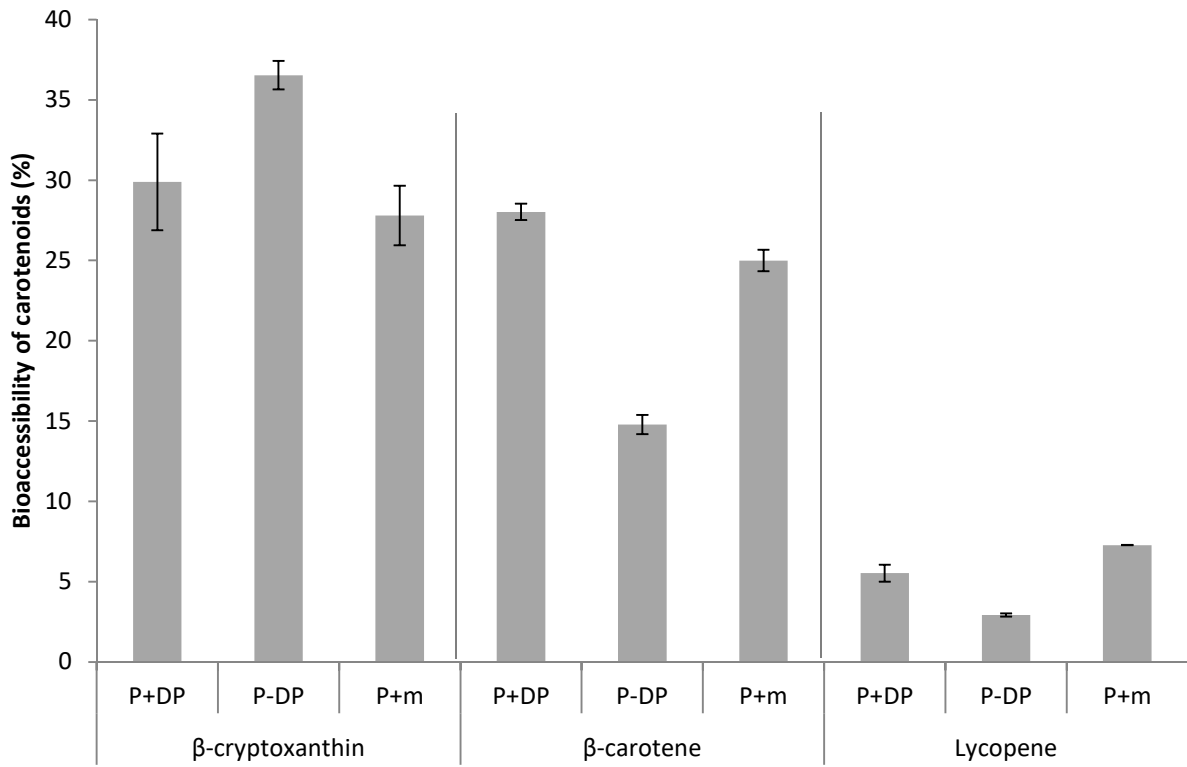
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529 **Figure 2**

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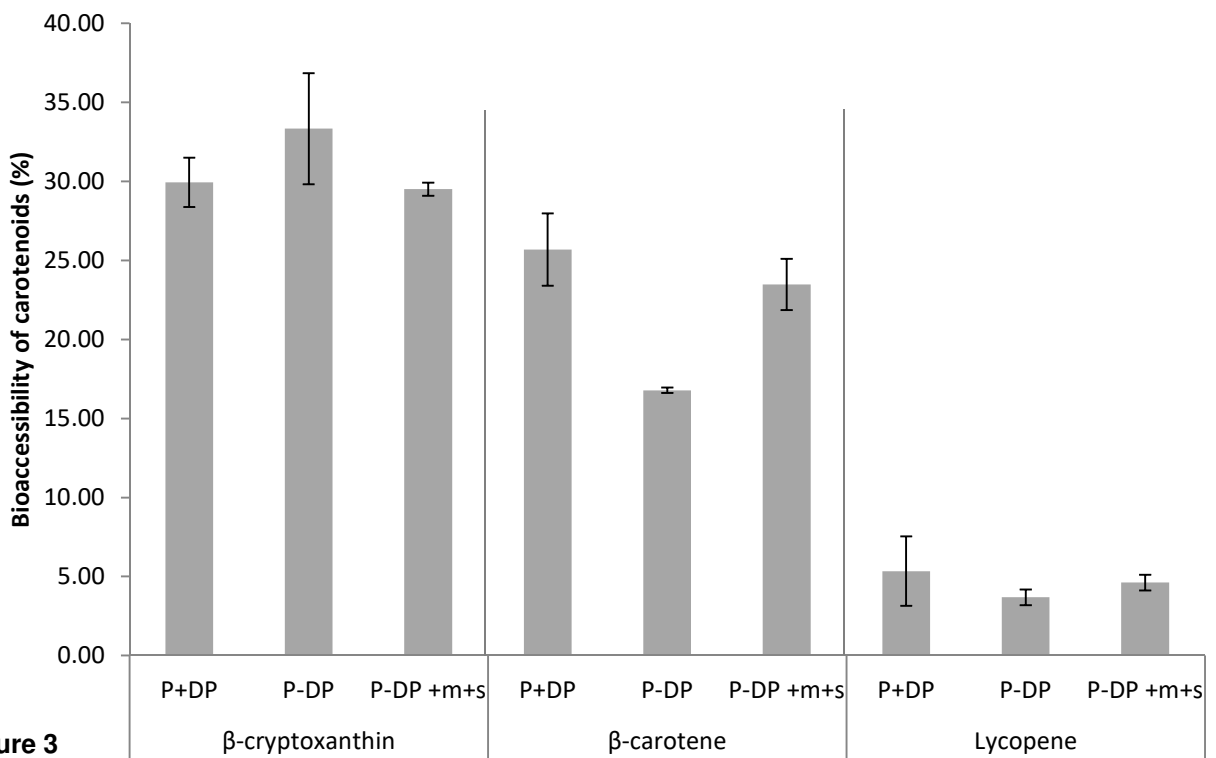
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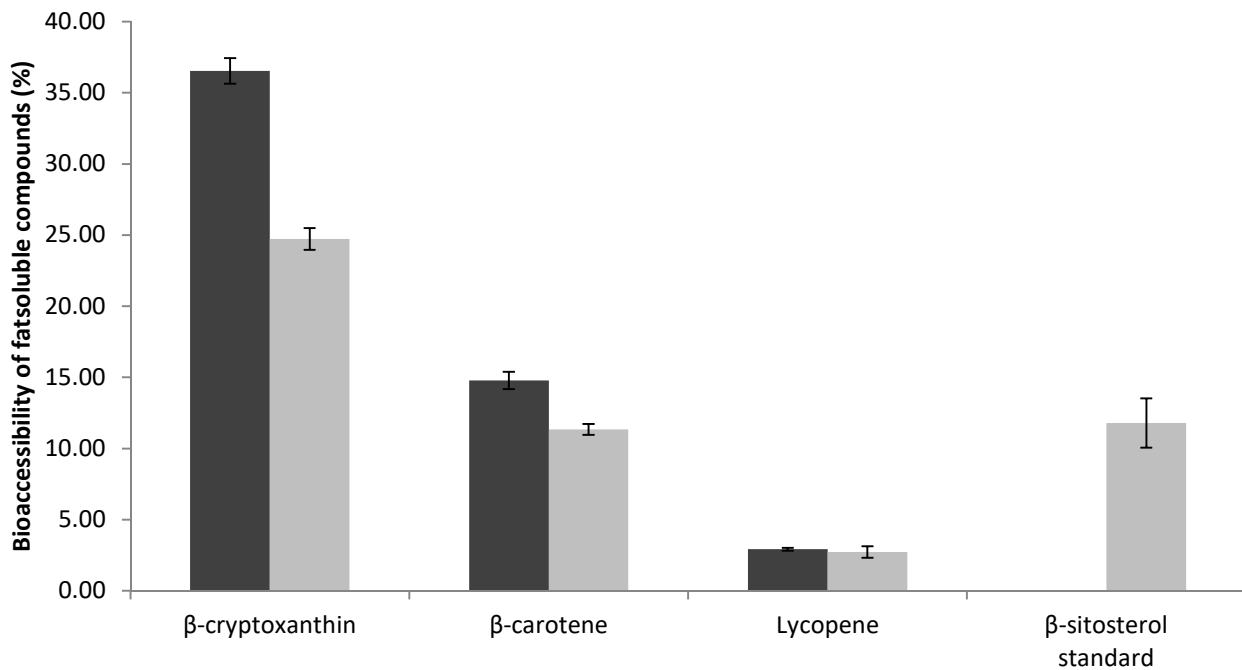
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**Figure 3**



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552 **Figure 4**

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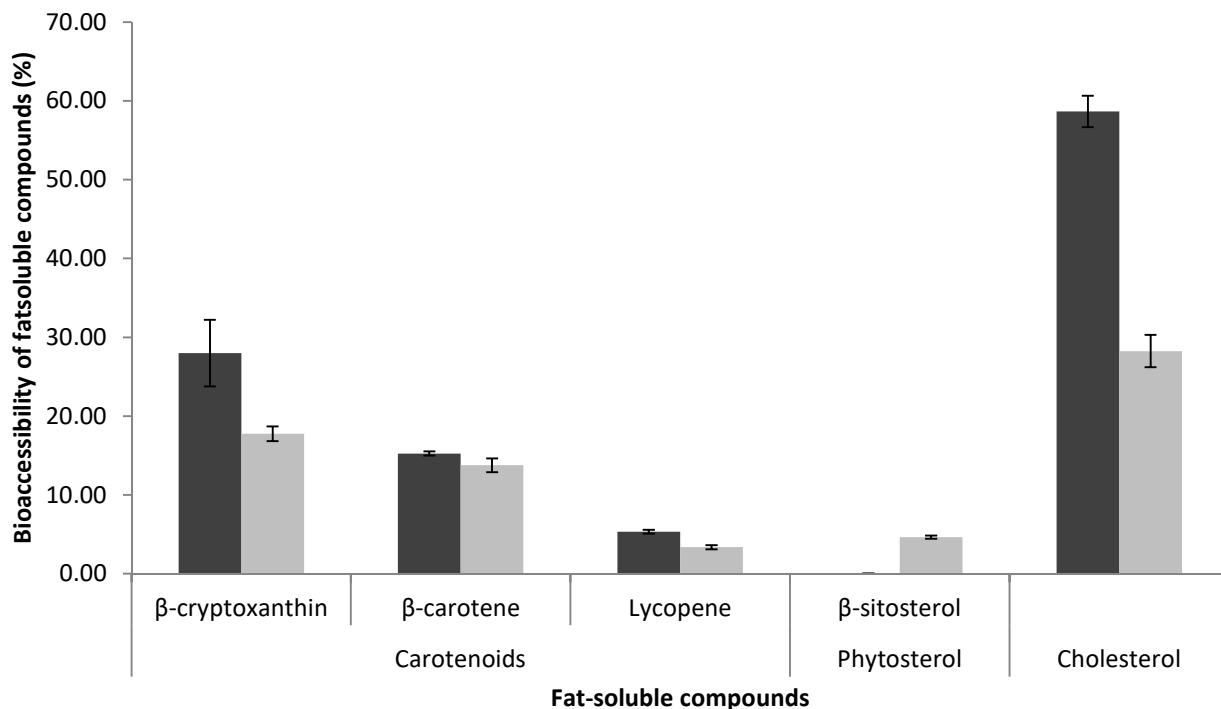
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566 **Table 1** : Fat-soluble compound contents of the fermented maize yogurt-like product

567

Phytochemicals	Content/kg	Content/serving portion
<b>Carotenoids</b>	<b>mg/kg</b>	<b>mg/125 g portion</b>
β-cryptoxanthin	1.90 ± 0.04	
β-carotene	14.55 ± 0.45	3.29 ± 0.10
Lycopene	9.85 ± 0.32	
<b>Tocopherols</b>	<b>mg/kg</b>	<b>mg/125 g portion</b>
α-tocopherol	1.53 ± 0.05	0.48 ± 0.04
γ-tocopherol	2.28 ± 0.06	
<b>Phytosterol</b>	<b>g/kg</b>	<b>g/125 g portion</b>
β-sitosterol	16.84 ± 0.46	2.11 ± 0.06

568 Values are means of 3 independent determinations ± standard deviation (SD)

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570

571 **Table 2:** Cellular uptake of fat-soluble compounds by Caco-2 cells

% UPTAKE	Bcx	Bc	Lyc	β-sitosterol	Cholesterol
<b>P</b>	18.9 ± 2.3 <sup>c</sup>	14.1 ± 1.6 <sup>de</sup>	7.2 ± 1.6 <sup>g</sup>	7.9 ± 1.3 <sup>g</sup>	-
<b>P without DP</b>	28.4 ± 2.4 <sup>a</sup>	21.3 ± 0.7 <sup>bc</sup>	10.4 ± 1.8 <sup>ef</sup>	-	-
<b>P + chol</b>	18.5 ± 0.8 <sup>cd</sup>	18.2 ± 0.6 <sup>cd</sup>	9.21 ± 1.9 <sup>fg</sup>	5.8 ± 0.4 <sup>h</sup>	0
<b>P without DP + chol</b>	24.9 ± 0.2 <sup>ab</sup>	25.4 ± 1.5 <sup>ab</sup>	12.31 ± 1.8 <sup>ef</sup>	-	2.1 ± 0.4 <sup>i</sup>

572 Values are means of 3 independent determinations ± standard deviation (SD)

573 Means with the same superscript (a–b) do not differ significantly (Tukey test, *p*-value ≤ 0.05)

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575

576 **Table 3:** Size of mixed micelles after the *in vitro* digestion of different products

	Size (mean radius, nm)	
	Mean	SEM
<b>P</b>	9.41 <sup>b</sup>	0.20
<b>P without DP</b>	8.33 <sup>c</sup>	0.20
<b>P without DP + vehicles</b>	10.61 <sup>a</sup>	0.24

577 P: Product; DP: Dispersible Phytosterols; vehicles: maltodextrin/sucroesters (10/3%)

578 Means with the same superscript (a–b) do not differ significantly (Tukey test, *p*-value ≤ 0.05)