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Phytoene and phytofluene isolated from a tomato extract are readily incorporated in mixed micelles and absorbed by Caco-2 cells, as compared to lycopene, and SR-BI is involved in their cellular uptake.

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**Abbreviations:** BLT1, block lipid transport-1; CD36, CD36 molecule; CDB, conjugated double bonds; FBS, fetal bovine serum; NPC1L1, NPC1 like intracellular cholesterol transporter 1; PT, phytoene; PTF, phytofluene; *SCARBI* (gene)/SR-BI (protein), scavenger receptor class B type I; SSO, sulfo-*N*-succinimidyl oleate.

## **ABSTRACT**

**Scope:** Absorption mechanisms of (PT) and phytofluene (PTF) are poorly known. Our main objectives were to measure their micellization and intestinal cell uptake efficiencies, and to compare them to that of commonly consumed carotenoids. Other objectives were to assess the involvement of protein(s) in their cellular uptake and whether they compete with other carotenoids for micellization and cellular uptake.

**Methods and results:** Tomato extract purified PT and PTF, mainly present as *cis*-isomers, were much better incorporated in synthetic mixed micelles than pure all-*trans* lycopene. PT impaired lycopene micellization (-56%,  $P < 0.05$ ) while PT and PTF did not significantly affect micellization of other carotenoids, and vice versa. At low concentration, Caco-2 PTF uptake was higher ( $P < 0.05$ ) than that of PT and lycopene (29%, 21% and not detectable). SR-BI, but not CD36 neither NPC1L1, was involved in PT and PTF uptake. PT and PTF impaired ( $P < 0.05$ )  $\beta$ -carotene uptake (-13 and -22%, respectively).

**Conclusions:** The high bioaccessibility of PT and PTF can be partly explained by their high micellization efficiency, which is likely due to their natural *cis* isomerization and/or to their high molecular flexibility. SR-BI is involved in their cellular uptake, which can explain competitions with other carotenoids.

## 1 1. Introduction

2 Phytoene (PT) and phytofluene (PTF) are carotenes, *i.e.* non-oxygenated carotenoids, which are  
3 found in a wide variety of fruits and vegetables, *e.g.* in tomatoes, carrots, and light orange apricots  
4 (at concentrations of around 1.4 and 0.4; 1.4 and 0.6; and 7.2 and 2.4 mg/100g edible portion, for  
5 PT and PTF, respectively).<sup>[1, 2]</sup> These carotenoids contain 3 (PT) and 5 (PTF) conjugated double  
6 bonds (CDB), while commonly consumed carotenoids contain at least 10 CDB (**Supporting**  
7 **Information Figure S1**). This provides them with a unique feature in the carotenoid kingdom: they  
8 do not absorb visible light and thus are colourless for human.<sup>[3]</sup> In addition, their lower number of  
9 CDB gives them a more twisted shape compared to commonly consumed carotenoids,<sup>[4, 5]</sup> which  
10 has been suggested to affect their bioavailability and biological actions.<sup>[2]</sup> Moreover, it could also  
11 be expected that their tendency to oxidation would be lower.<sup>[2]</sup>

12 PT and PTF are readily absorbed by the human body, being found in blood and several tissues.  
13 <sup>[6, 7]</sup> They have recently received increased interest because several studies have found positive  
14 associations between their consumption/blood concentration and some health benefits. The intake of  
15 PT and/or PTF could be related to an improvement of the immune system and a reduction in the risk  
16 to develop various diseases, including certain cancers.<sup>[2, 8, 9]</sup> Moreover, several studies have  
17 indicated that they could protect the skin against UV-damage and provide cosmetic benefits.<sup>[10–13]</sup>

18 To reach the bloodstream and then target tissues, carotenoids must first be released from the  
19 food matrix in which they are embedded and be incorporated into mixed micelles.<sup>[14, 15]</sup> PT and  
20 PTF have been shown to exhibit higher bioaccessibility than other carotenoids present in the same  
21 food matrices.<sup>[16–19]</sup> Indeed, a bioaccessibility ranking of carotenoid species seems to emerge  
22 regardless of the food matrix: PT and PTF > lutein >  $\beta$ -carotene > lycopene. However, available  
23 data do not allow us to conclude whether the high bioaccessibility of PT and PTF originates from a  
24 higher extraction efficiency from food matrices, due to specific intracellular localisation compared  
25 to other carotenoids,<sup>[20]</sup> or from a higher intrinsic solubility in mixed micelles,<sup>[21]</sup> due to their  
26 peculiar chemical and physical properties, or both. Once in mixed micelles, it is assumed that PT  
27 and PTF are taken up by enterocytes, transported to their basolateral side, and incorporated into  
28 chylomicrons before being secreted into the lymph.<sup>[14, 15, 22]</sup> These uptake and transport processes  
29 are apparently very efficient for the colourless carotenoids because *e.g.* PT is a major carotenoid in  
30 various tissues and its bioavailability has been shown to be nearly triple than that of lycopene.<sup>[23]</sup>  
31 Yet, their intestinal absorption mechanisms have not been studied and compared to those of  
32 commonly studied carotenoids. Nevertheless, studies in the last decade have allowed experts in this  
33 field to conclude that enterocyte uptake of commonly consumed carotenoids is not only passive but

34 facilitated by membrane proteins.<sup>[24-27]</sup> Indeed, it has been shown that CD36 molecule (CD36) is  
35 involved in cell uptake of provitamin A carotenoids<sup>[24]</sup> and lutein,<sup>[28]</sup> scavenger receptor class B  
36 type I (SR-BI) is involved in cell uptake of provitamin A carotenoids,<sup>[24, 29]</sup> lycopene<sup>[28]</sup> and lutein,  
37<sup>[26]</sup> and NPC1 like intracellular cholesterol transporter 1 (NPC1L1) is involved in lutein uptake.<sup>[30,</sup>  
38<sup>31]</sup> Yet, it is not known whether any of these proteins are involved in cell uptake of the colourless  
39 carotenoids.

40 Our main objective was to obtain fundamental data on two key steps that are assumed to govern  
41 the bioavailability of the colourless carotenoids, *i.e.* micellization and apical uptake by intestinal  
42 cells. For that, we first measured the incorporation efficiency of tomato extract purified PT and PTF  
43 in synthetic mixed micelles and compared it to that of pure commonly consumed carotenoids. We  
44 next assessed whether previously mentioned proteins involved in uptake of commonly consumed  
45 carotenoids were also involved in that of these colourless carotenoids. Finally, because these  
46 colourless carotenoids might be used as supplements in the future we assessed in all experiments  
47 whether they compete with the studied commonly consumed carotenoids.

48

## 49 **2. Materials and methods**

50

### 51 **2.1. Chemicals**

52 PT and PTF (99.6% and 99.8% pure as checked by HPLC) were isolated from a tomato extract  
53 as described previously.<sup>[19]</sup> Purified PT contained 96% of 15-*cis*-isomer and 4% of all-*trans*-isomer  
54 and purified PTF contained 94% of *cis*-isomers and 6% of all-*trans*-isomer. Note that in most foods,  
55 human tissues and biological fluids, PT and PTF are expected to be present as a mixture of isomers,  
56 the *cis* isomers assumed to be predominant.<sup>[5, 32]</sup> All-*trans*  $\alpha$ -carotene, lycopene and lutein ( $\geq 95\%$   
57 pure) were a gift from DSM Ltd. (Basel, Switzerland). All-*trans*  $\beta$ -carotene ( $\geq 97\%$  pure), 2-oleoyl-  
58 1-palmitoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-oleoyl-*rac*-glycerol  
59 (monoolein), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine), 3 $\beta$ -hydroxy-5-  
60 cholestene (free cholesterol), oleic acid, and sodium taurocholate were purchased from Sigma-  
61 Aldrich (Saint-Quentin-Fallavier, France). DMEM containing 4.5 g/L glucose, trypsin-EDTA (500  
62 mg/L and 200 mg/L, respectively), non-essential amino acids, penicillin/streptomycin and PBS  
63 were purchased from Life Technologies (Illkirch, France). Fetal bovine serum (FBS) came from  
64 PAA (Vélizy-Villacoublay, France). Block lipid transport-1 (BLT1), used as chemical inhibitor of  
65 SR-BI, was purchased from Sigma-Aldrich. Ezetimibe  $\beta$ -D-glucuronide, used as chemical inhibitor  
66 of NPC1L1, was purchased from Sequoia-Research (Pangbourne, UK). Sulfo-*N*-succinimidyl  
67 oleate (SSO), used as chemical inhibitor of CD36, was synthesized as previously published.<sup>[33]</sup>

68

## 69 **2.2. Preparation of carotenoid-rich mixed micelles**

70 Mixed micelles containing carotenoids were synthesized as previously described, [34] with  
71 minor modifications. In summary, we first mixed solvent solutions of carotenoids with solvent  
72 solutions of micelle lipids and evaporated the mixture. Then we added DMEM containing 5 mM  
73 sodium taurocholate and we synthesized mixed micelles by sonication. The mixed micelle fraction  
74 was optically clear and stored at -20 °C until Caco-2 cell experiments.

75

## 76 **2.3. Micellization experiments**

77

### 78 *2.3.1 Measurement of carotenoid micellization*

79 The amount of carotenoids that could be incorporated in the mixed micelle fraction was  
80 measured at three target carotenoid concentrations, 0.5, 2 and 10 µM. These concentrations are  
81 expected to be found in the human intestinal lumen after either low dietary, high dietary, or  
82 pharmacological intake of these carotenoids. They were estimated from a previous work [19] where  
83 carotenoid concentrations were measured in gastro-intestinal fluid following *in vitro* digestions of  
84 different doses of these compounds.

85

### 86 *2.3.2. Protocol to study competitions between carotenoids for micellization*

87 To study this competition, we compared the amount of carotenoid recovered in mixed micelles  
88 when only one carotenoid was added at 0.5 µM during mixed micelle synthesis (control) to the  
89 carotenoid amount recovered in micelles when 0.5 µM of another carotenoid was added to the  
90 previous one during mixed micelle synthesis.

91

## 92 **2.4. Caco-2 cell experiments**

93

### 94 *2.4.1. Culture of Caco-2 cells*

95 Caco-2 clone TC-7 cells were a gift from Dr. M. Rousset (UMR-S872, Paris, France). Cells  
96 were thawed at passage number 70 or higher and were cultured as previously described. [26] Three  
97 weeks before each experiment, the cells were seeded on Millicell® hanging cell culture inserts (1  
98 µm pore size polycarbonate membrane, Millipore S.A.S., Molsheim, France) in 6-well plates at a  
99 density of  $25 \times 10^4$  cells/well to allow for differentiation. Twelve hours before each experiment,  
100 media were changed to FBS-free medium at both sides. Based on preliminary results (**Supporting**

101 **Information Figure S2)** and unless otherwise stated, an incubation time of 2 h was selected for the  
102 following Caco-2 cells experiments.

103

104 *2.4.2. Protocol to evaluate the maximal amount of micellar PT and PTF that could be theoretically*  
105 *taken up by Caco-2 cells*

106 The apical side of Caco-2 cell monolayers received different concentrations of micellar PT and  
107 PTF, more precisely from 0.3 to 6.9  $\mu\text{M}$ . The amount of PT and PTF taken up by the cells was  
108 measured at the end of the incubation time (2 h).  $Q_{\text{max}}$ , which is the maximal amount of carotenoid  
109 that could be theoretically taken up by the cells, and apparent  $K$ , which is the micellar carotenoid  
110 concentration at which uptake is half the  $Q_{\text{max}}$ , were calculated.

111

112 *2.4.3. Comparison of uptake of different carotenoid species by Caco-2 cells*

113 0.5  $\mu\text{M}$  purified PT or PTF, or pure lutein, lycopene or  $\beta$ -carotene were incorporated in mixed  
114 micelles and added to the apical side of Caco-2 cells monolayers. The amount of carotenoids taken  
115 up by the cells was measured after 2 h incubation.

116

117 *2.4.4. Competitions between micellar carotenoids for their uptake by Caco-2 cells*

118 Cells were incubated with mixed micelles containing one carotenoid together with mixed  
119 micelles containing either no carotenoid (control) or another carotenoid. These experiments were  
120 carried out with micellar carotenoid concentrations of about 1  $\mu\text{M}$ .

121

122 *2.4.5. Apical efflux of micellar PT and PTF by Caco-2 cells*

123 Apical efflux was assessed as previously described.<sup>[34]</sup> First, the apical side of the cells was  
124 incubated during 4 h with carotenoid-rich mixed micelles that contained around 10  $\mu\text{M}$  carotenoids.  
125 Cells were then washed with PBS and incubated for 15 min with FBS-free medium. Lastly, cells  
126 were incubated during 30, 60, or 120 min with carotenoid-free mixed micelles at the apical side and  
127 the amount of carotenoid recovered in the apical medium was measured.

128

129 *2.4.6. Effect of NPC1L1 and SR-BI chemical inhibitors on micellar PT and PTF uptake by Caco-2*  
130 *cells*

131 Cells were first pre-incubated with either 10  $\mu\text{M}$  DMSO (control) or 10  $\mu\text{M}$  of the  
132 corresponding chemical inhibitor (ezetimibe glucuronide for NPC1L1 or BLT1 for SR-BI) for 1 h.  
133 The apical side then received 1 mL of carotenoid-rich mixed micelles (at 1.4  $\mu\text{M}$  of PT or 1.2  $\mu\text{M}$   
134 of PTF) supplemented with either 10  $\mu\text{M}$  DMSO (control) or 10  $\mu\text{M}$  of the corresponding chemical

135 inhibitor while the basolateral side received FBS-free medium. The cellular uptake of carotenoids  
136 was measured after 2 h incubation.

137

## 138 **2.5. HEK cell culture experiments**

139 To confirm previous results obtained on the protein apparently involved, or not, in PT and PTF  
140 uptake by Caco-2 cells, and to further assess the potential involvement of CD36, which is not  
141 expressed in Caco-2 TC-7 cells, [35] we performed uptake studies in GripTite™ cells, *i.e.* genetically  
142 engineered Human Embryonic Kidney cells (HEK 293-T cells).

143 HEK cells were cultured and transfected as previously described. [24] For transfection, 3 µg of  
144 DNA was used, *i.e.* empty pIRES plasmid or human *CD36* in pIRES plasmid to study the  
145 involvement of CD36; and empty pCDNA3.1 plasmid or human *SCARB1* in pCDNA3.1 plasmid to  
146 study the involvement of SR-BI. The transfections were checked by Western blot analysis. [33]

147 Carotenoids in mixed micelles were not used in these experiments because they exert toxic  
148 effects on HEK cells. Therefore, the carotenoids vehicles were prepared as follows: First,  
149 carotenoids in hexane were incorporated in a glass tube and, after evaporation of the solvent, 6 µL  
150 of ethanol were added to facilitate the subsequent solubilisation of carotenoids in FBS. Then, 1.2  
151 mL of FBS and 10.8 mL of DMEM were added and the final mixture was vortexed and sonicated  
152 for two min.

153 Before each experiment, carotenoid concentration in the complete medium was analysed by  
154 HPLC. Three conditions were tested: 1) HEK cells transfected with the empty plasmid (control  
155 condition), 2) HEK cells transfected with a plasmid containing either *SCARB1* or *CD36*, and 3)  
156 HEK cells transfected with a plasmid containing either *SCARB1* or *CD36* together with an inhibitor  
157 of the corresponding protein (BLT1 at 10 µM or SSO at 400 µM, [36] respectively). Thus, the cells  
158 received 1 mL of complete medium in which was added 5 µM of either PT or PTF, supplemented  
159 with either DMSO for the first and second conditions, or with the corresponding inhibitor for the  
160 third condition. After 3 h of incubation, carotenoid concentration was measured in the media and  
161 the scraped cells.

162

## 163 **2.6. Carotenoid extraction and HPLC analysis**

164 Carotenoid extraction was carried out as previously described, [33] using  $\alpha$ -carotene as internal  
165 standard. Carotenoid extracts were re-dissolved in 100 µL of ethyl acetate and 10-80 µL were  
166 injected. HPLC analyses were carried out on a Dionex system, [37] using a YMC-C<sub>30</sub> column (5 µm,  
167 4.6 × 250 mm) kept at 30 °C with a YMC-C<sub>30</sub> pre-column (5 µm, 10 × 4 mm). The mobile phase  
168 consisted of a mixture of methanol and methyl *tert*-butyl ether with an elution gradient that was

169 described previously. [37] The quantification was performed by considering the data extracted at 286  
170 (PT), 350 (PTF), 450 ( $\beta$ -carotene, lutein and  $\alpha$ -carotene), and 470 nm (lycopene), using  
171 Chromeleon software (version 6.50 SP4 Build 1000, Dionex) and external calibration curves.

172

## 173 **2.7. Calculations and statistics**

174 Carotenoid uptake efficiency by cells was expressed as the percentage of carotenoids recovered  
175 in the scraped cells at the end of the experiments relative to the sum of carotenoids recovered in the  
176 apical chamber plus those recovered in the scraped cells.

177 Carotenoid efflux efficiency by cells was calculated as the relative amount of carotenoid  
178 recovered in the apical medium at the end of the experiment compared to that measured in the cells  
179 after 4 h incubation.

180 When micellization and uptake experiments were done using the same mixed micelles than  
181 those used to measure carotenoid micellization, the percentage of theoretical bioavailability of a  
182 carotenoid was calculated as: micellization efficiency (%)  $\times$  uptake efficiency (%).

183 All experiments were done in triplicate, except those to study the implication of SR-BI and  
184 NPC1L1 in the uptake of PT and PTF by Caco-2 cells, which were performed on two different days  
185 and included 4 replicates per day. Results are expressed as means  $\pm$  SEM.

186 Statistical analyses were performed using SPSS (version 20, SPSS Inc., Chicago, IL, USA)  
187 statistical package. Prior to Student *t*-test or ANOVA, homogeneity of variances was checked by  
188 Levene's test and normality of distributions by Q-Q plots. When the *F*-test in ANOVA was significant,  
189 Tukey's test was used as a *post hoc* test for pairwise comparisons but Dunnett's test was used when  
190 comparing means from several experimental groups against a single control group mean. For all tests,  
191 the bilateral alpha risk was  $\alpha = 0.05$ .

192 Relationships between two continuous variables were examined by regression analysis on  
193 KaleidaGraph software (version 3.6, Synergy software, Reading, PA).

194

## 195 **3. Results**

196

### 197 **3.1. Incorporation efficiency of PT and PTF in synthetic mixed micelles as compared to that of** 198 **commonly consumed carotenoids**

199 Marked differences in incorporation efficiency of the investigated carotenoids were observed  
200 (**Figure 1-A**). PT and lutein displayed the highest incorporation efficiencies, which were linear over  
201 the three concentrations tested. PTF incorporation efficiency was similar to that of PT and lutein up  
202 to about 2  $\mu$ M, *i.e.* high dietary concentrations, but it then it apparently started to plateau when the

203 concentration increased. Lycopene exhibited the lowest incorporation efficiency with a maximum  
204 micellar concentration of 0.06  $\mu\text{M}$  at all three concentrations tested.

205

### 206 **3.2. Competition between colourless carotenoids and other carotenoids for micellization**

207 Neither did PT or PTF compete for their micellization when they were added concurrently at  
208 0.5  $\mu\text{M}$  during mixed micelle synthesis. The addition of 0.5  $\mu\text{M}$  PTF during mixed micelle  
209 synthesis did not significantly impair lutein or lycopene micellization. Concerning PT, its addition  
210 did not significantly impair lutein micellization whereas it significantly ( $P<0.05$ ) impaired that of  
211 lycopene (-55.6%). Finally, the incorporation efficiencies of PT and PTF were not significantly  
212 affected by the simultaneous addition of lutein,  $\beta$ -carotene or lycopene during mixed micelle  
213 synthesis (data not shown).

214

### 215 **3.3. Effect of the concentration of micellar PT and PTF on their uptake efficiency by Caco-2** 216 **cells**

217 PT and PTF uptake by Caco-2 cells as a function of their micellar concentration followed  
218 hyperbolic curves (**Figure 1-B**). Thus, their uptake efficiency decreased when their micellar  
219 concentration increased. More precisely, PT uptake efficiency decreased from 23.4% to 14.6% (at  
220 0.3 and 6.9  $\mu\text{M}$ , respectively) and that of PTF from 32.0% to 14.8% (at 0.4 and 2.2  $\mu\text{M}$ ,  
221 respectively). Calculated  $Q_{\text{max}}$  and  $K$  of PT were almost 6- and 10-fold higher than that of PTF,  
222 respectively (**Table 1**).

223

### 224 **3.4. Comparison of carotenoid uptake by Caco-2 cells**

225 The uptake efficiency of lutein,  $\beta$ -carotene and PTF was not significantly different ( $P= 0.121$ ).  
226 Conversely, PT uptake efficiency was significantly lower than that of PTF and  $\beta$ -carotene (**Table 2**).  
227 The uptake of lycopene was too low to be accurately measured and was therefore markedly lower  
228 than that of the other studied carotenoids.

229

### 230 **3.5. Competitions between micellar carotenoids for their uptake by Caco-2 cells**

231 The effect of the addition of either micellar PT or PTF on the cellular uptake of commonly  
232 consumed carotenoids is shown in **Figures 2-A** and **2-B**. Micellar lutein uptake was not  
233 significantly affected by the addition of either micellar PT or PTF (**Figure 2-A**). Conversely,  
234 micellar  $\beta$ -carotene uptake was significantly impaired by PT and PTF (-12.9% and -21.6%,  
235 respectively) (**Figure 2-B**). The effect of the addition of either micellar PT or PTF on lycopene  
236 uptake is not shown because it could not be accurately measured due to the very low amount of  
237 lycopene taken up by the cells.

238 The effect of the addition of the other studied carotenoids on PT and PTF uptake by Caco-2  
239 cells is shown in **Figures 2-C** and **2-D**. The uptake of micellar PT was significantly impaired when  
240 micellar PTF,  $\beta$ -carotene or lutein were added in the apical chamber (-30.8, -52.4 and -27.8%,  
241 respectively,  $P < 0.001$ ) (**Figure 2-C**). Conversely, only micellar lutein significantly impaired  
242 micellar PTF uptake (-40%,  $P < 0.001$ ) (**Figure 2-D**).

243

### 244 **3.6. Apical efflux of PT and PTF by Caco-2 cells**

245 The apical efflux of PT and PTF following their apical uptake was not significantly different, *i.e.*  
246 around  $14 \pm 2\%$  for both carotenoids ( $P = 0.649$ ), and it did not significantly vary from 30 to 120 min  
247 (data not shown).

248

### 249 **3.7. Effect of NPC1L1 and SR-BI chemical inhibitors on micellar PT and PTF uptake by Caco-2** 250 **cells**

251 Ezetimibe glucuronide, a chemical inhibitor of NPC1L1, did not significantly affect PT or PTF  
252 uptake (**Figure 3-A**). Conversely, uptake of PT and PTF was significantly decreased (-76.9 and -  
253 85.4%, respectively,  $P < 0.001$ ) when BLT1, a chemical inhibitor of SR-BI inhibitor, was added to  
254 the apical medium.

255

### 256 **3.8. Effect of transfection of membrane proteins on micellar PT and PTF uptake by HEK cells**

257 PT and PTF uptake was significantly higher in HEK cells transfected with *SCARB1*, which  
258 encodes for SR-BI, than in HEK cells transfected with an empty plasmid ( $P < 0.01$  and  $P < 0.05$  for  
259 PT and PTF, respectively). Furthermore, the addition of BLT1 to the *SCARB1* transfected cells led  
260 to abolish the higher uptake observed in these cells (**Figure 3-B**). In addition, transfection of HEK  
261 cells with *CD36* did not significantly change their PT and PTF uptake efficiency (data not shown).

262

## 263 **4. Discussion**

264

265 This study was based on the hypothesis that the high bioavailability of PT and PTF relative to  
266 that of other carotenoids found in the same food matrices is due, at least in part, to their peculiar  
267 molecular properties, which could lead to higher solubility in mixed micelles and/or to higher  
268 uptake efficiency by intestinal cells. To verify this hypothesis, we first purified PT and PTF from  
269 tomato extract and we compared their micellization and their cellular uptake efficiency to that of  
270 pure commonly consumed carotenoids. We observed that PT and PTF possess a much higher  
271 intrinsic ability to be incorporated into synthetic mixed micelles than lycopene, another linear non-  
272 oxygenated carotenoid. In fact, at low and high dietary concentrations, *i.e.* 0.5 and 2.0  $\mu\text{M}$ , their

273 micellization efficiency was similar to that of lutein, which is an oxygenated carotenoid  
274 acknowledged to have a higher micellization efficiency compared to carotenes.<sup>[38, 39]</sup> We  
275 hypothesize that this high intrinsic solubility in mixed micelles is due either to the fact that PT and  
276 PTF were mainly present in the form of *cis*-isomers, which is similar to their isomerization status in  
277 foods, and/or to the fact that these carotenoids have a higher molecular flexibility than the other  
278 studied carotenoids. Concerning the first hypothesis, although it is not known whether the *cis*-  
279 isomers of PT and PTF have higher solubility in micelles than their respective all-*trans* isomers, we  
280 hypothesize that this is very likely because this has been shown for another linear carotene, *i.e.*  
281 lycopene.<sup>[39, 40, 41]</sup> Concerning the second hypothesis, it has been shown that, due to their lower  
282 number of CDB (**Supporting Information Figure S1**), PT and PTF can fold more freely and adopt  
283 less rigid shapes than commonly consumed carotenoids.<sup>[32]</sup> Furthermore, the higher number of  
284 sigma bonds in these molecules, where rotation is possible,<sup>[43]</sup> leads to a more pronounced twist in  
285 the back-bone of these molecules.<sup>[4, 5]</sup> In fact, torsional energies of the linear carotenoids  
286 investigated rank as follows: PT (*ca.* 57 kcal/mol, 3 CDB) < PTF (*ca.* 61 kcal/mol, 5 CDB) <  
287 lycopene (*ca.* 73 kcal/mol, 11 CDB).<sup>[32]</sup> This higher flexibility and twist ability are assumed to  
288 translate into better insertion of these carotenoids between lipid molecules composing mixed  
289 micelles. However, we cannot conclude whether the high bioaccessibility of PT and PTF is due to  
290 their *cis*-isomerization, to their high molecular flexibility, or both. We secondly studied the uptake  
291 of PT and PTF by intestinal cells. The first key observation was that their uptake efficiency was  
292 much higher than that of lycopene. In fact, the uptake efficiency of PTF was equivalent to that of  
293 lutein and  $\beta$ -carotene. The second key observation was that the saturable uptake of PT and PTF  
294 strongly suggested a protein-mediated uptake. Another interesting observation was that PT uptake  
295 was higher than that of PTF at high dietary concentrations, *i.e.* > 2  $\mu$ M (**Supporting Information**  
296 **Figure S2**), while it was lower at low dietary concentrations (**Table 2**). This effect of the colourless  
297 carotenoid concentration on their relative cellular uptake efficiency was unexpected but it was in  
298 agreement with a previous study.<sup>[44]</sup> In this clinical study the bioavailability of PTF was higher than  
299 that of PT in the group of subjects who ingested the lowest concentrations of colourless carotenoids  
300 ( $\approx$ 0.9 mg of PT and PTF/day for 12 weeks), while it was the opposite in the group who ingested the  
301 highest concentrations (4.6 mg of PT and 3.2 mg of PTF/day for 12 weeks). We hypothesize that  
302 this concentration effect can be due to differences between PT and PTF regarding their relative  
303 affinity for membrane transporter(s). Indeed, the higher apparent  $Q_{max}$  of PT, as compared to that of  
304 PTF, could be explained by the hypothesis that PTF possesses a higher affinity for the main  
305 transporter of these colourless carotenoids than PT. This last hypothesis is supported by its lower  
306 apparent  $K$  and by the fact that PTF significantly inhibited PT uptake while the opposite was not

307 observed. The percentages of theoretical bioavailability of PT and PTF at 0.5  $\mu\text{M}$ , *i.e.* at a low  
308 dietary concentration, were 18.3 and 26.1%, respectively, which was in agreement with the results  
309 obtained in the previously mentioned study. [44]

310 After obtaining results suggesting that the colourless carotenoids uptake is protein-mediated,  
311 we evaluated whether proteins that have been shown to participate in the uptake of commonly  
312 consumed carotenoids, *i.e.* SR-BI, CD36, and NPC1L1, [27] are also involved in PT and PTF uptake.  
313 Overall, our results suggest that SR-BI is involved in the uptake of PT and PTF while CD36 and  
314 NPC1L1 are not. The involvement of SR-BI is in agreement with the results obtained for other  
315 carotenes, *i.e.* lycopene and  $\beta$ -carotene. [24, 25, 45] The lack of involvement of CD36 suggests that this  
316 protein is more specifically associated with the uptake of provitamin A carotenoids. [24] Another  
317 interesting finding was that about 14% of PT and PTF taken up by the cells was apparently effluxed  
318 back to their apical side. This is consistent with previous data suggesting that other fat-soluble  
319 micronutrients such as tocopherol, cholecalciferol or phyloquinone, [33, 46, 47] are partially effluxed  
320 by Caco-2. After having obtained key information on the mechanisms implicated in absorption of  
321 these carotenoids and because these phytochemicals might be used in the future as dietary  
322 supplements, whether they exhibit demonstrated benefits for health, we assessed whether they  
323 compete with commonly consumed carotenoids for either their micellization or intestinal cell  
324 uptake. Indeed, significant competitions at these key steps of carotenoid absorption could lead to a  
325 decrease in absorption of carotenoids that possess well-acknowledged health effects, *e.g.*  $\beta$ -carotene  
326 and lutein. Concerning micellization, only one competition was observed, *i.e.* PT significantly  
327 impaired lycopene micellization. It seems logical to observe that the carotenoid that has the highest  
328 ability to be incorporated in mixed micelles significantly impaired the micellar incorporation of the  
329 one that has the lowest ability to be incorporated in micelles. Concerning cellular uptake, our results  
330 suggest that PT and PTF can partially impair the intestinal uptake of  $\beta$ -carotene, and *vice versa*.  
331 This is in agreement with previous results showing that commonly consumed carotenoids compete  
332 for their intestinal uptake, [48] and this is likely explained by the fact that all these carotenoids share  
333 at least one common membrane transporter, in that case SR-BI.

334 In summary this study has provided results allowing us to suggest why the bioaccessibility of  
335 PT and PTF is unexpectedly high as compared to that of the other main linear dietary carotene, *i.e.*  
336 lycopene. Indeed, this is likely because these colourless carotenoids are present mainly as *cis*-  
337 isomers in foods and/or because of their high molecular flexibility. This study has also provided us  
338 data suggesting that SR-BI, which is involved in uptake of commonly consumed carotenoids, is also  
339 involved in cellular uptake of PT and PTF, which in turn explains competitions for cellular uptake.  
340 We have also data obtained data suggesting that a fraction of absorbed PT and PTF is effluxed back

341 to the intestinal lumen. We acknowledge some limitations of this study. First, we compared  
342 micellization of mainly *cis*-isomers of PT and PTF with that of all-*trans* isomers of commonly  
343 consumed carotenoids. Thus, we cannot conclude that all-*trans* PT and PTF are better incorporated  
344 in micelles than all-*trans* common carotenoids. Nevertheless, this would have a low interest for  
345 nutritionists because these colourless carotenoids are naturally present in foods as *cis*-isomers. Yet,  
346 this could be of interest for peoples who would like to chemically synthesize these compounds. The  
347 second main limitation is that we did not use an *in vitro* digestion model to assess bioaccessibility.  
348 Nevertheless, this model was used in previous studies and our aim was to go further by obtaining  
349 data on the intrinsic solubility of these carotenoids in micelles in order to understand why they are  
350 so bioaccessible.

### **Author contributions**

PB designed the research project with PMB, CD, and AMM; PB, CD and ER designed the protocol; PMB and VM conducted the micellization studies; PMB, MM, VM and MN conducted the cell studies; PMB and CH measured carotenoid concentrations by HPLC; PMB analysed the results with CD and ER; PMB and CD performed statistical analyses; PB, PMB and CD wrote the paper with consultation from ER; PB had primary responsibility for the final content of the manuscript. All authors have read and approved the final manuscript.

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### **Conflict of interest**

AMM is a member of the advisory board of IBR-Israeli Biotechnology Research, Ltd. (Yavne, Israel).

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## TABLES

**Table 1. Parameters of phytoene and phytofluene uptake by Caco-2 cells.**

Carotenoid	Apparent $Q_{\max}$ (nmol)	Apparent $K$ ( $\mu\text{M}$ )	$R^2$
Phytoene	$3.17 \pm 0.05^a$	$13.67 \pm 0.35^a$	1.000
Phytofluene	$0.53 \pm 0.04^b$	$1.44 \pm 0.25^b$	0.997

Caco-2 clone TC-7 cells were thawed at passage number 67. Three weeks before each experiment, the cells were seeded on culture inserts (1  $\mu\text{m}$  pore size polycarbonate membrane) in 6-well plates at a density of around  $25 \times 10^4$  cells/well to allow for differentiation. Twelve hours before each experiment, media were changed to FBS-free medium at both sides. Cells received 1 mL of carotenoid-rich synthetic mixed micelles at around 0.5  $\mu\text{M}$  on the apical side. Carotenoid uptake was measured after 2 h incubation. Results are shown in **Figure 1B**. Best fitting curves were hyperbolic ones:  $y=ax/(b+x)$ . Apparent  $Q_{\max}$  represents the maximal amount of carotenoid that could be taken up by cells. Apparent  $K$  is the micellar carotenoid concentration at which the amount taken up is half the  $Q_{\max}$ . Values represent means  $\pm$  SEM of 3 replicates. Mean values with unlike superscript letters within a column were significantly different ( $P < 0.05$ ).

**Table 2. Carotenoid uptake by Caco-2 cells at a micellar concentration corresponding to a low dietary intake of carotenoids.**

Carotenoid	Uptake (%)
Phytoene	20.8 ± 0.6 <sup>b</sup>
Phytofluene	28.9 ± 1.2 <sup>a</sup>
β-carotene	30.6 ± 0.7 <sup>a</sup>
Lutein	25.8 ± 2.1 <sup>ab</sup>

Caco-2 clone TC-7 cells were thawed at passage number 92. Three weeks before each experiment, the cells were seeded on culture inserts (1 µm pore size polycarbonate membrane) in 6-well plates at a density of about  $25 \times 10^4$  cells/well to allow for differentiation. Twelve hours before each experiment, media were changed to FBS-free medium at both sides. Cells received 1 mL of carotenoid-rich synthetic mixed micelles at around 0.5 µM on the apical side. Carotenoid uptake was measured after 2 h incubation. Values represent means ± SEM of 3 replicates. Lycopene uptake could not be accurately measured because it was lower than the HPLC detection limit. Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ).

## FIGURE LEGENDS

**Figure 1. Characterization of phytoene and phytofluene micellization and uptake by Caco-2 cells. (A) Incorporation of phytoene, lutein, phytofluene, and lycopene in synthetic mixed micelles.** Mixed micelles with varying concentrations of pure carotenoids were synthesized and their carotenoid concentration was measured by HPLC. Linear trend lines: Phytoene:  $y = 0.874x$  ( $R^2 = 0.999$ ); lutein:  $y = 0.823x$  ( $R^2 = 0.999$ ). Curvilinear trend lines: Phytofluene:  $y = -0.0734x^2 + 0.899x$  ( $R^2 = 1.000$ ); lycopene:  $y = -0.0008x^2 + 0.0137x$  ( $R^2 = 0.836$ ). **(B) Effect of micellar phytoene and phytofluene concentrations on their uptake by Caco-2 cells.** The apical side of the cells received mixed micelles that contained different concentrations of phytoene and phytofluene. Basolateral side received FBS-free medium. Carotenoid concentrations were measured in scraped cells after 2 h incubation. The best fit curves were hyperbolic ones:  $y = ax/(x+b)$ . Values represent means of 3 replicates and error bars indicate standard error of the mean.

**Figure 2. Competitions between micellar carotenoids for their uptake by Caco-2 cells. (A) Effect of phytoene and phytofluene on lutein uptake. (B) Effect of phytoene and phytofluene on  $\beta$ -carotene uptake. (C) Effect of phytofluene and commonly consumed carotenoids on phytoene uptake. (D) Effect of phytoene and commonly consumed carotenoids on phytofluene uptake.** The apical side of the cells received 1 mL of mixed micelles that contained the carotenoid of interest plus either carotenoid-free mixed micelles (control) or mixed micelles loaded with another carotenoid species. The target micellar concentration of each carotenoid in each competition conditions was 1  $\mu$ M. Carotenoid uptake was measured after 2 h incubation. The effect of phytoene and phytofluene on lycopene uptake could not be accurately measured because lycopene uptake was too low to be accurately measured in our experimental conditions. Values represent means of 3 replicates and error bars indicate SEM. Asterisks indicate significant differences from the control (absorption of the carotenoid of interest alone): \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

**Figure 3. Implication of NPC1L1 and SR-BI on phytoene and phytofluene uptake by cells. (A) Effect of chemical inhibitors of NPC1L1 and SR-BI on phytoene and phytofluene uptake by Caco-2 cells.** Cell apical sides were pre-incubated for 1 h with either 10  $\mu$ M DMSO (control) or 10  $\mu$ M chemical inhibitor (ezetimibe glucuronide for NPC1L1 or BLT1 for SR-BI). Apical sides received thereafter phytoene- or phytofluene-loaded synthetic mixed micelles at 1.4 and 1.2  $\mu$ M, respectively. Carotenoid uptake was assessed after 2 h incubation. The experiment was carried out twice, with 4 replicates in each case. This figure shows results of one experiment. Asterisks indicate

significant differences from the control (\*\*\*,  $P < 0.001$ ). **(B) Effect of transfection of HEK cells with SR-BI gene and further addition of SR-BI chemical inhibitor on phytoene and phytofluene uptake by these cells.** Cells were first transfected with either an empty plasmid (control) or a plasmid containing *SCARBI*, i.e. the gene encoding the SR-BI protein. Then cells received complete medium enriched with either micellar phytoene or phytofluene at 5  $\mu\text{M}$ , supplemented or not with 10  $\mu\text{M}$  DMSO or BLT1 (the chemical inhibitor of SR-BI). Incubation time was 3 h. For each carotenoid bars bearing unlike superscript letters are significantly different ( $P < 0.05$ ). In each figure values represent means of 3 replicates and error bars indicate SEM.