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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.

Paula Mapelli-Brahm[1†], Charles Desmarchelier[2†], Marielle Margier[2], Emmanuelle Reboul[2], Antonio J. Meléndez Martínez[1], Patrick Borel[2]*

[1]Food Colour & Quality Lab., Area of Nutrition & Food Science, Universidad de Sevilla, 41012 Seville, Spain.
[2]C2VN, Aix Marseille Univ, INRA, INSERM, Marseille, France.

[†]Paula Mapelli-Brahm and Charles Desmarchelier are joint first authors.

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[*]To whom correspondence should be addressed:
Patrick.Borel@univ-amu.fr
UMR 1260 INRA/1263 INSERM/Université d'Aix-Marseille
Centre CardioVasculaire et Nutrition (C2VN)
Faculté de Médecine
27, boulevard Jean Moulin
13385 Marseille cedex 05
France
Tel: +33-(0)4-91-32-42-77

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; *SCARB1* (gene)/SR-BI (protein), scavenger receptor class B type I; SSO, sulfo-*N*-succin-imidyl 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 ($P < 0.05$) β-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. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

2.2. Preparation of carotenoid-rich mixed micelles

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

2.3. Micellization experiments

2.3.1 Measurement of carotenoid micellization

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

2.3.2. Protocol to study competitions between carotenoids for micellization

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

2.4. Caco-2 cell experiments

2.4.1. Culture of Caco-2 cells

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

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

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

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

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

0.5 µM purified PT or PTF, or pure lutein, lycopene or β-carotene were incorporated in mixed micelles and added to the apical side of Caco-2 cells monolayers. The amount of carotenoids taken up by the cells was measured after 2 h incubation.

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

Cells were incubated with mixed micelles containing one carotenoid together with mixed micelles containing either no carotenoid (control) or another carotenoid. These experiments were carried out with micellar carotenoid concentrations of about 1 µM.

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

Apical efflux was assessed as previously described. [34] First, the apical side of the cells was incubated during 4 h with carotenoid-rich mixed micelles that contained around 10 µM carotenoids. Cells were then washed with PBS and incubated for 15 min with FBS-free medium. Lastly, cells were incubated during 30, 60, or 120 min with carotenoid-free mixed micelles at the apical side and the amount of carotenoid recovered in the apical medium was measured.

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

Cells were first pre-incubated with either 10 µM DMSO (control) or 10 µM of the corresponding chemical inhibitor (ezetimibe glucuronide for NPC1L1 or BLT1 for SR-BI) for 1 h. The apical side then received 1 mL of carotenoid-rich mixed micelles (at 1.4 µM of PT or 1.2 µM of PTF) supplemented with either 10 µM DMSO (control) or 10 µM of the corresponding chemical

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

2.5. HEK cell culture experiments

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

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

Carotenoids in mixed micelles were no used in these experiments because they exert toxic effects on HEK cells. Therefore, the carotenoids vehicles were prepared as follows: First, carotenoids in hexane were incorporated in a glass tube and, after evaporation of the solvent, 6 µL of ethanol were added to facilitate the subsequent solubilisation of carotenoids in FBS. Then, 1.2 mL of FBS and 10.8 mL of DMEM were added and the final mixture was vortexed and sonicated for two min.

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

2.6. Carotenoid extraction and HPLC analysis

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

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

2.7. Calculations and statistics

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

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

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

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

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

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

3. Results

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

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

concentration increased. Lycopene exhibited the lowest incorporation efficiency with a maximum micellar concentration of 0.06 µM at all three concentrations tested.

3.2. Competition between colourless carotenoids and other carotenoids for micellization

Neither did PT or PTF compete for their micellization when they were added concurrently at 0.5 µM during mixed micelle synthesis. The addition of 0.5 µM PTF during mixed micelle synthesis did not significantly impair lutein or lycopene micellization. Concerning PT, its addition did not significantly impair lutein micellization whereas it significantly (P<0.05) impaired that of lycopene (-55.6%). Finally, the incorporation efficiencies of PT and PTF were not significantly affected by the simultaneous addition of lutein, β-carotene or lycopene during mixed micelle synthesis (data not shown).

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

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

3.4. Comparison of carotenoid uptake by Caco-2 cells

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

observed. The percentages of theoretical bioavailability of PT and PTF at 0.5 μM, *i.e.* at a low dietary concentration, were 18.3 and 26.1%, respectively, which was in agreement with the results obtained in the previously mentioned study. [44]

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

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

to the intestinal lumen. We acknowledge some limitations of this study. First, we compared micellization of mainly *cis*-isomers of PT and PTF with that of all-*trans* isomers of commonly consumed carotenoids. Thus, we cannot conclude that all-*trans* PT and PTF are better incorporated in micelles than all-*trans* common carotenoids. Nevertheless, this would have a low interest for nutritionists because these colourless carotenoids are naturally present in foods as *cis*-isomers. Yet, this could be of interest for peoples who would like to chemically synthesize these compounds. The second main limitation is that we did not use an *in vitro* digestion model to assess bioaccessibility. Nevertheless, this model was used in previous studies and our aim was to go further by obtaining data on the intrinsic solubility of these carotenoids in micelles in order to understand why they are 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 (μM)	R^2
Phytoene	3.17 ± 0.05^a	13.67 ± 0.35^a	1.000
Phytofluene	0.53 ± 0.04^b	1.44 ± 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 μm pore size polycarbonate membrane) in 6-well plates at a density of around 25×10^4 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. 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 ± 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^{b}
Phytofluene	28.9 ± 1.2^{a}
β-carotene	30.6 ± 0.7^{a}
Lutein	25.8 ± 2.1^{ab}

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 25×10^{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 β-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 µM. 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 µM DMSO (control) or 10 µM 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 µM, 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 *SCARB1*, i.e. the gene encoding the SR-BI protein. Then cells received complete medium enriched with either micellar phytoene or phytofluene at 5 µM, supplemented or not with 10 µM 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.