COMPARATIVE STUDY OF THE COLORLESS CAROTENOIDS PHYTOENE AND PHYTOFLUENE BIOACCESSIBILITY IN POWDERS AND PULP OF TOMATO: MICROSTRUCTURAL ANALYSIS AND EFFECT OF ADDITION OF SUNFLOWER OIL

Paula Mapelli-Brahm, Carla M. Stinco, Antonio J. Meléndez-Martínez

Food Colour & Quality Lab., Area of Nutrition & Food Science, Universidad de Sevilla, Seville, Spain.

Abstract

The objective was to assess the potential bioavailability of phytoene (PT) and phytofluene (PTF) from tomato powders used as raw materials for supplements as compared to the pulp of a common and a cherry tomato. PT and PTF are attracting much interest nowadays as they can provide health and cosmetic benefits. PT and PTF levels in the more concentrated powder were up to 1000 times higher than that in the tomatoes. The bioaccessibility from the powders was lower as compared to the tomato fruits and increased markedly when sunflower oil was added. However, the best source of potentially absorbable PT and PTF (0.5 and 2 mg/g respectively) was by far the powder with higher levels of them. This result could be due to the higher carotenoid concentration in the powder, the reduction of the particle sizes, and the rupture of cell structures compared to the pulps.

Keywords: bioavailability, chromoplasts, colorless carotenoids, digestion, matrix effect, plastids.

1. INTRODUCTION

Carotenoids are lipophilic compounds responsible for the colouring in many fruits and vegetables that constitute everyday consumer foods. It is widely acknowledged that the proper intake of carotenoids can provide health benefits for humans. [1,2] In contrast to the other major bioavailable carotenoids in humans, the colourless phytoene (PT) and phytofluene (PTF) have been little studied in the context of the diet nutrition and health, although they are eliciting a great interest in the carotenoid field as evidence is accumulating that they may provide health and cosmetic benefits. [3] These carotenoids seem to have anti-inflammatory and anti-oxidant properties among others and their consumption may be related to the reduction of the risk of developing certain cancers and other diseases. [4–6] Importantly, PT and PTF are present in a wide variety of fruits and vegetables, as tomato and tomato-based food products, [7,8] carrots, citrus[9], apricots and several other common and exotic foods. [6] It has been estimated that the daily per capita intake of PT and PTF combined represents 16% of the total dietary intake of carotenoids in Luxembourg. [10] However, in addition to intake, carotenoid bioavailability must also be considered when it comes to gain insight into their biological effects.[11] One of the key factors in the bioavailability of lipophilic compounds like carotenoids is the release from the food matrix and their incorporation into mixed micelles. The bioaccessibility, i.e. the percentage of carotenoid content in a food that is incorporated into mixed micelles and is available for the absorption, can be evaluated by *in vitro* models. It is accepted that the carotenoid bioaccessibility data obtained with *in vitro* models are good to predict the bioavailability of these compounds in humans and so the *in vitro* models seems to be a good choice for previous studies and comparisons between different matrices. [12]

50 The processing and storage of food are important processes to take into account
51 since they can lead to chemical degradation or physical losses of the carotenoids while
52 they can also increase their bioaccessibility, and therefore their bioavailability, as a
53 consequence of the disruption of the cell walls of plant tissues, the dissociation of the
54 nutrient-matrix complexes, or the transformation into more active molecular
55 structures.[11]

56 Dehydration is a common technique that has been used for a long time to improve
57 the preservation, storage and transportation of foods. Such practice leads to the
58 concentration of the food constituents as a result of the elimination of water. On the
59 other hand, the preparation of powders from fresh foods results in a decrease in particle
60 size and microstructural changes, which are known to affect the release of carotenoids
61 from the matrix and, hence, increase their bioaccessibility. [13] The combination of these
62 two effects, i.e. increase of concentration and enhancement of the release from the
63 matrix can result in a marked increase of the actual amount of carotenoids that can be
64 absorbed and be available to exert their health-promoting biological actions. Due to all
65 these facts, dehydrated and ground foods offers many advantages to be used as raw
66 materials in the functional food, cosmetic or pharmaceutical industries. However,
67 surprisingly, the study of the potential bioavailability of carotenoids from powdered
68 food products has been ignored. More insight in this regard is important not only for
69 their importance as raw materials for other products, but also because dehydrated fruits
70 are increasingly common in supermarkets.

71 In this context, the aim of this study was to assess the bioaccessibility of PT and
72 PTF from two types of tomato powders used as raw materials for the development of
73 other products and to compare it with those from the pulp of a common tomato and a
74 cherry tomato commercially available from retailers. To gain further insight into the

75 factors involved in the bioaccessibility of these compounds, the effect of adding
76 sunflower oil as a source of fat to favour bioaccessibility was also studied. Sunflower
77 oil was chosen as a source of fat because it is widely consumed and does not contain
78 detectable amounts of carotenoids[14], as the presence of other carotenoids could affect
79 the bioaccessibility of PT and PTF. Additionally, this oil is rich in unsaturated fatty
80 acids, which seem to be related to a greater increase in the carotenes bioaccessibility in
81 comparison with the saturated fatty acids [15]

82 Furthermore, the samples were observed under the transmission electron
83 microscope and the epifluorescence microscope to assess differences in the structure
84 and integrity of the plastids and the distribution of the compounds of interests in the
85 different materials under study.

86

87 ## 2. MATERIALS AND METHODS

88 ### 2.1. Chemicals and standards

89 All the solvent used, which were of analytical grade or higher, were purchased from
90 Panreac (Barcelona, Spain) or Sigma-Aldrich (Bornem, Belgium). The digestive
91 enzymes, i.e. pepsin (porcine, 367 units/mg solid, measured as TCA- soluble products
92 using hemoglobin as substrate) and pancreatin (porcine, 8 × USP specifications of
93 amylase, lipase and protease), were also from Sigma-Aldrich. The standards of the
94 colourless carotenoids, i.e. phytoene and phytofluene, were isolated as previously
95 described [16] and their purity (> 95%) were checked by HPLC. The reagents for
96 obtaining the Transmission Electron Microscopy (TEM) images were acquired from
97 Ted Pella (Redding, USA) or Electron Microscopy Sciences (PA, USA).

98 ### 2.2. Samples

Two types of tomato powders were analysed. One had a higher carotenoid concentration (TPH) and the other a lower concentration (TPL). Tomato powders tested are produced from proprietary, non-GMO tomatoes (courtesy of IBR. LTD), which undergo concentration processes followed by freeze-drying and grinding, resulting in a carotenoid-rich natural tomato powder. Differences in carotenoid contents between the different products derives from the different crops combined into the respective tomato powder grades. The pulps from ripe fruits of a common tomato (*Solanum lycopersicum* var. *daniela,* hereafter named as tomato) and of a vine cherry tomato (*Solanum lycopersicum* var. *cerasiforme,* hereafter named as cherry) were also evaluated for comparison. The fruits were obtained from a supermarket located in Seville (Spain).

2.3. Carotenoid extraction from matrices

Whenever possible, the samples were kept on ice and the extraction was carried out in dim light. The extraction method was based on the protocol of Stinco et al. [17] with some minor modifications as follows. An specific amount of each sample (0.1 g for cherries, tomatoes and TPL and 0.005 g for TPH) was weighed into a Eppendorf tube and 750 µL of a mixture of trichloromethane (TCM) : methanol (2:1, v/v) and 250 µL of distilled water was added. This solution was shaken vigorously for c.a. 1 min, sonicated for 5 minutes, and centrifuged at 14,000 rpm (Microfuge 22R, Beckman Coulter, Madrid, Spain) for 5 min, and at 4 °C to promote phase separation. Then, the lower organic phase was transferred to a new Eppendorf tube. The aqueous phase was re-extracted with 500 µL of TCM until colourless of the extraction solvent. The lower phases were combined all together. The combined organic phase was washed with water and a solution of saturated sodium chloride. The extract was concentrated to dryness in a rotating evaporator at 25 °C (Eppendorf Concentrator Plus, Hamburg, Germany) and

123 was kept at - 80 °C under nitrogen until the HPLC analysis (for no more than one
124 week).

125 2.4. *In vitro* gastrointestinal digestion

126 The digestion protocol followed was based on that described by Mapelli et al. [16]
127 and is detailed below. Moreover, to study the effect of the addition of fat on the
128 bioaccessibility, the same digestion protocol was carried out with the tomato powder
129 samples but adding 200 µL of sunflower oil to the samples immediately before adding
130 the gastric juice. Taking into account that tomatoes usually have a water content of *ca.*
131 95%, the amount of oil added corresponded to approximately 5% volume/wet weight. [18]

132 *2.4.1. Gastric phase*

133 A specific amount of each sample was weighed (specifically 0.25 g of the powders
134 samples and 2 g of the cherry and tomato pulp) in a 50 mL Falcon tube. Fifteen
135 millilitres of physiological saline and two millilitres of a pepsin solution (40 mg/mL in
136 0.1 M HCl, prepared the day of usage) were added. The pH was then adjusted to 3. The
137 sample was incubated in a rotating incubator (Max Q5000 shaker, Labware, Madrid,
138 Spain) at 37 °C at 100 strokes per minute (spm) for 1 h to simulate the gastric digestion.

139 *2.4.2. Intestinal phase*

140 The sample was placed in ice and a mixture of 4 mg/mL of pancreatin and 24
141 mg/mL of bile extract in 0.1 M of $NaHCO_3$ (prepared the day of the analysis) was
142 incorporated into the gastric digesta. The pH was adjusted to 7 and after the sample was
143 brought with physiological saline to a volume of 50 mL. Then, the intestinal phase was
144 simulated by incubation the samples during 2 h at 37 °C and at 100 spm in the rotating
145 incubator. Twelve millilitres of the digesta were pipetted into a Falcon tube and were
146 centrifuged during 1 h at 5000 *g* and at 20 °C. Six millilitre aliquot of the aqueous
147 micellar phase was taken with a syringe and was filtered (0.2-µm nylon membrane

148 filter) in order to isolated the mixed micelles. Then, a 4 mL aliquot was stored in
149 another Falcon tube under a nitrogen atmosphere at -80 °C for the subsequent
150 carotenoid extraction on the following day.

151 *2.4.3. Carotenoid extraction from digesta*

152 For the carotenoid extraction 4 mL of a mixture of TCM : methanol (2:1, v/v) was
153 added to the 4 mL aliquot of the isolated mixed micelles solution. After stirring for 1
154 min and centrifuging at 4000 rpm at 4 °C for 5 min, the lower phase with carotenoids
155 was transferred to a new 50 mL Falcon tube. The extraction was repeated with 4 mL of
156 TCM until the colour exhaustion of the extraction solvent. The lower phases were
157 combined all together and then this combined phase was washed with water and a
158 solution of saturated sodium chloride. Lastly, the organic phase was concentrated to
159 dryness and the extract was kept at – 80 °C under nitrogen until the HPLC analysis.

160 2.5. HPLC analysis

161 Carotenoid concentrations were determined by High Performance Liquid
162 Chromatography (HPLC) by using an Agilent Technologies 1100 system. The
163 separation was carried out by gradient elution with methanol (MeOH), methyl-*tert*-butyl
164 ether (MTBE), and water according to Stinco et al. [19] with minor modifications.
165 Ammonium acetate was added to the methanol at a concentration of 0.1% (p/v) to avoid
166 carotenoid losses during the analysis. A 3 µm-C_{30} column (150 × 4.6 mm, YMC
167 America, Inc., Allentown, PA, USA) was used. The column was kept at 30 °C and the
168 flow rate was 1 mL/min. The diode array detector was set at 286 and 350 nm for the
169 detection of phytoene and phytofluene, respectively. To identify the geometrical
170 isomers of PT and PTF, their spectral and chromatographic characteristics were
171 compared with those reported in a previous work. [20] The quantification was carried out
172 by external calibration.

173 2.6.Transmission Electronic Microscopy (TEM)

174 The protocol followed to prepare the samples to obtain the images by TEM was
175 similar to that described by Mapelli et al. [21] with slight modifications. For the fruits, a
176 small piece of the pulp was taken in an Eppendorf tube, was centrifuged (microfuge
177 22R, Beckman Coulter, Krefeld, Germany) at 2000 rpm for 1 min at 4 °C and the
178 supernatant was discarded. The sample was fixed with 1 mL of Karnovsky fixative
179 (0.5% glutaraldehyde, 2.5% formaldehyde) for 5 h at room temperature in the dark.
180 Then, the sample was centrifuged at 2000 rpm for 1 min and the upper phase was
181 discarded. The matrix was washed with 0.1 M sodium cacodylate buffer (pH 7.4) three
182 times. The sample was post-fixed with 2% osmium tetraoxide in the buffer for 1 h at
183 25 °C after which it was washed with MilliQ water three times for 20 min at 4 °C. The
184 staining was carried out with 2% uranyl acetate for 2 h at 25 °C. After that, the sample
185 was dehydrated with acetone in series at 25 °C (starting with 50% acetone in ethanol
186 and ending with 100% acetone). Samples were embedded in Spurr resin in series at
187 25 °C (starting with a 3: 1 mixture of acetone: Spurr and ending with 100% Spurr). The
188 sample was polymerized for 13 and a half hours in an oven at 70 °C. To select the areas
189 of interest semi-thin sections of approximate 350 nm were observed under a
190 microscope. Subsequently, ultrathin sections (70 nm) were cut with an ultramicrotom
191 (Leica UC7, Wetzlar, Germany). As a sample holder, 200-mesh carbon-coated copper
192 grids were used. The images were taken in a Zeiss Libra 120 microscope (Oberkochen,
193 Germany) equipped with a digital SSCCD camera. The same protocol was followed for
194 the powders but, before the post-fixation, cacodylate buffer with 3% agarose was added
195 to the sample.

196 2.7. Epifluorescence Microscopy

PTF can be detected by fluorescence microscopy as it emits light at approximately 510 nm when is excited with near-UV light. [6] The procedure and equipment used to obtain the images were the same as recently described by Mapelli et al. [21]

2.8. Statistical analysis and calculations

All samples were extracted and digested in triplicate. The samples were digested in order to determine the bioaccessibility and the amount of carotenoid per gram of product that is potentially absorbable. Although there is still not a consensus to name this latter parameter, in this paper it will be referred to as carotenoid bioaccessible content (CBC). CBC were calculated by determining the absolute carotenoid levels in the micellar fractions. Bioaccessibility was calculated as the percentage of carotenoids content that remained in the micellar aqueous fraction after centrifugation in relation to the respective initial content in the food matrices.

One-way analysis of variance (ANOVA) were performed in order to detect statistically significant differences among samples and, when it was necessary, Bonferroni *post hoc* test were used to determine between which samples were these differences occurred. For the statistical analysis the IBM SPSS Statistics 20® software (SPSS Inc., 2012) was used.

3. RESULTS AND DISCUSSIONS

3.1. Carotenoid profile and content

The main isomer of PT in all the samples was the 15-*cis*-PT, the predominant one in most carotenogenic organisms. [22] The percentage of the 15-*cis* isomer of PT in relation to the total content of PT was as follows: 100% for tomato and cherry, 98.5% for TPL, and 95.7% for TPH.

221 The occurrence of several isomers of PTF agrees well with the findings of other
222 researchers in tomatoes. [23] The percentage of the *cis* isomers of PTF in relation to the
223 total content of PTF was as follows: 100% for tomato and cherry, 77.5% for TPL, and
224 70.3% for TPH.

225 PT content was higher than that of PTF in all samples, which is a common
226 characteristic in fruits and vegetables accumulating detectable levels of these
227 compounds. [6] More specifically, PT concentration was about twice that of PTF in both
228 the powder samples and in the tomato fruits (tomato and cherry) (Table 1). Similar
229 carotenoids concentrations were found in other common and cherry tomatoes. [10,24,25]
230 The PT and PTF concentrations from the powders was much higher as compared to that
231 from tomato fruits (Table 1). The highest differences were found between TPH and
232 tomato pulp, being the level of colourless carotenoids in TPH (10.6 mg PT+PTF/g) up
233 to 1000 times higher than that in the tomatoes fruits (8.3 µg PT+PTF /g).

234 **Table 1.** Carotenoid concentration in the matrices tested (µg/g).

Sample	Phytoene	Phytofluene
TPH	7478.27 ± 232.38 [a]	3114.82 ± 60.62 [a]
TPL	947.45 ± 10.41 [b]	440.10 ± 3.43 [b]
Tomato	5.36 ± 0.11 [c]	2.99 ± 0.08 [c]
Cherry	17.06 ± 3.63 [c]	9.21 ± 1.40 [c]

235 TPH, Tomato Powder with Higher carotenoid concentration; TPL, Tomato Powder with Lower
236 carotenoid concentration; Tomato, common tomato pulp; Cherry, cherry tomato pulp. Values are the
237 mean ± SD of 3 independent measures. Values within a column with different letters indicate statistically
238 significant differences ($P \leq 0.05$).

239

240 3.2.Transmission Electron Microscopy (TEM)

241 The TEM images (Figure 1) showed intact chromoplasts in the tomato and cherry
242 samples. Within these chromoplasts, crystals and plastoglobules, which are very
243 common substructures in tomato samples, were detected. It is well known that lycopene
244 tends to accumulate in these crystal-like structures in tomato. Due to the leaching out of
245 the lycopene during the dehydration process that takes place during the sample
246 preparation for TEM [26] these crystals were observed as membranes with undulating
247 shape in empty spaces (crystals remnants). Plastoglobules are regarded as deposition
248 sinks for the stable storage of carotenoids such as phytoene and β-carotene, [27,28]
249 although the latter can also be found as crystals in very rich sources like carrots. [26]
250 Although tomato is one of the richest sources of PTF and there are several studies on the
251 deposition form of carotenoids in this fruit, there is not information available in relation
252 to the deposition form of PTF. However, taken into account that this linear carotenoid
253 has fewer conjugated double bonds than lycopene, the shape is expected to be less rigid
254 than that of this compound and therefore it is not expected to aggregate to form crystals.
255 Besides, tomato phytofluene is in the form of several *cis/trans* isomers, the *cis* being
256 thought to be predominant. These isomers are thought to be more soluble and less prone
257 to aggregation, [29] so it seems reasonable to expect that phytofluene does not aggregate
258 and is mainly found in plastoglobules.

259 On the other hand, the microscopic analysis revealed that degraded cellular
260 structures rather than intact ones were predominant in the dehydrated and powdered
261 tomato materials. Thus, by comparing Figures 1-C and 1-D with 1-A and 1-B it can be
262 readily observed that the carotenoid-accumulating sub-structures were almost
263 indistinguishable and were dispersed in the matrix in the powders.

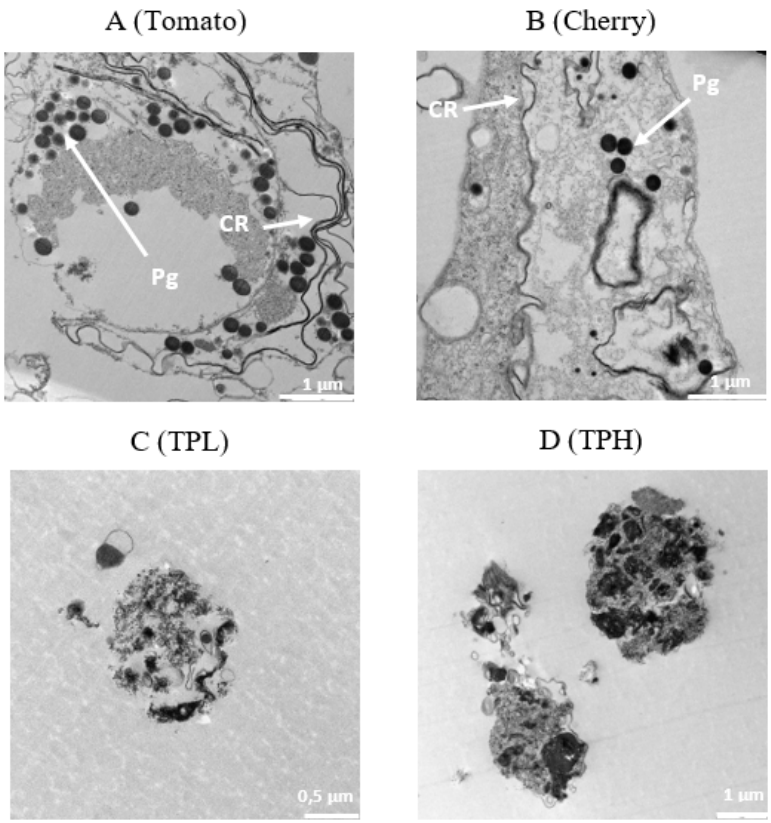

Figure 1. Representative micrographs obtained by Transmission Electron Microscopy (TEM). (A) Common tomato pulp; (B) Cherry tomato pulp; (C) Tomato powder with lower carotenoid concentration; (D) Tomato powder with higher carotenoid concentration. CR, crystal remnants; Pg, plastoglobuli.

3.3.Epifluorescence microscopy

In a previous work, the epifluorescence microscopy was used to analyse the distribution of PTF in orange pulps and juices. [21] In this study, the conclusions obtained from the epifluorescence images agreed well with the results obtained on the concentration and the bioaccessibility of this carotenoid. Therefore, it seems that epifluorescence is a simple technique that could be used for these purposes. However, certain problems are associated with the use of this technique. Due to the photobleaching showed by the PTF, the epifluorescence images had to be taken with little exposure time to avoid the destruction of the PTF and, in some cases, it was complicated to obtain the images.

Cells walls were easily detected in the tomato fruits (Figure 2) and PTF seemed to be accumulated within the cells in these samples. On the other hand, in the powder samples the PTF was more easily detected and was found more dispersed in the matrix.

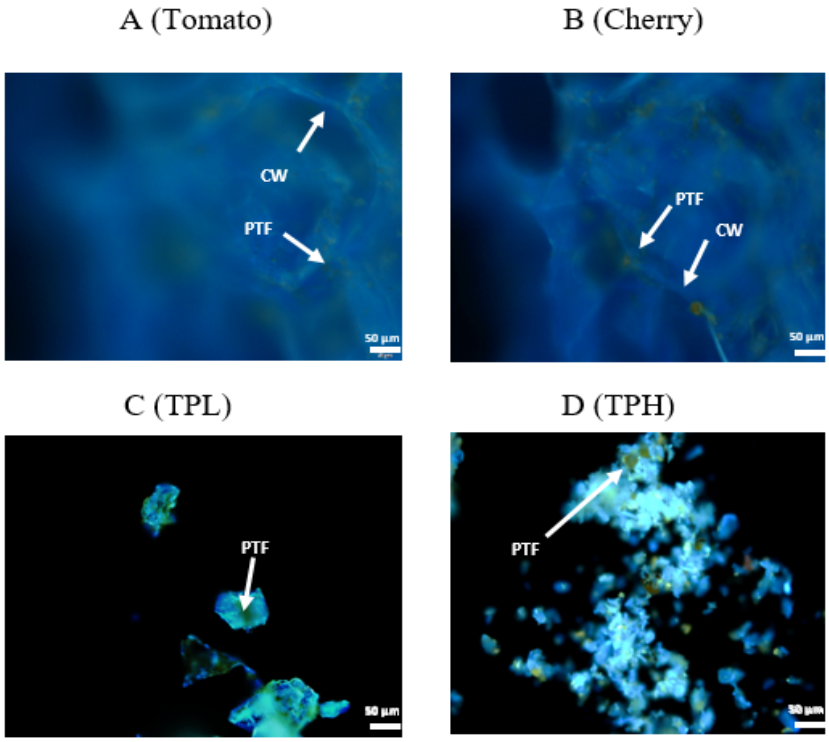

Figure 2. Representative micrographs of the samples obtained by epifluorescence microscopy. (A) Common tomato pulp; (B) Cherry tomato pulp; (C) Tomato powder with lower carotenoid concentration; (D) Tomato powder with higher carotenoid concentration. CW, Cell Wall; PTF, Phytofluene.

3.4. *In vitro* bioaccessibility

The bioavailability of carotenoids is affected by a variety of factors commonly referred to by the acronym SLAMENGHI. Such factors are: Species of carotene; molecular Linkage; Amount of carotene ingested; effects of food-Matrix; Effectors of absorption; Nutrient status; Genetic factors; Host related factors; and Interactions. [30] Among them, all except the nutrient status, genetic factors and host related factors, also

297 affect the bioaccessibility. In this regard, the substantial differences found in the
298 carotenoid bioaccessibility values (Table 2) across carotenoids and matrices could be
299 expected beforehand. Thus, it was observed differences due to the species of carotene;
300 the bioaccessibility of PT was higher than that of PTF in all the samples. Specifically,
301 the differences ranged between 1.03-fold (in cherry pulp) and 1.67-fold (in TPH). This
302 could be due to some extent to the differences in the number of conjugated double
303 bonds, which is thought to have an impact in the shape of the molecules, their rigidity
304 and susceptibility to aggregation. [29] These results are in line with those obtained by
305 other authors who have compared the bioaccessibility of phytoene and/or phytofluene
306 respect to other carotenoids. [9,16,31,32]

307 Apart from the physicochemical properties of the carotenoids itself, the type of
308 food matrix in which they are contained is considered one of the main factors that
309 govern their bioaccessibility [33] since incorporation of a carotenoid into mixed micelles
310 requires first their release from the food matrix. It is well known that the cell walls are
311 great barriers that hinders the carotenoids release and hence their bioaccessibility. [34]As
312 TEM images showed how the cell structures were markedly disrupted in the powders
313 samples (Figure 1) it was at first thought that the differences in the microstructure of the
314 matrices would be responsible of a possible higher carotenoid bioaccessibility from the
315 powders in comparison with that from the tomato fruits. Also, the disruption of the
316 matrix in the powder samples would lead to a decrease in the particle sizes and it is
317 known that the lower particle sizes, the easier the carotenoid release from the matrix and
318 the higher their bioaccessibility. [35] However, although the disruption of the cells
319 structures and the smaller particle size in the powders may have favoured the release of
320 carotenoids, the enormous differences found in the carotenoid concentrations among the
321 matrices (Table 1) seems to play a more important role in the differences in the

322 bioaccessibility. Given the much larger concentration of carotenoids in the powder
323 samples, important competition phenomena between molecules to be incorporated into
324 micelles might occur, and this fact may be one of the main reasons why the
325 bioaccessibility of PT and PTF from the powders was lower as compared to the tomato
326 fruits (Table 2). On the other hand, the dehydration process used in the manufacturing
327 of the powders increases the concentration of the food constituents as a result of the
328 elimination of water. This high concentration of food constituents could lead to
329 interactions during digestion which could impede the micelle formation and, therefore,
330 decrease the bioaccessibility of carotenoids in the powders.[36] Thus, it is known that the
331 dehydration of tomatoes leads to an increase in the concentration of sugars. [37] This
332 increase in sugars could be related to the crystalline matrix observed in the powders
333 (Figure 2). In addition, the proportion of *cis* isomers of each carotenoid in each sample
334 could also help to explain the differences in the bioaccessibility of each carotenoid
335 among the samples. It is known that the all-*trans* isomers are more prone to aggregate
336 and crystallize than their *cis* counterparts due to their more linear shape, which explain
337 why they usually have a lower bioaccessibility.[16,38,39] Therefore, the greater proportion
338 of *cis* isomers of both PT and PTF of the pulp samples in relation to that of the powder
339 samples could also help to explain the greater bioaccessibility of carotenoids from
340 tomato pulps. In regard to this aspect, it is interesting to note that the greater proportion
341 of *cis* isomers of PT and PTF could also be one of the main reasons why their
342 bioaccessibility is usually superior to that of other common carotenoids such as
343 lycopene or β-carotene. [9,16,20,38]

344 However, when 5% of sunflower oil was added to the samples as a source of fat,
345 the bioaccessibility of the colourless carotenoids in the powders was increased to similar
346 values, and in some cases even superior, to those found in the cherry and tomato pulp.

347 Thus, the increases in the bioaccessibility due to the addition of 5% oil ranged between
348 ca. 3.1 and 3.2-fold times for PT and between 3.5 and 4.2-fold times for PTF. Similarly,
349 Schweiggert et al. observed that the bioaccessibility of lycopene from tomato pulp
350 increased 3.3-fold times when sunflower oil was added to the sample.[40] A significant
351 increase in the bioaccessibility of β-carotene from tomato with the addition of the oil
352 was also found in this study. Also, in agreement with our results, it has been observed
353 that the bioaccessibility of β-carotene and lutein from drumstick leaves powders was
354 greatly increased by adding peanut oil. More specifically, increments of 84.2-fold and
355 of 1.8-fold were found for β-carotene and for lutein, respectively. [41] Similar results were
356 also found with lutein and zeaxanthin from the microalgae *Scenedesmus almeriensis*
357 when olive oil was added. [18]

358 Recently, it has been observed that the increase in the bioaccessibility of
359 carotenoids with the addition of fat depends on the type of fat. [42] Thus, the increase in
360 the bioaccessibility of the carotenes seems to be higher when the added fat is rich in
361 unsaturated fatty acids. This fact agrees with the great increase in the bioaccessibility of
362 PT and PTF observed in this work, since sunflower oil is rich in unsaturated fatty acids.
363 [15] Nevertheless, taking into account that the bioaccessibility of β-carotene and lutein has
364 been shown to increase more with oleic acid (main fatty acid of olive oil) than with
365 linoleic acid (main fatty acid of sunflower oil), [43] even higher increases in the
366 bioaccessibility of PT and PTF might be expected if olive oil had been added instead of
367 sunflower oil to the powder samples.

368 The clear increases in the bioaccessibility of the carotenoids observed when
369 sunflower oil was added is related to the fact that fat is needed to form the micelles
370 required for the transport of carotenoids from the matrix to the enterocytes. [44]
371 Physiologically, fat also stimulates digestive secretions that favour the formation of

micelles. [45] In other words, when fat is added, there is more "micellar material" for the incorporation and transport of carotenoids. Thus, taking into account that dietary fats can increase the carotenoids bioavailability in humans by increasing absorption, possibly by enhancing micellization in the small intestine,[36] it is possible that the addition of sunflower oil led also to an increase in the bioavailability of PT and PTF from the samples.

Table 2. Bioaccessibility of phytoene and phytofluene.

Sample	Phytoene	Phytofluene
TPH	27.43 ± 0.52^{d}	16.37 ± 0.52^{c}
TPL	29.59 ± 1.69^{d}	21.32 ± 1.55^{c}
TPH + Sunflower oil	86.65 ± 1.77^{bc}	69.41 ± 1.16^{b}
TPL + Sunflower oil	92.09 ± 0.86^{b}	74.90 ± 1.08^{b}
Tomato	102.22 ± 0.33^{a}	95.77 ± 0.72^{a}
Cherry	82.15 ± 7.13^{c}	79.38 ± 7.00^{b}

TPH, Tomato Powder with Higher carotenoid concentration; TPL, Tomato Powder with Lower carotenoid concentration; Tomato, common tomato pulp; Cherry, cherry tomato pulp. Values are the mean ± SD of 3 independent measures. Values within a column with different letters indicate statistically significant differences ($P \leq 0.05$).

3.5. Carotenoid bioaccessible content (CBC)

As stated before (Section 3.4.), the bioaccessibility of PT and PTF in the powders were lower than that in tomatoes fruit, however, it is to be considered that bioaccessibility was expressed as a percentage and that it is much more interesting to

assess which is the actual amount of a carotenoid that is incorporated into micelles and potentially absorbable (CBC).

By analysing the results obtained without adding sunflower oil to the samples, it was concluded that the source leading potentially to a higher absorption of PT and PTF was by far TPH, with 2 and 0.5 mg of potentially absorbable PT and PTF per gram of product, respectively (data not shown). As TPH was the sample with the lowest bioaccessibility (Table 1) it can be concluded that this sample has the highest CBC mainly due to its very high carotenoid content. These differences were even more evident when sunflower oil was added to the powders because the bioaccessibility increased considerably as it was discussed in the previous section. As a self-explanatory example, the PT and PTF bioaccessible contents of the sample TPH with oil (the sample with overall highest CBC) were 1149 and 753-fold higher relative to the tomato sample (the one with overall lowest CBC).

To know which matrices are better sources of colourless carotenoids from a more realistic point of view, the amount of bioaccessible carotenoid per food ration was compared. The rations chosen were the same chosen by Granado-Lorencio et al., [46] that is, 1 g of powders, 10 g of powders with oil, and 100 g of fruits. Also in this case, TPH was the best source of PT and PTF (Figure 3). In particular, when sunflower oil was added to this sample the difference between their CBC and that of the other samples were astounding (Figure 3). The bioaccessible contents of PT and PTF per ration of TPH with oil was 173 and 60-fold higher respectively than that per ration of a fresh juice (250 mL) of a mutant orange (Pinalate) with a very high concentration of colourless carotenoids, [21] and 18 and 19-fold higher than that per ration of a tomato juice (250 mL), respectively. [16]

With all this, it can be concluded that, in principle, the tomato powders are by far better sources of colourless carotenoids compared to tomato and cherry pulps. Although it is though that carotenoid bioaccessibility is well correlated with bioavailability, [11,36,47] further studies *in vivo* are necessary to confirm that, indeed, tomato powders can lead to higher increases in circulating carotenoids than tomato fruits. As it can be readily observed in Table 3, all the tomato and tomato-based products analysed are better sources of potentially absorbable PT than of PTF.

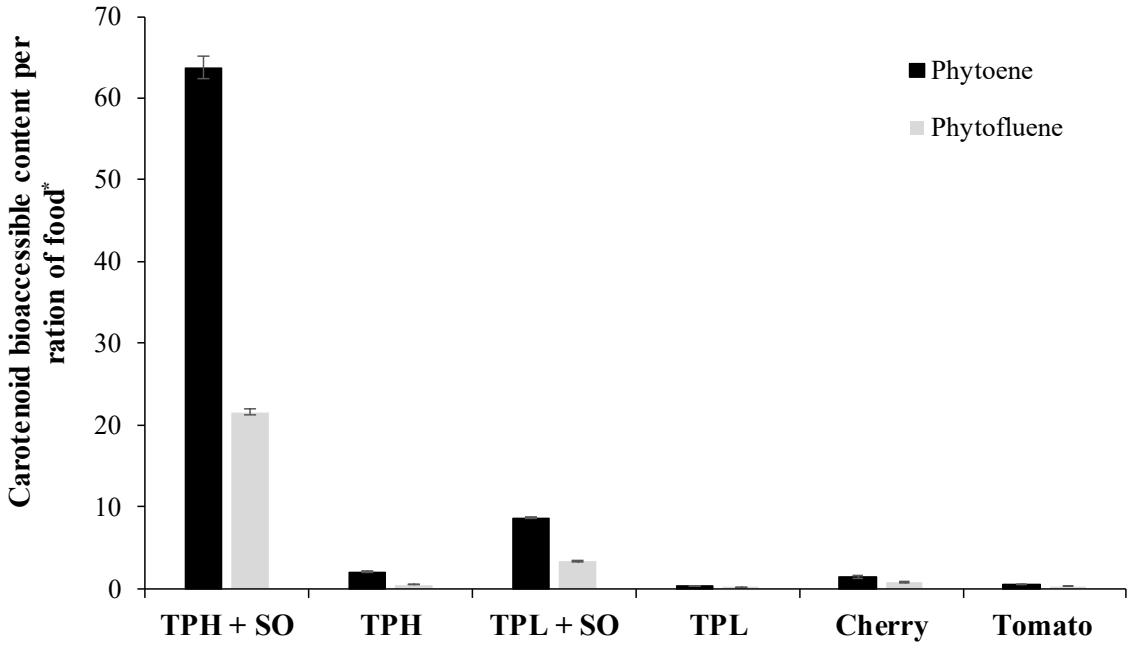

Figure 3. Carotenoid bioaccessible content of phytoene and phytofluene (in mg) per ration of food*. TPH, Tomato Powder with Higher carotenoid concentration; TPL, Tomato Powder with Lower carotenoid concentration; Tomato, common tomato pulp; Cherry, cherry tomato pulp; SO, Sunflower Oil. Values are the mean ± SD of 3 independent measures. * Ration of food: 1 g powders (TPH and TPL), or 10 g powders with oil, or 100 g fruits (tomato or cherry).

4. CONCLUSIONS

Despite the lower carotenoid bioaccessibility of the powders in comparison with the commercial tomato pulps studied, their bioaccessible content is much higher. Both the disruption of the cell structures that suffered the samples as a result of the dehydration

435 and powdering and the higher concentration of PT and PTF in the powders compared to
436 the pulps may contribute to this. Interestingly, adding sunflower oil to the samples can
437 increase the already high carotenoid bioaccessibility of such concentrated sources of PT
438 and PTF, and hence their bioavailability. With all this, tomato powders, which could be
439 used as ingredients of supplements (or related compounds) or incorporated in a
440 functional food, seem to be a markedly richer source of potentially absorbable
441 colourless carotenoids in comparison with fresh common and cherry tomatoes.
442

443 **CONFLICT OF INTEREST**

444 Antonio J. Meléndez-Martínez is a member of the advisory board of IBR –
445 Israeli Biotechnology Research, Ltd. (Yavne, Israel).

446

447 **ACKNOWLEDGEMENTS**

448 This work was partially funded by IBR – Israeli Biotechnology Research, Ltd.,
449 Yavne, Israel. The authors also acknowledge funding from the Andalusian Council of
450 Economy, Innovation, Science and Employment (ref. CAROTINCO-P12-AGR-1287)
451 and Carotenoid Network: from microbia and plants to food and health (CaRed), funded
452 by the Spanish Ministry of Economy and Competitiveness (BIO2015-71703-REDT).
453 We thank the technical staff of the services of Biology and Microscopy (SGI,
454 Universidad de Sevilla).

455 **REFERENCES**

456 1 G. Britton, S. Liaaen-Jensen and H. Pfander, in *Carotenoids*, eds. G. Britton, S.
457 Liaaen-Jensen and H. Pfander, Birkhäuser, Basel, Switzerland, 2009, vol. 5.

2 M. Rodriguez-Concepcion, J. Avalos, M. L. Bonet, A. Boronat, L. Gomez-Gomez, D. Hornero-Mendez, M. C. Limon, A. J. Meléndez-Martínez, B. Olmedilla-Alonso, A. Palou, J. Ribot, M. J. Rodrigo, L. Zacarias and C. Zhu, *Prog. Lipid Res.*, 2018, **70**, 62–93.

3 A. J. Meléndez-Martínez, P. Mapelli-Brahm and C. M. Stinco, *J. Food Compos. Anal.*, 2018, **67**, 91–103.

4 A. Kaulmann and T. Bohn, *Nutr. Res.*, 2014, **34**, 907–929.

5 E. Bony, F. Boudard, P. Brat, E. Dussossoy, K. Portet, P. Poucheret, J. Giaimis and A. Michel, *Fitoterapia*, 2012, **83**, 33–43.

6 A. J. Meléndez-Martínez, P. Mapelli-Brahm, A. Benítez-González and C. M. Stinco, *Arch. Biochem. Biophys.*, 2015, **572**, 188–200.

7 F. Khachik, L. Carvalho, P. S. Bernstein, G. J. Muir, D.-Y. Zhao and N. B. Katz, *Exp. Biol. Med.*, 2002, **227**, 845–851.

8 A. J. Meléndez-Martínez, P. D. Fraser and P. M. Bramley, *Phytochemistry*, 2010, **71**, 1104–1114.

9 M. J. Rodrigo, A. Cilla, B. Reyes and L. Zacarías, *Food Funct.*, 2015, **6**, 1950–1959.

10 E. Biehler, A. Alkerwi, L. Hoffmann, E. Krause, M. Guillaume, M.-L. Lair and T. Bohn, *J. Food Compos. Anal.*, 2012, **25**, 56–65.

11 A. Cilla, L. Bosch, R. Barberá and A. Alegría, *J. Food Compos. Anal.*, 2018, **68**, 3–15.

12 E. Reboul, M. Richelle, E. Perrot, C. Desmoulins-Malezet, V. Pirisi and P. Borel,

480 *J. Agric. Food Chem.*, 2006, **54**, 8749–8755.

481 13 S. Van Buggenhout, M. Alminger, L. Lemmens, I. Colle, G. Knockaert, K.
482 Moelants, A. Van Loey and M. Hendrickx, *Trends Food Sci. Technol.*, 2010, **21**,
483 607–618.

484 14 S. Franke, K. Fröhlich, S. Werner, V. Böhm and F. Schöne, *Eur. J. Lipid Sci.*
485 *Technol.*, 2010, **112**, 1122–1129.

486 15 A. Thomas, *Ullmann's Encycl. Ind. Chem.*, 2012, 547–572.

487 16 P. Mapelli-Brahm, J. Corte-Real, A. J. Meléndez-Martínez and T. Bohn, *Food*
488 *Chem.*, 2017, **229**, 304–311.

489 17 C. M. Stinco, F. J. Rodríguez-Pulido, M. L. Escudero-Gilete, B. Gordillo, I. M.
490 Vicario and A. J. Meléndez-Martínez, *Food Res. Int.*, 2013, **50**, 111–120.

491 18 R. Pullakhandam and M. L. Failla, *J. Med. Food*, 2007, **10**, 252–257.

492 19 C. M. Stinco, F. J. Rodríguez-Pulido, M. L. Escudero-Gilete, B. Gordillo, I. M.
493 Vicario and A. J. Meléndez-Martínez, *Food Res. Int.*, 2013, **50**, 111–120.

494 20 A. J. Meléndez-Martínez, C. M. Stinco, C. Liu and X.-D. Wang, *Food Chem.*,
495 2013, **138**, 1341–50.

496 21 P. Mapelli-Brahm, C. M. Stinco, M. J. Rodrigo, L. Zacarías and A. J. Meléndez-
497 Martínez, *J. Funct. Foods*, 2018, **46**, 38–47.

498 22 A. Than, P. M. Bramley, B. H. Davies and A. F. Rees, *Phytochemistry*, 1972, **11**,
499 3187–3192.

500 23 J. L. Cooperstone, D. M. Francis and S. J. Schwartz, *Food Chem.*, 2016, **210**,
501 466–472.

24 P. Flores, E. Sánchez, J. Fenoll and P. Hellín, *Food Res. Int.*, 2017, **100**, 510–516.

25 A. Raffo, C. Leonardi, V. Fogliano, P. Ambrosino, M. Salucci, L. Gennaro, R. Bugianesi, F. Giuffrida and G. Quaglia, *J. Agric. Food Chem.*, 2002, **50**, 6550–6556.

26 R. M. Schweiggert and R. Carle, *Crit. Rev. Food Sci. Nutr.*, 2017, **57**, 1807–1830.

27 M. Nogueira, L. Mora, E. M. A. Enfissi, P. M. Bramley and P. D. Fraser, *Plant Cell*, 2013, **25**, 4560–4579.

28 J. L. Cooperstone, R. A. Ralston, K. M. Riedl, T. C. Haufe, R. M. Schweiggert, S. A. King, C. D. Timmers, D. M. Francis, G. B. Lesinski, S. K. Clinton and S. J. Schwartz, *Mol. Nutr. Food Res.*, 2015, **59**, 658–669.

29 A. J. Meléndez-Martínez, M. Paulino, C. M. Stinco, P. Mapelli-Brahm and X. D. Wang, *J. Agric. Food Chem.*, 2014, **62**, 12399–12406.

30 S. De Pee and C. E. West, *Eur. J. Clin. Nutr.*, 1996, **50**, S38–S53.

31 J. L. Jeffery, N. D. Turner and S. R. King, *J. Sci. Food Agric.*, 2012, **92**, 2603–10.

32 D. B. Rodrigues, C. Chitchumroonchokchai, L. R. B. Mariutti, A. Z. Mercadante and M. L. Failla, *J. Agric. Food Chem.*, 2017, **65**, 11220−11228.

33 L. Lemmens, I. Colle, S. Van Buggenhout, P. Palmero, A. Van Loey and M. Hendrickx, *Trends Food Sci. Technol.*, 2014, **38**, 125–135.

34 P. Palmero, A. Panozzo, D. Simatupang, M. Hendrickx and A. Van Loey, *Food*

Res. Int., 2014, **64**, 831–838.

35 J. Parada and J. M. Aguilera, *J. Food Sci.*, 2007, **72**, 21–32.

36 T. Bohn, G. J. Mcdougall, A. Alegría, M. Alminger, E. Arrigoni, A. M. Aura, C. Brito, A. Cilla, S. N. El, S. Karakaya, M. C. Martínez-Cuesta and C. N. Santos, *Mol. Nutr. Food Res.*, 2015, **59**, 1307–1323.

37 T. L. Maung and Y. Y. Win, *Univ. Res. J.*, 2011, **4**, 401–413.

38 M. L. Failla, C. Chitchumroonchokchai and B. K. Ishida, *J. Nutr.*, 2008, **138**, 482–486.

39 G. Britton, in *Carotenoids*, eds. G. Britton, S. Liaaen-Jensen and H. Pfander, Birkhäuser, Basel, Switzerland, 1995, p. Vol. 1B, 13–62.

40 R. M. Schweiggert, D. Mezger, F. Schimpf, C. B. Steingass and R. Carle, *Food Chem.*, 2012, **135**, 2736–2742.

41 F. Granado-Lorencio, C. Herrero-Barbudo, G. Acién-Fernández, E. Molina-Grima, J. M. Fernández-Sevilla, B. Pérez-Sacristán and I. Blanco-Navarro, *Food Chem.*, 2009, **114**, 747–752.

42 M. L. Failla, C. Chitchumronchokchai, M. G. Ferruzzi, S. R. Goltz and W. W. Campbell, *Food Funct.*, 2014, **5**, 1101–1112.

43 A. Nagao, E. Kotake-Nara and M. HASE, *Biosci. Biotechnol. Biochem.*, 2013, **77**, 1055–1060.

44 J. de J. Meléndez-Martínez, A.J., Pérez-Gálvez, A., Roca, M., Estévez-Santiago, R., Olmedilla-Alonso, B., Mercadante, A.Z., Ornelas-Paz, in *Carotenoides En Agroalimentación Y Salud.*, ed. A. J. Meléndez-Martínez, Editorial Terracota,

Ciudad de México, México, 2017, pp. 574–608.

45 A. Pérez-Gálvez, Y. M. Pacheco, B. Bermúdez, S. López, R. Abia, F. J. G. Muriana, J. Villar and J. Garrido-Fernández, *Food Res. Int.*, 2005, **38**, 1097–1102.

46 F. Granado-Lorencio, C. Herrero-Barbudo, I. Blanco-Navarro, B. Pérez-Sacristán and B. Olmedilla-Alonso, *Br. J. Nutr.*, 2009, **101**, 576–582.

47 T. Bohn, F. Carriere, L. Day, A. Deglaire, L. Egger, D. Freitas, M. Golding, S. Le Feunteun, A. Macierzanka, O. Menard, B. Miralles, A. Moscovici, R. Portmann, I. Recio, D. Rémond, V. Santé-Lhoutelier, T. J. Wooster, U. Lesmes, A. R. Mackie and D. Dupont, *Crit. Rev. Food Sci. Nutr.*, 2017, **8398**, 00–00.