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1 **The effect of pulsed electric fields on carotenoids bioaccessibility: the role of tomato matrix**

2

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15

16 **Abstract**

17 Tomato fractions (tissue, cells clusters, single cells, chromoplasts) were subjected to PEF treatment  
18 combined or not with heating. Results show that PEF and heating applied in combination or  
19 individually induced permeabilization of cell membranes in the tomato fractions. However, in tissue  
20 no changes in  $\beta$ -carotene bioaccessibility were found upon combined and individual PEF and  
21 heating, while a decrease in lycopene bioaccessibility upon combined PEF and heating and heating  
22 only was observed. In cells clusters and single cells, carotenoids bioaccessibility did not change  
23 upon the treatments. In chromoplasts both  $\beta$ -carotene and lycopene bioaccessibility significantly  
24 decreased upon combined PEF and heating. Differences in the effects of PEF on carotenoids  
25 bioaccessibility were related to the structure complexity of the tomato fractions. In particular, for  
26 chromoplasts the reduction in bioaccessibility was attributed to the lower protection against PEF

27 treatment present in this fraction compared to multiple physical barriers present in the other  
28 fractions.

29

30 **Keywords:** pulsed electric fields, heating, tomato, food matrix, structural barriers, carotenoids  
31 bioaccessibility

32

### 33 **1. Introduction**

34 Several studies relate a high intake of bioactive compounds present in fruit and vegetables to human  
35 health benefits. Among the large spectrum of bioactive compounds, carotenoids are a widespread  
36 family of fat-soluble plant pigments giving yellow, orange and red colour to many plant foods.  
37 Lycopene and  $\beta$ -carotene, the major carotenoids present in tomato and derived products, play an  
38 important role in human health because of their powerful antioxidant properties and pro-vitamin A  
39 activity. Moreover, they are associated with a decreased risk of cardiovascular diseases and cancer  
40 (Giovannucci, 1999). To study the carotenoids health related functions, their bioavailability needs  
41 to be evaluated. However, the bioavailability is strongly related to their bioaccessibility, that is the  
42 fraction released from the matrix and available for the intestinal absorption (Parada & Aguilera,  
43 2007). The specific localization of carotenoids into the chromoplasts as well as the structural  
44 barriers within the cell govern carotenoids bioaccessibility. In particular, chromoplasts and cell  
45 membrane as well as cell wall are the limiting factors for both  $\beta$ -carotene and lycopene  
46 bioaccessibility in tomato and carrot (Jeffery, Holzenburg, & King, 2012; Palmero et al., 2013).

47 Several studies investigated the effect of thermal treatment, high pressure homogenization or high  
48 power ultrasounds on carotenoids bioaccessibility in tomato juice (Anese, Mirolo, Beraldo, & Lippe  
49 2013; Colle, Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010a; Colle, Van Buggenhout,  
50 Van Loey, & Hendrickx, 2010b). However, the structural complexity of the tomato matrix did not  
51 allow to disentangle the effect of the various processes on the different cell barriers thus to  
52 understand which are the key factors governing the bioaccessibility. To tackle this issue, Palmero

53 and co-authors used tomato fractions posing different physical barriers to carotenoids  
54 bioaccessibility (i.e. chromoplasts and cells clusters) and applied thermal or high pressure  
55 homogenization treatments (Palmero et al., 2013; Palmero, Lemmens, Hendrickx, & Van Loey,  
56 2014; Palmero, Panozzo, Colle, Chigwedere, Hendrickx, & Van Loey, 2016). Upon thermal  
57 treatment the carotenoids bioaccessibility decreased in cluster cells. It has been suggested that  
58 carotenoids became entrapped by a new formed network consisting of cell wall material (Palmero et  
59 al., 2014). On the other hand, high pressure homogenization treatment induced an increase of  
60 carotenoids bioaccessibility due to the disruption of the cell structure present in chromoplasts and  
61 cells clusters (Palmero et al., 2016).

62 Pulsed electric fields (PEF) is a widely explored technology for inducing the permeabilization of  
63 cell membranes. The exposure of a plant tissue to an electric field for short voltage pulses, typically  
64 in the range of  $\mu\text{s}$ , induces the formation of pores on the membrane (electroporation phenomena).  
65 More specifically, when the cells are exposed to an external electric field, the accumulation of  
66 oppositely charged ions on both sides of membrane causes membrane thickness reduction. Further  
67 increases of the electric field up to the critical values (0.5-5 kV/cm for plant cells) cause pore  
68 formation and loss of semi-permeability of the cell membrane. However, depending on electric field  
69 strength and treatment intensity, electroporation may be either reversible or irreversible  
70 (Zimmermann, 1986).

71 The effect of PEF at low electric fields applied individually or in combination with heating has been  
72 investigated in order to improve the extraction yield of intracellular compounds present in fruits and  
73 vegetables tissue (Donsì, Ferrari, & Pataro, 2010). Several studies found that PEF treatments at 0.1-  
74 10 kV/cm increased the extraction of hydrophilic compounds, such as sugar from sugar beet,  
75 betaine from red beet and anthocyanins from grapes, red cabbage or purple fleshed potatoes  
76 (Eshtiaghi & Knorr, 2002; Gachovska, Cassada, Subbiah, Hanna, Thippareddi, & Snow, 2010;  
77 López, Puértolas, Condón, Raso, & Alvarez, 2009; Puértolas, Cregenzán, Luengo, Álvarez, &  
78 Raso, 2013). By contrast, only a few studies investigated the effect of PEF on the extraction of

79 lipophilic compounds, such as carotenoids (Luengo, Álvarez, & Raso, 2014; Wiktor et al., 2015).  
80 Both an increase or no effect in carotenoids concentration was found, depending on the origin of the  
81 matrix (skin or tissue) (Luengo, Álvarez, & Raso, 2014; Jayathunge, Stratakos, Cregenzán-Albertia,  
82 Grant, Lyng, & Koidis, 2017). Recently, Jayathunge et al. (2017) investigated the effect of PEF as  
83 pre-treatment in on carotenoids bioaccessibility in whole tomato fruit during storage. These  
84 treatments, while causing an increase in carotenoids concentration, induced both an increase or a  
85 decrease of carotenoids bioaccessibility depending on the number of pulses and storage time. On  
86 the other hand, to the best of our knowledge, there is a lack of information on the effect of PEF on  
87 tomato fractions characterized by different structural barriers. Therefore, the aim of this study was  
88 to investigate the effect of PEF on carotenoids bioaccessibility in tomato fractions. To this purpose  
89 tomato tissue, cells clusters, single cells and chromoplasts were isolated and subjected to PEF and  
90 heating, in combination or individually. Contextually, microstructure, conductivity and carotenoids  
91 concentration of untreated and treated samples were determined.

92

## 93 **2. Materials and methods**

### 94 *2.1. Materials*

95 A 30 kg batch of red tomatoes (*Lycopersicon esculentum*) was purchased in a local store in the  
96 Netherlands and stored at 7 °C until their use for the experiment. Tomato samples were prepared  
97 fresh for every trial from the same batch of fruits to minimize the influence of the matrix.

98

### 99 *2.2 Experimental set-up*

100 Four fractions with different level of structural barriers corresponding to tissue, cells clusters, single  
101 cells and chromoplasts were isolated from tomato and subjected to PEF (PEF), heating (HEAT) or  
102 combined PEF and heating (PEF+HEAT). Microstructure, conductivity, carotenoids concentration  
103 and *in vitro* bioaccessibility analyses were performed to each fraction. Fig. 1 depicts schematically  
104 the experimental set-up performed in the present study.

105

### 106 *2.3. Preparation of tomato fractions*

#### 107 *2.3.1 Tissue*

108 Tomato tissue was prepared by removing skin and placental tissue and cutting the mesocarp into  
109 cubes of 5 cm length.

110

#### 111 *2.3.2 Cells clusters*

112 Tomato cubes, obtained by previous discard of skin and placental tissue, were blended in a kitchen  
113 blender (5 s for 3 times). The cells clusters were obtained by separating the juice with the use of wet  
114 sieving equipment (Analysette 3 Spartan, Idar-Oberstein, Germany). The size of the fraction  
115 considered in this study ranged between 71-350  $\mu\text{m}$ .

116

#### 117 *2.3.3. Single cells*

118 Single cells were obtained based on the procedure of McAtee, Hallett, Johnston, & Schaffer (2009)  
119 with minor modifications. Tomato cubes, obtained by previous discard of skin and placental tissue,  
120 were immersed in a 0.05 M  $\text{Na}_2\text{CO}_3$  in 0.3 M mannitol solution. The solution was heated at 90 °C  
121 for 30 minutes under continuous stirring, and filtered with a sieve (1 mm). The cells were isolated  
122 by filtering the solution through a cheesecloth.

123

#### 124 *2.3.4. Chromoplasts*

125 Chromoplasts were isolated based on the procedure of Hansen and Chiu (2005) as described by  
126 Palmero et al. (2013). Tomato cubes, obtained by previous discard of skin and placental tissue, were  
127 blended in a kitchen blender (5 s for 5 times) with 0.05 M EDTA solution (1:1 ratio). The obtained  
128 juice was filtrated using a cheesecloth and the filtrate was centrifuged (Beckman Coulter Avanti J-  
129 26XP centrifuge, Palo Alto, CA, USA) at 27250 g and 4 °C for 30 min. The pellet, consisting of the  
130 chromoplasts, was re-dissolved in 5 mg/mL NaCl.

131

## 132 2.4. Treatments

### 133 2.4.1. Pulsed electric fields treatments

134 Pulsed electric fields (PEF) treatment was carried out using a NP110-60 system (IXL Netherlands  
135 B.V.) with an output voltage of 3.8 kV. The system provided monopolar, rectangular shaped pulses  
136 of average 350  $\mu$ s width. The treatment chamber consisted of a batch chamber with two circular  
137 stainless steel electrodes with a surface area of 28.3 cm<sup>2</sup>, resulting in a 56.5 cm<sup>3</sup> total volume. The  
138 distance between the electrodes was 2.0 cm. Aliquots of 57 g of tomato fractions were put into the  
139 chamber subjected to PEF treatments characterized by a total energy input ( $Q$ ) (MJ/kg) of 7.6  
140 MJ/kg. The latter was calculated according to Zhang, Barbosa-Cánovas and Swanson (1995) (eq.  
141 1), by using the following equation:

$$142 \quad Q = \frac{V^2 t}{R m} \quad (\text{eq. 1})$$

143 where  $V$  is the voltage (kV),  $t$  is the total treatment time (s),  $R$  is the resistance (Ohm) and  $m$  is the  
144 sample mass (kg). Two PEF treatments were performed: (i) 90 pulses at 1Hz repetition rate in order  
145 to reach an initial temperature of 90 °C. Afterwards, 210 pulses where delivered at 0.167 Hz s  
146 within 30 minutes at an equilibrium temperature in the range of 85-90 °C. This treatment was  
147 indicated as PEF+HEAT; (ii) 600 pulses with an interval time of 3 s was applied. The equilibrium  
148 temperature of this treatment was within the range of 40-45 °C. This treatment was indicated as  
149 PEF.

150 The temperature was measured at the end of the treatment by using a copper-constantan  
151 thermocouple probe connected to a data logger (YC 727UD, TMS Europe Ltd, Hope Valley, United  
152 Kingdom). After the treatments, the samples were cooled in a water-ice bath.

153

154 *2.4.2. Heating*

155 The total temperature–time combination received by the samples during PEF+HEAT was applied to  
156 the sample in absence of the electric field. To this purpose, aliquots of 57 g of tomato fractions were  
157 heated in a thermostatic water bath (Ika Werke, MST BC, Staufen, Germany) under continuous  
158 stirring. The sample reached 90 °C within 5 min and the temperature was maintained constant at 90  
159 °C for 25 min. This treatment was indicated as HEAT. The temperature was recorded using a  
160 copper-constantan thermocouple probe connected to a data logger (YC 727UD, TMS Europe Ltd,  
161 Hope Valley, United Kingdom). After the treatment, the samples were cooled in a water-ice bath.

162

163 *2.5. Microscopy analysis*

164 Microstructure of tomato fractions was analysed using an optical microscope (Axioskop Zeiss,  
165 Göttingen Germany). The pictures were taken by a digital camera (AxioCam HCR, Göttingen,  
166 Germany). Tissue, cells clusters and single cells were analysed with 10x lens, while 100x objective  
167 lens was used for chromoplasts.

168

169 *2.6. Impedance measurement*

170 Measurement of electrical complex impedance was used to characterized tissue permeabilization  
171 after treatments (Donsì, Ferrari, & Pataro, 2010). The measurement was conducted by loading the  
172 sample in a test vessel between two 0.3 mm platinum wires separated 1.0 cm and inserted 1.0 cm  
173 deep into the sample. The platinum wires were connected to an impedance analyser which consisted  
174 of a Rigol DG1022 function generator and a Rigol DS1054Z oscilloscope. The generator produced  
175 a sinusoidal voltage of 2 V peak-peak with a frequency ranging between 1 kHz and 1 MHz. All the  
176 measurement were carried out at 20 °C. The electrical conductivity is the ratio of the current  
177 through the sample and the voltage drop across the sample, multiplied by the cell constant ( $1\text{cm}^{-1}$ )  
178 of the wire electrode set-up. Conductivities, given in the unit S/m, are corrected for evaporation in

179 the heated samples (leading to an increase in ion concentration) by normalizing to the sample  
180 weight i.e. conductivity is expressed in units of S/m·g.

181

## 182 2.7. Cell disintegration index

183 Cell disintegration index ( $Z_p$ ) was computed according to Angersbach, Heinz and Knorr (1999).

184 This index indicate the proportion of permeabilized cells based on the frequency dependence of

185 conductivity of intact and permeabilized plant tissue. The  $Z_p$  was calculated by using the following

186 equation (eq. 2):

$$187 Z_p = 1 - \left( \frac{K_h}{K_h'} \right) \cdot \frac{(K_h' - K_l')}{(K_h - K_l)} \quad (eq. 2)$$

188 Where  $K_l$ , and  $K_l'$  are the conductivities of untreated and treated tomato fraction at 1 kHz,

189 respectively, and  $K_h$  and  $K_h'$  are the electrical conductivities of untreated and treated tomato fraction

190 at 1 MHz, respectively. The  $Z_p$  varies between 0 for intact tissue and 1 for a tissue with all the cells

191 permeabilized.

192

## 193 2.9. Carotenoids concentration

194 The extraction of carotenoids from tomato fractions or micelles was performed following the

195 procedure of Sadler, Davis and Dezman (1990) with minor modifications. The analysis was

196 carried out under subdued light to prevent carotenoid degradation and isomerisation. 0.5 g NaCl

197 and 50 mL extraction solution (hexane:acetone:ethanol, 2:1:1 v/v/v) were added to 2 g of tomato

198 sample or supernatant containing micelle fraction. After 20 min of stirring at room temperature,

199 15 mL of reagent grade water was added and stirring was continued for 10 min. The apolar

200 phase, containing carotenoids, was collected, filtered (Chromafil PET filters, Düren, Germany;

201 0.20  $\mu\text{m}$  pore size, 25 mm diameter) and transferred to an amber HPLC vial. The HPLC analyses

202 were performed on a Ultimate 3000 Rapid Separation LC System (Thermo Fisher Scientific,

203 Sunnyvale, CA, USA) equipped with a Ultimate 300 RS photodiode Diode array detector

204 (DAD-3000RS, Thermo Fisher Scientific, Sunnyvale, CA, USA).  $\beta$ -carotene and all-*trans*  
205 lycopene were separated at 25 °C on a reversed phase C<sub>30</sub> column (250 x 4.6 mm, particle size 5  
206  $\mu$ m, YMC Europe, Dinslaken, Germany) with a gradient of two methanol:methyl tert-butyl ether  
207 eluents (eluent A 90:10, eluent B 10:90) containing 0.1% BHT. The gradient was as follow: 0  
208 min: 88% A, 12% B; 2-4 min: 73% A, 26% B; 4-6 min: 57% A, 43% B; 6-8 min: 20% A, 80%  
209 B; 9-14 min: 0% A and 100% B; 15-25 min: 88% A, 12% B. The flow rate was 1.05 mL/min  
210 with an injection volume of 20  $\mu$ L.  $\beta$ -carotene and all-*trans* lycopene were identified based on  
211 retention times and spectral characteristics compared to the standards (data not shown). To  
212 quantify the carotenoids, HPLC-DAD responses were measured at 450 nm for  $\beta$ -carotene and at  
213 470 nm for all-*trans* lycopene. The carotenoids content was calculated based on their calibration  
214 curves and expressed as  $\mu$ g/g tomato fraction dry weight ( $\mu$ g/g dw).

215

#### 216 2.10. Carotenoids *in vitro* bioaccessibility

217 The carotenoids *in vitro* bioaccessibility was measured the day after the processing following the  
218 procedure described by Minekus et al. (2014) with minor modifications. Five g of sample was  
219 weighed into a 50 mL capacity falcon tube. The sample was diluted with 4 mL of Simulated  
220 Salivary Fluid (SSF: 15.1 mL of 0.5 M KCl, 3.7 mL of 0.5 M KH<sub>2</sub>PO<sub>4</sub>, 6.8 mL of 1 M NaHCO<sub>3</sub>,  
221 0.5 mL of 0.15 M MgCl<sub>2</sub>(H<sub>2</sub>O)<sub>6</sub>, 0.06 mL of 0.5 M (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>), 975  $\mu$ L miliQ water, 7.24 mL of  
222 Simulated Gastric Fluid (SGF: 6.9 mL of 0.5 M KCl, 0.9 mL of 0.5 M KH<sub>2</sub>PO<sub>4</sub>, 12.5 mL of 1 M  
223 NaHCO<sub>3</sub>, 11.8 mL of 2 M NaCl, 0.4 mL of 0.15 M MgCl<sub>2</sub>(H<sub>2</sub>O)<sub>6</sub>, 0.5 mL of 0.5 M of (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>),  
224 50  $\mu$ L of 0.3 M CaCl<sub>2</sub> solution, 260  $\mu$ L of freshly prepared L- $\alpha$ -phosphatidylcholine solution (10  
225 mg/mL in SGF). The latter was obtained by preparing 50 mg/mL L- $\alpha$ -phosphatidylcholine (Sigma-  
226 Aldrich) solution in chloroform:methanol (1:1 v:v). To simulate the gastric digestion, the pH of the  
227 mixture was adjusted to  $3 \pm 0.05$  with 1 M HCl or 1 M NaOH and 1.6 mL of porcine pepsin  
228 solution (25000 U/mL) was added. After flushing sample headspace with nitrogen for 10 s, the  
229 mixture was incubated at 37 °C for 2 hours while shaking end-over-end. Afterwards, to mimic the

230 passage through the small intestine, 11 mL of Simulated Intestinal Fluid (SIF: 6.8 mL of 0.5 M  
231 KCl, 0.8 mL of 0.5 M KH<sub>2</sub>PO<sub>4</sub>, 42.5 mL of 1 M NaHCO<sub>3</sub>, 9.6 mL of 2 M NaCl, 1.1 mL of 0.15 M  
232 MgCl<sub>2</sub>(H<sub>2</sub>O)<sub>6</sub>, 2.5 mL of fresh bile (0.089 g/ml in SIF), 40 µL of 0.3 M CaCl<sub>2</sub> and 5.0 mL of  
233 pancreatin (0.33 g/ml in SIF) were added. The amount of pancreatin added to SIF was calculated  
234 based on a trypsin activity measured in the pancreatin according to the assay described in the  
235 harmonized protocol (Minekus et al., 2014). The pH of the solution was adjusted to 7 ± 0.05 with 1  
236 M HCl or 1 M NaOH. Finally, the sample headspace was flushed with nitrogen for 10 seconds and  
237 the solution was incubated at 37 °C for 2 hours while shaking end-over-end. The digest was  
238 centrifuged (Beckman L-60 Ultracentrifuge, Palo Alto, CA, USA) at 162000 g for 67 min at 4 °C to  
239 separate the micelles containing the carotenoids. The supernatant was collected and carotenoids  
240 quantified according to the method described above. After the extraction procedure, the carotenoids  
241 extract was up-concentrated under vacuum using a rotary evaporator at 35 °C and re-dissolved in  
242 hexane:dichlorometane (4:1 v:v). The concentration factor was calculated by adding a specific  
243 amount of β-apo-8'-carotenale prior to evaporation. The carotenoid *in vitro* bioaccessibility (B/C) in  
244 each fraction and for each treatment was defined as the percentage ratio between the carotenoids  
245 concentration in the micelles (B) and the carotenoid concentration (C) of the corresponding fraction  
246 and treatment before digestion.

247

#### 248 2.11. Total solids content

249 The total solids content was measured by gravimetric method (AOAC, 1995).

250

#### 251 2.12. Data analysis

252 The results are the average of at least two measurements carried out on two replicated experiments  
253 ( $n \geq 4$ ). Data are reported as mean value ± standard error. Statistical analysis was performed using R  
254 v.2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the

255 homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine  
256 statistically significant differences among means ( $p<0.05$ ).

257

### 258 **3. Results and discussion**

#### 259 *3.1 Effect of PEF on microstructure and conductivity of tomato fractions*

260 Fig. 2 shows the microstructure of untreated and PEF+HEAT treated tissue, cells clusters, single  
261 cells and chromoplasts. In tomato tissue, carotenoids were dispersed within the cells glued together  
262 through the middle lamella (Moelants, Cardinaels, Van Buggenhout, Van Loey, Moldenaers, &  
263 Hendrickx, 2014). The isolation procedure allowed to obtain in the cells clusters fraction a mixture  
264 of intact and broken cells and debris with carotenoids homogenously dispersed, while in single cells  
265 intact membranes can be observed. In chromoplasts fraction, a single thin membrane layer  
266 enveloped carotenoid crystals (Jeffery, Holzemburg, & King, 2012). PEF+HEAT caused cell  
267 detachment in tomato tissue, while in single cells, a large number of cells were damaged. In cells  
268 clusters and chromoplasts, no visual differences were found among untreated and PEF+HEAT  
269 treated samples. No microstructural differences were found among the samples subjected to  
270 PEF+HEAT, PEF and HEAT (data not shown).

271 To understand the effect of PEF+HEAT, PEF and HEAT on modification of cell membranes,  
272 conductivity in a frequency range between 1 kHz and 1 MHz was measured. Fig. 3 shows the  
273 conductivity spectra of untreated and PEF+HEAT treated tomato tissue as well as those relevant to  
274 samples subjected to PEF and HEAT treatments. In untreated tomato tissue low conductivity values  
275 were found at low frequency because the cell membrane acts as capacitor, preventing the current  
276 electric flow into the medium. As expected, by increasing the frequency, higher conductivity values  
277 were found because the cell membrane became less resistant to the current flow applied during the  
278 conductivity measurement. Upon PEF+HEAT, PEF and HEAT treatments, high conductivity values  
279 were found in the whole frequency range, indicating a modification of the membrane permeability.  
280 In particular, at low frequency the higher conductivity level indicated the irreversible membrane

281 electroporation (Donsì et al., 2010). However, no significant differences ( $p>0.05$ ) in conductivity  
282 were found among the treated samples. In order to obtain an indication of the proportion of  
283 permeabilized cells due to treatments, the disintegration index ( $Z_p$ ) was calculated (Angersbach,  
284 Heinz, & Knorr, 1999). Upon PEF+HEAT,  $Z_p$  reached the value of 0.8, indicating that most of the  
285 cells membrane were damaged. Similar  $Z_p$  values were found for PEF and HEAT (data not shown).  
286 Thus, although these results give an indication that cell membrane modification actually occurred as  
287 a consequence of processing, they do not allow to discriminate among the different cell damages,  
288 which have been described in the literature. For instance, it has been reported that PEF treatments  
289 cause membrane pores formation, while thermal treatments induce pectin depolymerisation in the  
290 cell wall and disruption of cell membrane (Moelants et al., 2014; Zimmermann, 1986).  
291 In cells clusters, single cells and chromoplasts, the conductivity did not increase upon the treatments  
292 as compared to the untreated counterparts (data not shown). It is likely that samples preparation  
293 steps (e.g. blending and heating at 90 °C for 30 min) have already damaged cell membranes,  
294 causing an increase in conductivity to a maximum value. Therefore, no further conductivity  
295 increase was found for the treated tomato fractions, although membrane damage can be  
296 hypothesised as occurring under our process conditions. In fact, according to Zhang, Chang and  
297 Barbosa-Cánovas (1994), membrane electroporation depends not only on process parameters but  
298 also on cell size. Moreover, Janositz and Knorr (2010) found that PEF treatments at 0.25 to 7.5  
299 kV/cm caused electroporation of tobacco cells, which are characterized by cell size similar to  
300 chromoplasts (~ 10 $\mu$ m).

301

### 302 *3.2 Effect of PEF on carotenoids concentration and bioaccessibility*

303 In order to understand the effect of PEF+HEAT treatment on bioactive compounds, carotenoids  
304 concentration and bioaccessibility were determined. Table 1 shows  $\beta$ -carotene and all-*trans*  
305 lycopene concentrations of untreated and treated tomato fractions. It can be observed that the  
306 application of PEF+HEAT did not change  $\beta$ -carotene concentration in tomato tissue, cells clusters

307 and isolated cells. However, a decrease of  $\beta$ -carotene concentration was found upon PEF or HEAT  
308 in cells clusters and single cells, likely due to oxidation and isomerization phenomena favoured by  
309 electrochemical reactions and metal release (Morren, Roodenburg, & de Haan, 2003; Pataro,  
310 Falcone, Donsi, & Ferrari, 2014; Roodenburg, Morren, Berg, & de Haan, 2005). By contrast, no  
311 significant differences ( $p>0.05$ ) in all-*trans* lycopene concentration were found in tissue, cells  
312 clusters and single cells upon the application of the above technologies, probably due to the higher  
313 stability to oxidation and isomerization phenomena of lycopene compared to  $\beta$ -carotene (Lemmens,  
314 Tchuente, Van Loey, & Hendrickx, 2013; Seybold, Fröchlich, Bitsch, Otto, & Böhm, 2004).  
315 These results are in agreement with the literature in the framework of PEF and heating. In  
316 particular, PEF treatment at 3-7 kV/cm did not affect the lycopene concentration in tomato tissue  
317 (Luengo et al., 2014). Similarly, temperature below 100 °C did not induce lycopene isomerization  
318 and degradation in tomato derivatives (Nguyen & Schwartz, 1998; Colle et al., 2010a). It is  
319 noteworthy that under our experimental conditions, temperature never exceeded 90 °C. In  
320 chromoplasts fraction, HEAT caused a decrease of about 21% in  $\beta$ -carotene concentration, while no  
321 changes were found in all-*trans* lycopene concentration. By contrast, both  $\beta$ -carotene and all-*trans*  
322 lycopene concentrations decreased by 47% and 36%, respectively, upon the application of  
323 PEF+HEAT or PEF. Such a decrease that is attributable to carotenoids oxidation and/or  
324 isomerization could be favoured by the higher susceptibility of carotenoids, enveloped in a thin  
325 membrane layer, towards oxygen permeability, metal release by the electrodes and electrochemical  
326 reaction occurring during PEF treatment (Jeffery et al., 2012; Morren et al., 2003; Pataro et al.,  
327 2014; Roodenburg et al., 2005). In order to understand the effect of PEF+HEAT, PEF and HEAT on  
328 the functional properties of the tomato fractions, carotenoids bioaccessibility was investigated.  
329 Carotenoids bioaccessibility indicates the carotenoids fraction released from the matrix and  
330 incorporated into micelles during the *in vitro* digestion. In our study, bioaccessibility was calculated  
331 as the ratio of the carotenoids incorporated into micelles after the digestion to the initial carotenoid  
332 concentration in the same processed sample. Fig. 4 shows  $\beta$ -carotene and all-*trans* lycopene

333 bioaccessibility in tomato fractions upon PEF+HEAT, PEF and HEAT. In untreated tissue, cells  
334 clusters and single cells the  $\beta$ -carotene and all-*trans* lycopene bioaccessibility ranged between 2%  
335 and 5%, in agreement with the literature (Palafox-Carlos et al., 2011; Palmero et al., 2013). As  
336 expected, lower  $\beta$ -carotene and all-*trans* lycopene bioaccessibility values were found in untreated  
337 tissue, cells clusters and single cells than in chromoplasts. It can be inferred that intact cell wall  
338 polysaccharides may prevent the digestive enzymes, bile salts and surfactants (i.e. phospholipids)  
339 reaching the bioactive compounds within the chromoplasts and hampered micelle formation during  
340 intestinal step (Palafox-Carlos et al., 2011). Moreover, the higher carotenoids bioaccessibility in  
341 tissue compared to cells clusters and single cells could be attributed to the presence of chromoplast  
342 leaked from the broken cells along the surface during the preparation step (i.e. cutting). Moreover,  
343 from Fig. 4A it can be also observed that in tissue  $\beta$ -carotene bioaccessibility did not change upon  
344 PEF+HEAT, PEF and HEAT. It is likely that, although the treatments caused the modification of  
345 cell permeability (Fig. 1), the presence of multiple encapsulation barriers is enough to prevent the  
346 carotenoids release (Palafox-Carlos et al., 2011). All-*trans* lycopene bioaccessibility in tomato  
347 tissue were not affected by PEF, in agreement with Jayathunge et al. (2017), while a significant  
348 decrease was found in PEF+HEAT and HEAT treated samples. It can be inferred that heating  
349 induced the formation of a barrier consisting of cell wall and cell membrane that hinders the  
350 lycopene release (Palmero et al., 2014). Differences in  $\beta$ -carotene and all-*trans* lycopene  
351 bioaccessibility upon the treatments can be attributable to their different molecular structure. In fact,  
352 the linear isoprenoid chain confers to lycopene lower solubility capacity compared to  $\beta$ -carotene and  
353 thus lower bioaccessibility (Tyssandier, Lyan, & Borel, 2001; Van het Hof, West, Weststrate, &  
354 Hautvast, 2000).

355 In cells clusters and single cells (Fig. 4B and 4C),  $\beta$ -carotene and all-*trans* lycopene  
356 bioaccessibility did not change upon PEF+HEAT, PEF and HEAT. So we concluded that these  
357 treatments did not affect the carotenoids bioaccessibility due to the presence of cell wall  
358 polysaccharides in cells clusters and cell barriers induced by thermal treatment during samples

359 preparation in isolated cells (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011; Palmero et  
360 al., 2014). To understand the role of membranes, carotenoids bioaccessibility was studied in the  
361 chromoplasts fraction (Fig. 4D), which has a single membrane layer. In untreated chromoplasts  
362 fraction,  $\beta$ -carotene and all-*trans* lycopene bioaccessibility ranged between 16% and 20%. The  
363 application of PEF+HEAT caused a significant reduction of carotenoids bioaccessibility to 5%.  
364 Similar results were obtained for the PEF treated chromoplasts. On the contrary, HEAT did not  
365 cause significant modifications ( $p>0.05$ ) of carotenoids *in vitro* bioaccessibility, in agreement with  
366 Colle et al. (2010a).

367 The reduction in carotenoids bioaccessibility at chromoplasts level is attributable to the effect of  
368 PEF rather than to HEAT. This decrease in the bioaccessibility might suggest that PEF induced  
369 modifications not only in the membrane but also in the carotenoids-protein complexes. In fact, it is  
370 well known that carotenoids are tightly bound to subcellular lipids and binding proteins within the  
371 chromoplast structure (Faulks & Southon, 2005) and that complexes constitute a further structural  
372 barrier for carotenoids bioaccessibility. Moreover, Perez and Pilosof (2004) found that PEF  
373 treatment can induce modification in protein conformation. Although further research is needed,  
374 these results clearly show a role of PEF on carotenoids bioaccessibility.

375

#### 376 **4. Conclusions**

377 The results of this study show that PEF treatment applied individually or in combination with  
378 heating induced changes of the microstructure and membrane permeability of tomato fractions.  
379 These treatments caused slight or no changes in  $\beta$ -carotene concentration and bioaccessibility in  
380 tissue. Similarly, all-*trans* lycopene concentration and bioaccessibility did not change upon PEF. A  
381 decrease of all-*trans* lycopene bioaccessibility upon PEF+HEAT and HEAT was found, suggesting  
382 the formation of a barrier that hinders carotenoids release. In cells clusters and single cells, slight or  
383 no changes were found in carotenoids concentration and bioaccessibility. On the contrary, in  
384 chromoplasts both  $\beta$ -carotene and all-*trans* lycopene concentration and bioaccessibility significantly

385 decreased upon PEF with or without heating due to PEF related phenomena. This effect was  
386 attributed to the comparatively higher susceptibility of the chromoplasts fraction towards PEF  
387 treatment compared to tomato fractions which possess further structural barriers. It can be  
388 concluded that the effect of PEF on carotenoids bioaccessibility strongly depends on the vegetable  
389 structure complexity and presence of physical barriers naturally present or induced by the process  
390 that impede the carotenoids release. The results of this study clearly indicate individual PEF can be  
391 applied to tomato based products, mainly constituted by tissue, cells clusters and single cells,  
392 without impairing carotenoids functionality.

393

#### 394 **Conflict of interest**

395 Hennie Mastwijk has been involved as a consultant in the high voltage engineering of the NP110-60  
396 pulsed electrical field system.

397

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401

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538 **Figure captions**

539 **Fig. 1.** Experimental set-up.

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541 **Fig. 2.** Micrographs of untreated and PEF+HEAT treated tissue, cells clusters, single cells and  
542 chromoplasts fractions obtained from tomatoes.

543

544 **Fig. 3.** Conductivity (S/m·g) spectra of tomato tissue subjected to combined pulsed electric fields  
545 and heating (PEF+HEAT), pulsed electric fields (PEF) and heating (HEAT) only. Data relevant to  
546 untreated samples are also shown.

547

548 **Fig. 4.**  $\beta$ -carotene (■) and all-*trans* lycopene (□) bioaccessibility (B/C %) in tissue (A), cells  
549 clusters (B), single cells (C) and chromoplasts (D) subjected to combined pulsed electric fields and  
550 heating (PEF+HEAT), pulsed electric fields (PEF) or heating (HEAT) only. Data relevant to  
551 untreated samples are also shown.

552 <sup>a, b</sup> : Means with different letters within each tomato fraction indicate significant differences  
553 ( $p < 0.05$ ) for  $\beta$ -carotene.

554 <sup>a', b'</sup> : Means with different letters within each tomato fraction indicate significant differences  
555 ( $p < 0.05$ ) for all-*trans* lycopene.

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564 **Table 1.**  $\beta$ -carotene and all-*trans* lycopene concentrations ( $\mu\text{g/g dw}$ ) in tomato tissue, cluster cells,  
 565 single cells and chromoplasts subjected to combined pulsed electric fields and heating  
 566 (PEF+HEAT), pulsed electric fields (PEF) or heating (HEAT) only. Data relevant to untreated  
 567 samples are also shown.

568

| <b>Concentration (<math>\mu\text{g/g dw}</math>)</b> |                  |                     |                            |
|--|------------------|---------------------|----------------------------|
| <b>Sample</b>  | <b>Treatment</b> | $\beta$ -carotene   | all- <i>trans</i> lycopene |
| Tissue   | Untreated        | $32.2 \pm 5.2^a$    | $647.6 \pm 65.9^{a'}$      |
|  | PEF+HEAT         | $40.6 \pm 3.1^a$    | $557.1 \pm 58.1^{a'}$      |
|  | PEF              | $26.5 \pm 2.3^a$    | $463.8 \pm 53.2^{a'}$      |
|  | HEAT             | $25.9 \pm 2.2^a$    | $588.2 \pm 51.0^{a'}$      |
| Cells cluster  | Untreated        | $32.2 \pm 1.3^a$    | $346.8 \pm 47.8^{a'}$      |
|  | PEF+HEAT         | $30.4 \pm 0.9^a$    | $345.5 \pm 5.6^{a'}$       |
|  | PEF              | $16.9 \pm 0.5^c$    | $395.3 \pm 2.8^{a'}$       |
|  | HEAT             | $24.2 \pm 0.4^b$    | $304.3 \pm 1.0^{a'}$       |
| Single cells   | Untreated        | $75.7 \pm 4.2^a$    | $839.4 \pm 119.4^{a'b'}$   |
|  | PEF+HEAT         | $68.4 \pm 6.4^{ab}$ | $825.9 \pm 134.2^{a'b'}$   |
|  | PEF              | $52.6 \pm 0.7^{bc}$ | $1096.8 \pm 31.7^{a'}$     |
|  | HEAT             | $45.5 \pm 3.1^c$    | $569.8 \pm 69.1^{b'}$      |
| Chromoplasts   | Untreated        | $341.6 \pm 5.9^a$   | $2970.1 \pm 44.0^{a'}$     |
|  | PEF+HEAT         | $187.3 \pm 7.7^c$   | $1881.7 \pm 43.9^{b'}$     |
|  | PEF              | $255.8 \pm 18.4^b$  | $2191.7 \pm 227.2^{b'}$    |
|  | HEAT             | $283.6 \pm 1.3^b$   | $2794.1 \pm 46.3^{a'}$     |

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570 <sup>a, b</sup> : means with different letters within each tomato fraction indicate significant differences  
 571 ( $p < 0.05$ ) for  $\beta$ -carotene.

572 a', b' : means with different letters within each tomato fraction indicate significant differences  
573 (p<0.05) for all-*trans* lycopene.