The Effect of Novel Processing Technologies on the Bioaccessibility and Caco-2 Cell Uptake of Carotenoids from Tomato and Kale-based Juice

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Abstract

This research aimed to measure the impact of novel food processing techniques, i.e. pulsed electric field (PEF), ohmic heating (OH), and high-pressure processing (HPP), on carotenoid bioaccessibility and Caco-2 cell uptake from tomato juice, and HPP and PEF on the same attributes from kale-based juices, as compared with raw (non-processed) and conventional thermal treated (TT) juices. Lycopene, β-carotene, and lutein were quantitated in juices and the micelle fraction using HPLC-DAD, and in Caco-2 cells using HPLC-MS/MS. *Tomato juice results:* PEF increased lycopene bioaccessibility by 150%, but reduced β-carotene bioaccessibility by 44%, relative to raw juice. All processing methods increased lutein uptake. *Kale-based juice results:* TT and PEF degraded β-carotene and lutein in the juice. No difference in bioaccessibility or cell uptake were observed. Total delivery of lycopene, β-carotene, and lutein was independent of type of processing. Taken together, PEF and OH enhanced total lycopene and lutein delivery from tomato juice to Caco-2 cells just as well as TT, and may produce a more desirable product due to other factors (i.e. conservation of heat-labile micronutrients, fresher organoleptic profile). HPP best conserved carotenoid content and color in kale-based juice, and merits further consideration.

Keywords: tomato juice, kale-based juice, pulsed electric field, ohmic heating, high pressure processing, thermal processing, carotenoids, lycopene, β-carotene, lutein, bioaccessibility, Caco-2 cell uptake

Introduction

Food preservation technology is used to ensure food safety, and for extending shelf life, during storage, retail, and delivery. Conventional thermal treatment has been the standard used for years to pasteurize processed foods. Unfortunately, heat treatment at 90-95 °C can also damage heat-labile vitamins, flavor, color, and texture[1,2]. In contrast, novel processing technologies aim to deliver the same extended shelf-life (or shelf-stable) product with improved nutrient and sensory profiles[3]. Some of these novel processing methods include pulsed electric field (PEF) and ohmic heating (OH), which use different proportions of electric fields and heat to inactivate microorganisms, while high-pressure processing (HPP) uses hydrostatic pressure to produce a pasteurization effect[4]. Notably, the non-thermal methods (HPP and PEF) by themselves, are incapable of inactivating bacterial spores without the use of heat, and thus are not suitable for foods of pH > 4.6, for which commercial sterilization is a necessity. OH, by contrast is able to do so because of the resultant heating effect.

Phytochemicals are plant secondary metabolites that may have biological activities in humans. Multiple studies of novel processing techniques have determined the effect of these technologies on nutrient and phytochemical content[2,5,6]. OH and PEF have been shown to preserve the bright color[7], flavor[6] compounds, and viscosity[7] of tomato products. HPP has also been shown to preserve carotenoids and vitamin C with tomato products and green vegetables[8,9].

These processing technologies have also been studied for their ability to create lipid-soluble phytochemicals which are more bioaccessible[10,11] (i.e. the transfer of carotenoids and fat-soluble vitamins to mixed micelles, a necessary preliminary step for absorption). *In vitro* studies have demonstrated that PEF without heat increases lycopene bioaccessibility up to 40% as compared to raw tomato juice[12–14]. Combination techniques,

like TT + PEF[12] and TT + HPP[11] can improve total lycopene bioaccessibility from tomatoes up to 238% relative to raw juice[12]. HPP without added heat increases the bioaccessibility of lutein in green beans by 20%, but did not affect the bioaccessibility of lutein in broccoli[5,15].

To the best of our knowledge, no studies have observed the effect of OH on nutrient or phytochemical bioaccessibility from any food. Furthermore, previous studies of carotenoid bioaccessibility after PEF and HPP treatment used raw juice as a negative control[9,12,14]. Because the carotenoids found in tomato products are largely conserved during traditional thermal processing (TT)[10,16], and multiple studies have demonstrated the ability of TT to improve carotenoid bioaccessibility and bioavailability (i.e., release into the simulated or actual blood compartment)[17,18], comparison with TT as a positive control is warranted. A great number of commercially available green vegetable juices are produced using HPP, and thus we chose to compare HPP (instead of OH) with TT and PEF treatment of kale-based juice. Finally, no studies have investigated the effects of PEF, HPP, and OH on the second step of carotenoid absorption in the intestine-delivery to small intestinal cells. Thus, we set out to determine the impact of PEF, OH, HPP on the bioaccessibility and Caco-2 human intestinal cell delivery of lutein, lycopene and β-carotene following *in vitro* digestion of a tomato juice (pH 3.9) and a kale-based juice (pH 3.9). The effects of these novel technologies were compared to a modeled conventional TT and raw juice (RAW).

Material and Methods

Chemicals and reagents

Kale, olive oil, and 100% frozen concentrated apple juice were purchased from a local supermarket (Columbus, Ohio). Porcine pancreatin (Lot SLBT2247), pepsin (Lot SLBR2349V), lipase (Lot SLBS3971), and bile extract (Lot SLBT0867) were purchased

from Sigma Aldrich (St. Louis, MO). Dulbecco's Modified Eagle's Medium with high glucose, L-glutamine and sodium pyruvate were produced by Caisson labs (Smithfield, UT). Fetal bovine serum, non-essential amino acid solution (NEAA), penicillin-streptomycin, gentamicin, and phosphate-buffered saline (PBS) were purchased from Fisher Scientific (Waltham, MA). Malt extract agar for microbiology was purchased from Sigma Aldrich (St. Louis, MO). Lycopene (*all-trans*-lycopene, ≥85% pure) and chlorophyll a and b standards (≥ 90% pure) were purchased from Sigma Aldrich (St. Louis, MO). Lutein (*all-trans*-lutein, ≥95% pure) and β-carotene (*all-trans*-β-carotene, ≥95% pure) were purchased from Cayman Chemicals (Ann Arbor, MI). HPLC grade methanol, hexane, acetone, dichloromethane (DCM), methyl-tert-butyl-ether (MTBE), and formic acid (98% pure) were purchased from Fisher Scientific (Waltham, MA).

Juice preparation

Tomato juice - Tomatoes (variety) were grown at Ohio State University North Central Agricultural Research Station near Fremont, Ohio and were mechanically harvested at breaker stage. Tomatoes were processed in the pilot plant of the Ohio State University Food Industries center. Fruit was sorted and washed in a tank with warm water to remove any field dirt, chopped in a pulper (Model CLE-360-D28, Chisholm-Ryder Co., fitted with a 1/64" screen, Niagara Falls, NY) into coarse particles that were further filtered through a mesh of 0.23 cm to separate juice from skin and seeds. Tomato juice was then aliquoted in plastic bags and kept frozen at – 20 ºC prior to use. Before processing, tomato juice was thawed with cold running water. Lemon juice from concentrate was added to reduce pH to 3.9, as preliminary experiments revealed that no PEF treatments were capable of inactivating *B. coagulans* in juice with a pH = 4.3 (data not shown). After pH adjustment, the juice was degassed under vacuum. Conductivity, pH and °Brix of the RAW juice were measured before treatment.

116 *Kale-based juice* - Kale leaves (125 g) were blended using an Oster 6843 blender on the
117 highest speed for 2 min, with 1L deionized water then filtered with a 0.23 cm mesh screen.
118 Frozen apple juice concentrate was mixed with the filtered kale juice (1 v:9 v), and lemon
119 juice added to adjust the pH to 3.9. Aliquots (100 mL) aliquots were measured into
120 individual plastic bags and stored at -20 °C until processing.
121 *Juice processing experiments*
122 *Pulsed Electric Field (PEF)* - PEF treatment was carried out using a continuous-flow
123 bench-scale system that provides square-wave pulses (OSU-4C, The Ohio State
124 University., Columbus, OH, USA). Four collinear chambers were serially connected with
125 each chamber consisting of two stainless steel electrodes separated by 0.29 cm with a
126 treatment volume of 0.012 cm^3. The flow rate was adjusted to 80 mL/min using a gear
127 pump (Pump head 74012-51, Drive unit 75211-10; Cole-Palmer Instrument Company,
128 Vernon Hills, IL, USA). A cooling coil was submerged into an ice-water bath and the
129 inlet and outlet temperatures of each pair of chambers were kept below 35 ºC. Treatment
130 temperature and time were monitored using a data logger (Campbell scientific 21X). A
131 digital oscilloscope was used to monitor the pulse waveform, peak voltage and intensity
132 of current (Tektronix TDS3014B, Tektronix Inc., Oregon, USA). A pulse generator
133 controlled the pulse width and frequency (Pulse Generator 9612 model, Quantum
134 Composers, Inc., Bozeman, MT, USA). Tomato juice was treated at 35 kV/cm for 1000
135 μs using 2 μs bipolar pulses at a frequency of 250 Hz, providing an energy input of 6860
136 MJ/m^3. Kale-based juice was treated at 35 kV/cm for 1000 μs using 2 μs bipolar pulses
137 at a frequency of 500 Hz, providing an energy input of 4655 MJ/m^3. With risk of *B.*
138 *coagulans* growth eliminated due to juice pH of 3.9, the electric field, pulse width, and
139 frequency conditions were selected based on the maximum values that could be applied
140 to the tomato and kale-based juices due to sample and equipment limitations, in addition

141 to those conditions which maximized total carotenoid bioaccessibility in tomato juice
142 from previous pilot studies (unpublished data from parameters also used in Ref. 19[19]).
143
144 *Ohmic heating (OH)* - Ohmic heating was performed in a treatment cell connected to an
145 AC power source (public supply) and a voltage control unit. Tomato juice was placed in
146 a treatment cell composed of two platinized-titanium electrodes separated by 4.8 cm. A
147 magnetic stirrer was placed into the cell to ensure that the juice was homogeneously
148 treated. Three thermocouples were inserted to the top of the cell and temperature was
149 monitored using a data logger (Campbell Scientific 21X). The same data logger was used
150 to record the treatment time, voltage, and current. Treatment temperature was controlled
151 by adjusting the input voltage during the holding time. Once treated, tomato juice samples
152 were rapidly cooled down by pulling the treatment cell into an ice-water bath. Ohmic
153 conditions were set up at 60 Hz and 13 V/cm based on the study of Somavat et al.[20].
154 Treatment time and temperature were set to 90 ºC and 30 s, based on typical juice
155 pasteurization conditions, which vary from 90 to 95 ºC for 15 to 60 s[4]. The average come-
156 up time was 8 min and 13 s. The OH treatment conditions were selected to ensure a 5-log
157 reduction in *B. coagulans*, following previously published literature[20].
158
159 *Thermal treatment (TT)* - For processing using traditional thermal treatment, the tomato
160 or kale-based juice was held in a Pyrex bottle and placed into an oil bath (~ 93 ºC). After
161 the holding time, the Pyrex bottle was transferred to an ice-water bath to cool down the
162 tomato juice sample. Treatment time and temperature were measured using three
163 thermocouples and monitored with a data logger (Campbell Scientific 21X). To achieve
164 a uniform temperature, the juice was continuously homogenized with a magnetic stirrer.
165 Tomato and kale-based juice were processed at 90 ºC for 30 s as a conventional

166 pasteurization treatment[20]. The average come up time of the tomato juice was 12 min 48
167 s, and the kale-based juice was 11 min 10 s. The TT treatment conditions were selected
168 to ensure a 5-log reduction in *B. coagulans*, following previously published literature[20].
169
170 *High Pressure Processing (HPP)* - A custom laboratory-scale hydrostatic pressure system
171 (model 26190, Harwood Engineering, Wapole, MA, USA) was used. This equipment
172 provides up to 1000 MPa of pressure coupled with a pressure intensifier (model SA-10-
173 6-.875FGD-150 K, Harwood Engineering, Wapole, MA, USA). Pressure transmitting
174 medium was propylene glycol (food-grade, 0.6 m^3/m^3, SAFE-T-THERM, Houghton Intl.
175 Inc., Valley Forge, PA, USA). Pressurization rate was approximately 14 MPa/s. Kale-
176 based juice was held in a cylindrical vessel of a nominal diameter and length of 2.54 cm
177 and 15.24 cm, respectively. Thermocouples were inserted to the top closure to monitor
178 the treatment temperature. Treatment time and pressure data was collected and monitored
179 using a data logger (Agilent 34970A). Kale-based juice samples were frozen and kept at
180 – 80 ºC. HPP treatment was conducted at 500 MPa for 3 min, and temperature did not
181 exceed 35 ºC. Processing conditions were selected on typical high-acid juice HPP-
182 pasteurization according to industry recommendations. HPP-treatment conditions of 500
183 MPa / 2 min were sufficient to reach a 5 log reduction of *E. coli* ATCC 117755 in apple
184 juice[21].
185 *Yeast and Molds* - RAW and PEF processed juices were taken and cultured in triplicate.
186 Endogenous yeasts & molds were cultured on malt extract agar medium acidified at pH
187 4.0 with tartaric acid, and incubated at 25 °C for 72 h.
188 *In vitro gastrointestinal digestion*
189 Tomato juice and kale-based juice were digested following a static *in vitro* procedure
190 according to Garrett et al.[22] with a few modifications. Juice (10 mL) was mixed with olive

191 oil (47.6 μL), using a ratio based on the tomato juice particle phase volume (i.e. 4% of
192 the solid phase mass of the juice was added as oil). Simulated digestion of the gastric and
193 intestinal phase was followed as described detail in Garrett et al.[22]. The aqueous fraction
194 was passed through a sterile 0.22 μm syringe filter to obtain the mixed micelle fraction.
195 An aliquot of this fraction was flushed with argon gas and stored at -80 ºC until further
196 analysis. A second aliquot was used for the Caco-2 cell uptake experiments.
197 The impact of *in vitro* digestion procedure was examined on the recovery of carotenoids
198 from the un-digested juice in the digested chyme. Tomato juices were diluted (1:4, *v*/*v*)
199 using salt solution in the *in vitro* digestion. The carotenoids in both the digested chyme
200 and diluted tomato juice were extracted and analyzed using the method motioned below.
201 The recovery rate for the carotenoids was 1.19± 0.2%, indicating that these carotenoids
202 were not destroyed during the digestion procedure.
203 The bioaccessibilities of the carotenoids were expressed as the fraction of carotenoids in
204 the juice (both tomato and kale-based) that were micellarized (%), relative to the initial
205 carotenoid concentration in the RAW juice sample used for that replicate.
206 *Cellular uptake of bioaccessibile carotenoids*
207 The Caco-2 cellular model described by Garrett *et al.*[22] was used to study the apical
208 uptake of carotenoids. Caco-2 cells (HTB 37) were obtained from the American Type
209 Cell Culture Collection (Rockville, MD) and grown and differentiated in T75 flasks at 37
210 ºC in a humidified atmosphere of 95% air: 5 % CO_2. The complete DMEM (cDMEM)
211 used during cell growth contained 15 % FBS and the media was changed every second
212 day. Once confluent, the serum content was decreased to 7.5 %. Differentiated
213 monolayers were used at passage 27 at 13 days post-confluency. Complete medium was
214 changed every other day and medium was changed 24 h before cell uptake study. The
215 filtered aqueous fraction of the freshly-prepared digesta was mixed with basal DMEM

(1:3) to prepare the test media. 12.5 mL of test media was gently mixed and added to wash monolayers. Caco-2 cell cultures were incubated at 37 ºC for 4 h in cell culture condition. After complete 4h incubation, the monolayer was washed once with cold PBS containing 2 g/L albumin and twice with cold PBS. Cells were harvested in cold PBS and placed in a 15 mL test tube. Tubes were centrifuged (894 × *g* for 10 min) and the supernatant was removed from the cell pellet. Tubes were flushed with argon gas, sealed and stored at -80 ºC until analysis.

Carotenoid and chlorophyll extraction and analysis

From Juice - Carotenoids (lycopene, β-carotene, lutein) and chlorophylls (a and b) were extracted from food as described previously[23] with modifications. Instead of using probe sonicator, a multivortex (IKA VIBRAX VXR basic, IKA Works, Inc., NC, level 1500) was used for organic extraction. Hexane/acetone (1:1, *v/v*, 5 mL) was added to the remaining pellet and vortexed for 5 min. Final aliquots (2 mL) were dried under argon gas and stored at -80 °C until analysis.

Mixed Micelle Fraction - Carotenoids and chlorophylls in the filtered aqueous fraction prepared from chyme were extracted using the method described previously[24].

Caco-2 cells - Carotenoid and chlorophyll extraction from the monolayers was published by Kopec. et al.[25]. The final extract was dried under argon gas and stored at -80 °C until analysis.

HPLC-DAD and HPLC-MS/MS analysis

Samples were dissolved in MTBE/MeOH (1:1) and 20 µL was injected onto a YMC C30 column for analysis. Carotenoids were separated using a 150 × 4.6 mm column (3 µm particle size) and chlorophylls were separated using a 250 × 4.6 mm column (5 µm particle size). All separations were conducted on a 1200 series HPLC (Agilent, Inc. Santa Clara, CA). For tomato juice carotenoid analysis, mobile phase A (MeOH/H_2O, 80:20,

v:v, with 0.1% formic acid) and mobile phase B (MTBE/MeOH/ H_2O, 78:20:2, *v:v*, with 0.1% formic acid) were used with a gradient as follows: 0% B at 0 min, linearly increasing to 100% B over 16 min, and returning and holding at 0% B for 2 min, with a flow rate of 1.2 mL/min and column temperature at 40 °C. Chlorophylls a and b were analyzed based on a modified LC method published by Ferruzzi et al.[26]. The gradient began with 30% B, linearly increasing to 80% over 15 min, before linearly increasing to 100% B over 5 min, and returning to 30% band holding over 5 min. A constant flow rate of 1.5 mL/min and column temperature of 35 °C were used and 10µL was injected. Identification and quantification of β-carotene, lutein, lycopene, chlorophyll a and b were based on external calibration curves made using commercial standards. Quantification of phytoene and phytofluene was made using the lycopene calibration curve, considering their relative response at their λ_{max} (287 and 350, respectively), adjusted for for differences in their molar extinction coefficients. Due to limited sensitivity of the PDA, lutein, β-carotene, and lycopene in Caco-2 cell extracts were analyzed by HPLC-MS/MS (Quantiva, Thermo Scientific) using the method reported previously by Kopec et al.[25], with the following source parameters: an APCI probe operated in positive ion mode, ion transfer tube = 300 °C, vaporizer temperature = 300 °C, sweep gas =1, auxillary gas = 5, sheath gas = 45, spray current = 5.3 µA, collision gas = 1.5 mTorr, Q1 resolution = 0.7, Q3 resolution = 0.7. The ion transitions monitored for carotenoids are shown in **Table 1**. Bioaccessibility, cell uptake and total carotenoid delivered were calculated as follows:

Bioaccessibility *(%)* = $\frac{micellerized\ compound\ (nmol)}{amount\ in\ juice\ (nmol)} \times 100\%$

Cell uptake *(%)* = $\frac{amount\ detected\ in\ cell\ (nmol)}{micellerized\ amount\ in\ medium} \times 100\%$

Carotenoid delivered *(mg/100g)* = $\frac{\text{amount detected in cell (nmol)}}{\text{amount in juice (nmol)}} \times carotenoid\ in\ juice\ (mg/100g)$

Statistics

Data were analyzed with R studio Version 1.1.456 (Boston, MA)[27]. Tomato juice processing was replicated three times for each processing type (i.e. PEF, OH, TT). Each replicate was digested once, and each digesta was used in one of the T75 flasks for the cell uptake experiment, resulting in each experiment conducted in triplicate. One-way ANOVA was conducted, followed by pairwise comparisons for the means of groups which were significantly different using Tukey's post-hoc test. A P <0.05 was considered statistically significant.

3. Results and Discussion

Yeasts and Molds

No previous studies were found regarding the pasteurization effect of the PEF-selected parameters in tomato juice, thus a microbiological analysis was conducted. A juice of pH 3.9 is known to be too low for *B. coagulans* survival, and thus yeast and mold count was performed instead. The raw tomato juice had 3.70 log CFU/mL of colony forming units, and the analysis, performed in triplicate, showed that the PEF treatment inactivated the tomato juice endogenous yeasts and molds to a level below the detection limit (see **Figure 1S** in Online Supplemental Material).

Effect of processing on the concentration of carotenoids in tomato and kale-based juice

At 25 ºC, the conductivity (S/m) of tomato juice adjusted to pH 3.9 was 0.56, and the soluble solids content (ºBrix) was 5.23. Color values of the pH adjusted RAW juice were L =33.92, a = 18.56, and b = 9.11, respectively. The kale-based juice adjusted to pH 3.9 had a conductivity (S/m) at 25 ºC of 0.38 ± 0.0 and the ºBrix was 8.17 ± 0.06.

Tomato Juice - The concentration of carotenoids in processed and non-processed tomato juices are shown in **Table 2.** Relative to the RAW juice, TT, OH, PEF processing did not significantly alter the concentration of total lycopene, *all-trans*-lycopene, *5-cis*-lycopene, other-*cis*-lycopene, β-carotene, lutein, phytoene and phytofluene (P> 0.05).

290 This result coincides with previous studies which have demonstrated that lycopene is
291 stable after TT, i.e. blanching at 90 °C[10,19,28], and heat treatment of up to 30 min increases
292 lycopene recovery[29]. Because lycopene in tomatoes is stored as needle-shape crystalized
293 particles in chloroplasts[30], the physical-chemical structure is believed to confer stability
294 during TT. These results also confirm those of OH treated tomato juice and grapefruit
295 juice, where no change in the lycopene content was observed[31,32]. There was a non-
296 significant trend towards increased lycopene concentration after PEF processing
297 compared to TT (P= 0.086). Previous work by Odriozola-Serrano *et al*. reported that the
298 lycopene content in tomato juice was increased after PEF treatment (35 kV/cm, 100 Hz,
299 1500 µs)[28] than raw juice and TT (90 °C, 30s or 60s). This may have resulted from longer
300 treatment in the former study compared to the current study (1500 µs vs 1000 µs).
301 Moreover, the lower field strength might have also increased lycopene synthesis by
302 endogenous enzymes (further discussed below).
303 The percent of lycopene isomerized in this study is shown in **Table 2** and **Figure 3A**.
304 Total *cis*-lycopene and *all-trans*-lycopene was significantly increased after PEF
305 processing, relative to the TT juice (P = 0.029 and 0.04, respectively), but no changes
306 were observed between RAW, TT, or OH processed tomato juice in (**Table 2**, $P > 0.05$).
307 A previous study with tomato juice treated with milder PEF conditions (i.e. lower field
308 strength and lower voltage) found an increase in *all-trans*- and *other-cis*-lyocopene
309 isomers (but not 5-*cis*-lycopene)[14], suggesting that PEF may increase synthesis of
310 carotenoids in this juice during processing, potentially via activation of carotenoid
311 desaturase and isomerase triggered by a tissue stress response. This study also showed
312 that the isomerization occurred in tomato fruit processed at a higher temperature[16] and a
313 higher frequency PEF in the tomato[14], relative to the conditions used for the study
314 reported herein. The phytoene concentration was non-significantly decreased during the

315 TT treatment relative to RAW (*P*= 0.072), but no trend was observed with other
316 processing methods, suggesting that the treatments did not activate enzymes earlier in the
317 carotenoid synthesis pathway (i.e. phytoene synthase and phytoene desaturase).
318 The concentration of β-carotene was not changed after TT, OH, or PEF processing in
319 tomato juice. Likewise, a previous study demonstrated that the β-carotene content of
320 canned tomato sauce was decreased only after prolonged heating[33]. In contrast, previous
321 work reported a significant increase in β-carotene concentration after TT (90 °C for 30 s
322 or 60s) and PEF (35 kV/cm for 1500 μs at 100 Hz) treatment for both whole tomato fruit
323 and tomato juice[14,28], using treatment times significantly greater than the current study. A
324 much lower PEF field strength and voltage (0.4kV/cm at 0.1Hz for 30 pulses, and 1kV/cm
325 at 0.1 Hz for 16 pulses) also increased β-carotene content in tomato fruit compared to raw
326 fruit[13,14]. The results herein coincide with previous studies of OH treatment on tomato
327 juice, where no significant difference in lycopene concentration was observed[31].
328 Lutein content was not changed in the tomato juice after TT, OH, and PEF treatment.
329 Likewise, TT (95 °C for 60 s) or high voltage PEF (i.e. 35 kv/cm, 100 Hz) also have been
330 shown to have no effect on lutein levels in tomato fruit or juice[12,14]. Oxidative degradation
331 kinetics of carotenoids under heat demonstrate that lutein has a lower rate of loss than
332 lycopene and all-*trans*-β-carotene[34]. Thus, because lycopene and β-carotene were largely
333 stable during these processing methods, one would also expect lutein concentrations to
334 be retained. In contrast, lower frequency PEF treatments (1 kV/cm, 0.1 Hz and 4- 20
335 kV/cm, 0.1 Hz) increase lutein concentration[13,14], suggesting that enzyme activation
336 occurs under these milder conditions.
337 *Kale-based Juice* - The effects of processing on kale-based juice carotenoid and
338 chlorophyll concentrations are shown in **Table 3**. Processed kale-based juice L*, a*, and
339 b* color values are showed in **Table 4**.

PEF and TT processing degraded β-carotene content by ~60% and ~25%, respectively, relative to RAW (**Table 3**), whereas the greatest concentration of β-carotene was retained by HHP processing. Likewise, ~44% loss of β-carotene was observed in broccoli after 30 s boiling[42]. Although investigations with kale-based juice are limited, studies of β-carotene retention in processed spinach have revealed ~50% loss after 4 min of boiling (i.e. 100 °C) [35]. Compared with tomato juice, the β-carotene in kale-based juice was more readily degraded. It is possible that pectin in tomato juice facilitated hydrocolloidal formation to protect carotenoids from the PEF generated H_2O_2 and OH- species, and this protection has been previously suggested for tomato paste carotenoids[37], but no such protection was conferred by the kale-based juice matrix. The HPP results coincide with previous work on spinach puree, where only 5% of the β-carotene was degraded after 600 MPa was applied[8].

Approximately 30% of the lutein was degraded in the kale-based juice after TT and PEF treatment relative to RAW. In contrast, lutein and chlorophyll a and b concentrations were maintained during HPP treatment of kale-based juice. This was anticipated, as HPP has been shown to conserve carotenoids and other color compounds better than thermally processed fruit and vegetable juices[3,5,8].

Treatment effect on bioaccessibility

Tomato juice - Bioaccessibility of the carotenoids in the mixed micelle fraction after digestion is shown in **Figure 1A**. The efficiency (%) of micellarization for carotenoids in tomato juice was inversely dependent on hydrophobicity, i.e., lutein > β-carotene> lycopene. This aligns with the physical-chemical behavior of these carotenoids[24] and previous *in vitro* studies of the bioaccessibility of carotenoids in tomatoes[38].

Micellarization of lycopene was 2.5 times as efficient in digested PEF-treated tomato juice than in digested raw tomato juice (1.46 ± 0.40% vs 0.59 ± 0.14%, respectively) (*P*

365 = 0.009, **Figure 2B**). This was largely due to the increased micellarization of *all-trans*-
366 lycopene compared to that of TT and OH. This phenomenon was previously observed
367 after PEF treatment of tomato juice, in which 50% of the total micellarized lycopene was
368 *all-trans*-lycopene[12], hypothesized to occur because PEF facilitates the release of
369 carotenoid from the food matrix by disrupting cell walls. There was no difference between
370 the bioaccessibility of lycopene in digested RAW, TT or OH treated tomato juices. This
371 observation was surprising considering the number of studies which demonstrate
372 improved bioaccessibility after TT[11,15,39]. However, many of these studies have used
373 significantly stronger thermal conditions (i.e. 60 ℃ for 40 min, 90 ℃ for 4 min) that are
374 likely to better destroy the integrity of cell organelles, releasing more lycopene from the
375 food matrix[10,11].
376 PEF decreased the micellarization of β-carotene by ~50% relative to RAW (P= 0.02), but
377 OH and TT had no significant change compared with RAW. The thermal conditions (90
378 °C for 30 s) used with OH in the current study have been reported to inactivate
379 lipoxygenase[6], while PEF cannot completely inactivate this enzymes. Thus, it is possible
380 that β-carotene bioaccessibility was decreased due to incomplete inactivation of
381 lipoxygenase after PEF treatment, whereas β-carotene was protected in raw juice by
382 lipoxygenase remaining sequestered, and OH and TT inactivation of lipoxygenase
383 eliminating activity altogether. It is also plausible that PEF released proteins and cell wall
384 components that sequestered β-carotene to prevent micellarization[10,39]. Lutein
385 bioaccessibility of tomato juice was not altered by different processing methods.
386 *Kale-based juice* - In kale-based juice, the bioaccessibility of β-carotene and lutein were
387 not significantly different between treatment groups (**Figure 2A**, $P > 0.05$). Although β-
388 carotene was the most abundant carotenoid in the kale-based juice, only ~10% was
389 micellarized, whereas almost 100% of the lutein was micellarized. These results are

similar to previous reports in kale where *all-trans*-β-carotene was micellarized after digestion of boiled leaves[40]. The high efficiency of micellarization of lutein in digested kale-based juice was similar to that reported for other leafy greens (e.g., ~80% from cooked spinach[41] and a mixed vegetable salad[42]). However, these results contrast with the 27-32% micellarization of lutein in cooked spinach reported in another other study[43]. Removal of large particles in the study reported herein likely reduced the fiber content of the kale-based juice, and the presence of only smaller particles likely provided greater access to digestive enzymes and bile acids for micellarization[44].

Effect of processing method of juice on cell-uptake of micellarized carotenoids

To the best of our knowledge, this is the first assessment of the uptake of carotenoids from digested OH and PEF treated tomato juice by Caco-2 intestinal cells. It should be noted that the % carotenoid uptake has been normalized to the % micellarization in **Figures 1B and 2B,** to eliminate differences in bioaccessibility.

Tomato juice - Across all processing methods, 6.04% of lycopene, ~ 0.76% of β-carotene, and 9.45% of lutein in the mixed micelle fraction was taken up by the intestinal cells. This result is supported by human studies in subjects fed pure β-carotene and lutein, measuring 5-fold higher bioavailability of lutein than β-carotene[45], and 5% bioavailability of lycopene from red tomato in humans[46]. There was significantly less uptake of lycopene from mixed micelles generated after the digestion of PEF treated tomato juice, compared to that from RAW (**Figure 1B**). This result is likely due to the lycopene isomer composition, as *in vitro* and *in vivo* studies have demonstrated that *cis*-lycopene is more bioavailable than *all-trans*-lycopene[46,47]. Indeed, ~54% of the PEF micellarized lycopene was in the *all-trans*- form, whereas *all-trans*-lycopene only accounted for 20% of total lycopene in micelles generated during digestion of RAW, TT, and OH juices. No significant difference was observed for lycopene uptake by cells incubated with the mixed

415 micelle fraction generated during digestion of RAW, TT, and OH (**Figure 1B**),
416 presumably because the profile of lycopene isomers was very similar. Also, thermal
417 processing time used for TT and OH treated tomato juice herein (30 sec) was significantly
418 less than those used in a previous TT study (100 °C for 1 h) where increased human
419 uptake of lycopene was reported[48].
420 PEF treatment significantly increased the percentage of β-carotene taken up by the cells,
421 relative to RAW, but not TT or OH, juices (**Figure 1B**). Relative to digested RAW tomato
422 juice, TT, OH and PEF treatments all increased cell uptake of lutein by ~91%. Studies
423 have shown that the cell wall material of TT tomato juice appeared to leak out to the
424 liquid phase during boiling[49], suggesting that phospholipid components of the cell wall
425 can also be released and promote carotenoid micellarization and absorption[50,51]. It is also
426 plausible that increased phospholipid released by TT, OH and PEF treatments may
427 potentially impact the size and phospholipid composition of micelles, resulting in
428 increased apical uptake by absorptive intestinal cells[50].
429 *Kale-based juice* - In the kale-based juice, there was no significant difference between
430 the cellular uptake of β-carotene and lutein, regardless of TT, HPP, or PEF treatment
431 (**Figure 2B**). The percentage of β-carotene taken up by cells was higher than that of lutein,
432 in contrast to previous studies of leafy greens[52,53]. Expression of results as a percentage
433 that has been normalized to percent micellarization likely accounts for this difference, as
434 almost 100% of lutein was micellarized, whereas only a small fraction of β-carotene was
435 micellarized.
436 *Treatment effect on overall carotenoid delivery*
437 Incorporating differences in processing, bioaccessibility, and uptake together, one-way
438 ANOVA shows that PEF had a non-significant trend towards increased total lycopene
439 delivery (P = 0.10) in the tomato juice (**Figure 1C**). A significant difference between the

440 treatment groups in lutein total delivery content (P = 0.048) was observed, although
441 subsequent pairwise comparisons revealed a non-significant trend between RAW and
442 PEF treatment (P = 0.07), and RAW and OH treatment (P = 0.09).
443 **Figure 2C** shows kale-based juice treated with TT delivered 43% more β-carotene to
444 cells than digested PEF treated juice (P = 0.011), whereas HPP provided no delivery
445 advantage compared to TT and PEF treated juice (P > 0.05). In contrast, there was no
446 significant difference observed for lutein delivery among the different treatments of the
447 kale-based juice. Although β-carotene concentration was three times higher than lutein,
448 less was delivered to the cell as compared to lutein.
449 Overall, this study suggests that the bioaccessibility and Caco-2 cell uptake of carotenoids
450 from tomato and kale-based juices can be altered depending on the chosen pasteurization
451 treatment. It should be noted that the comparison between OH, TT, PEF and HPP were
452 done using different criteria for microbial inactivation: *B. coagulans* inactivation was the
453 basis for OH and TT; *E. coli* ATCC 117755 inactivation the basis for HPP, and yeast and
454 molds being the criteria for PEF. This resulted in more severe processes for OH and TT
455 than for HPP, and for HPP in comparison for PEF. A major difficulty in this regard is
456 lack of data on inactivation of the same microorganisms, enabling an equal basis for
457 comparison of thermal and nonthermal technologies. Nevertheless, to our knowledge, this
458 represents the first study that compares all three approaches (PEF, OH and HPP against
459 TT and RAW) samples. Furthermore, from a nutritional perspective, these four
460 processing methods (i.e. PEF, TT, OH, and HPP) largely preserve carotenoids in the
461 tomato and kale-based juice, permitting comparable delivery, while also producing a
462 pasteurized product. With regards to the kale-based juice, HPP best preserved the color,
463 and thus may be preferred.

Abbreviations Used

DCM- dichloromethane

HPP- high pressure processing

MeOH- methanol

MTBE- methyl tertiary-butyl ether

OH-ohmic heating

PEF- pulsed electric fields

TT-thermal treatment

RAW- raw juice

NEAA- non-essential amino acid solution

PBS- phosphate- buffered saline

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Figure 1. Effect of non-processed (raw), thermal processing (TT; 90 ºC, 30 s), ohmic heating (OH; 13 V/cm, 60 Hz, 90 ºC, 30 s) and pulsed electric fields (PEF; 35 kV/cm, 1000 μs, 2 μs, 250 Hz) on (**A**) bioaccessibility, (**B**) Caco-2 cellular uptake, and (**C**) summation of lycopene, β-carotene and lutein delivered from tomato juice. Data are displayed as the mean ± standard deviation for n=3 replicates per processing treatment. Within a carotenoid, means with the same letter are not statistically different as determined by one-way ANOVA followed by Tukey's post-hoc test ($P < 0.05$).

Figure 2. Lycopene isomers in (A) raw and processed tomato juice, and (B) mixed micelles generated during simulated digestion. Data are displayed as the mean for n=3 replicates per processing treatment.

Figure 3. Effect of thermal processing (TT; 90 ºC, 30 s), high pressure processing (HPP; 500 MPa, 3 min, >35 ºC) and pulsed electric fields (PEF; 35 kV/cm, 1000 μs, 2 μs, 500 Hz) on (**A**) bioaccessibility, (**B**) cellular uptake and (**C**) summation of β-carotene and lutein delivered from kale-based juice. Within a carotenoid, means with the same letter are not statistically different as determined by one-way ANOVA followed by Tukey's post-hoc test ($P < 0.05$).

696 **Table 1**. HPLC-MS/MS ion transitions monitored for carotenoids tested

Compound	Precursor > Product ion transitions (m/z)	Collision energy (V)
Lutein	551.3 >119.0[a], 121.0[a], 145.0[a]	10, 10, 10
β-carotene	537.4 > 95.0[a], 105.0[a], 177.0[a]	20, 30, 15
lycopene	537.4 > 69.2, 413.0, 455.3[a], 537.4	25, 20, 15, 0

697 [a]All ion transitions noted were summed and used for quantification

698

699 **Table 2**. Effect of processing on measured carotenoids in the tomato juice.

Compound	RAW (mg/100g)	TT (mg/100g)	OH (mg/100g)	PEF (mg/100g)
all-trans-lycopene	3.33±0.22[a,b]	2.71±0.07[a]	3.09±0.47[a,b]	3.45±0.15[b]
5-*cis*-lycopene	0.75±0.16[a]	0.87±0.29[a]	0.73±0.04[a]	0.85±0.17[a]
other *cis*-lycopene	0.69±0.056[a,b]	0.56±0.056[a]	0.61±0.069[a,b]	0.72±0.019[b]
total lycopene	4.77±0.38[a]	4.15±0.39[a]	4.40±0.49[a]	5.01±0.20[a]
β-carotene	0.15±0.006[a]	0.14±0.003[a]	0.14±0.005[a]	0.15±0.006[a]
lutein	0.033±0.002[a]	0.028±0.003[a]	0.030±0.007[a]	0.036±0.001[a]
α-tocopherol	0.67 ± 0.13[a]	0.65 ± 0.20[a]	0.62 ± 0.07[a]	0.89 ± 0.09[a]
phytoene	0.55±0.009[a]	0.48±0.001[a]	0.51±0.057[a]	0.53±0.026[a]
phytofluene	1.39±0.061[a]	1.26±0.049[a]	1.29±0.120[a]	1.36±0.043[a]

700 Values are displayed as the mean ± standard deviation for n=3 replicates per processing treatment. Within
701 a carotenoid, values with different letters are significantly different, as determined by one-way ANOVA
702 followed by Tukey's post-hoc test ($P < 0.05$).
703
704

705 **Table 3.** Effect of processing on carotenoids measured in kale-based juice

Compounds	Raw (*mg/100 g*)	TT (*mg/100 g*)	HPP *(mg/100 g)*	PEF *(mg/100g)*
β-carotene	1.435±0.059[a]	1.081±0.025[c]	1.192±0.179[a, c]	0.587±0.015[b]
lutein	0.674±0.041[a]	0.447±0.030[b]	0.739±0.058[a]	0.497±0.001[b]
chlorophyll a + b	19.560±1.435[a]	1.222±0.091[b]	18.047±2.403 [a]	8.056±2.405[c]

706 Values are displayed as the mean ± standard deviation for n=3 replicates per processing treatment. Within
707 a carotenoid, values with different letters are significantly different, as determined by one-way ANOVA
708 followed by Tukey's post-hoc test ($P < 0.05$).
709

710 **Table 4.** Color characteristics of raw and processed kale-based juice

Color	RAW	TT	PEF	HPP
L*	31.40 ± 0.14[a]	34.32 ± 0.02[b]	33.53 ± 0.02[c]	32.68 ± 0.02[d]
a*	(-)6.41 ± 0.05[a]	(-)1.38 ± 0.02[b]	(-)6.08 ± 0.04[c]	(-)7.17 ± 0.04[d]
b*	8.80 ± 0.14[a]	11.77 ± 0.04[b]	11.35 ± 0.04[c]	10.46 ± 0.02[d]

711 Values are displayed as the mean ± standard deviation for n=3 replicates per processing treatment. Within
712 a row, values with different letters are significantly different, as determined by one-way ANOVA
713 followed by Tukey's post-hoc test ($P < 0.05$).
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