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Effect of pulsed electric fields on carotenoid and phenolic bioaccessibility and their relationship with carrot structure

Gloria López-Gámez, ^a Pedro Elez-Martínez,^a Amparo Quiles-Chuliá, ^b Olga Martín-Belloso, ^a Isabel Hernando-Hernando, ^b Robert Soliva-Fortuny ^{*a}

^{a.} *Department of Food Technology, Agrotecnio Centre, University of Lleida, Av. Alcalde Rovira Roure, 191, 25198 Lleida, Spain.*

^{b.} *Department of Food Technology, University Polytechnic of Valencia, Camino de Vera s/n, 46022 Valencia, Spain.*

*robert.soliva@udl.cat

Abstract

Phenolic compounds (PC) and carotenoids from carrots are bound to dietary fibre or stored in vacuoles and chromoplasts, respectively. To exert their antioxidant effects these compounds must be released during digestion, which is hindered by such barriers. Pulsed electric fields (PEF) modify cell membrane permeability, thus enhancing their bioaccessibility. The effect of PEF on the carrot carotenoid and PC content and bioaccessibility was investigated. With this purpose, PEF-treated carrots (5 pulses of 3.5 kV cm^{-1}) were stored for 24 h at 4°C and microstructure was evaluated before subjecting them to in vitro digestion. PEF did not affect carotenoid content, whereas their bioaccessibility improved (11.9 %). Likewise, PEF increased the content of some PC, e.g. coumaric acid (163.2 %), probably caused by their better extractability. Conversely, caffeic acid derivatives decreased, which may be associated to greater contact with oxidative enzymes. Total PC bioaccessibility (20.8 %) and some derivatives increased, e.g. caffeoylshikimic (68.9 %), whereas some decreased (e.g. ferulic acid). Structural changes caused by PEF may improve bioaccessibility of carotenoids and PC by favouring their release and easy access to digestive enzymes. However, other antioxidants may be further degraded or entrapped during digestion. Therefore, PEF is an effective technology for obtaining carrots with enhanced carotenoids and phenolic bioaccessibility.

1. Introduction

Carrot is one of the most consumed roots worldwide and a significant source of natural antioxidants, including vitamin C, carotenoids (e.g. α -carotene, β -carotene, lutein) and phenolic compounds (e.g. chlorogenic, p-hydroxybenzoic or ferulic acid).[1] High dietary intakes of carotenoids and phenolic compounds have been associated with protective effects against some types of cancer and cardiovascular diseases [2] due to their antioxidant, anti-inflammatory and antitumoral activities.[3] However, bioactive compounds must be bioaccessible in order to exert their positive effects. Bioaccessibility is defined as the fraction of the ingested food constituent that is released from the food matrix in the gastrointestinal tract during digestion, thus becoming available for intestinal absorption. [4] Therefore, bioaccessibility of phytochemicals is even more important than their content in food products.

Upon ingestion, the bioaccessibility of carotenoids and polyphenols depends on several factors.[5,6] Carotenoids are lipophilic compounds that need to be released from the food matrix, solubilized and incorporated into micelles for absorption.[7] In carrots, these compounds are stored in crystalline form in chromoplasts. Hence, together with cell walls, chromoplasts are the main barriers limiting carotenoids bioaccessibility.[8] On the other hand, polyphenols are generally stored in vacuoles or associated to dietary fibre. Likewise, they must be released from the food matrix to be available for absorption, although incorporation to micelles is not required. Their bioaccessibility also depends on their polarity and interactions with cell wall polysaccharides, proteins or starch. To the best of our knowledge, scarce information is available on the bioaccessibility of carrot polyphenols or the effect of their interaction with carotenoids during digestion. A study carried out by Anh et al.[9] demonstrated that antioxidant properties of carotenoids may avoid phenolic degradation in the gastrointestinal tract.

It is known that food processing may facilitate the disruption of food matrix structures, thus leading to the release of carotenoids and phenolic compounds and enhancing their bioaccessibility.[10–13] Extensive studies have been carried out regarding the effect of processing or cooking methods on carotenoids bioaccessibility from carrots and derived products. Mechanical and thermal processing are essential for cell wall rupture and the increase in all-E- β -carotene bioaccessibility,[14] though degradation has also been reported.[10,15] In addition, it has been demonstrated that the presence of cell fragments or structural networks formed by pectin or fibre may prevent an efficient transfer to micelles.[16–19]

Effects of processing on phenols bioaccessibility vary depending on food matrix, processing parameters or the chemical characteristics of individual compounds.[20] It has been reported that chemical or physical changes in the food matrix may lead to a better release of phenolic compounds.[21]

Pulsed electric fields (PEF) is a non-thermal technology based on the application of short duration pulses of external electric fields that induce electroporation of cell membranes. Modification of cell membrane permeability has been applied to more efficiently extraction of lipophilic[22–24] or hydrophilic compounds.[25–27] In addition, some authors have separately investigated the effect of PEF on the bioaccessibility of both types of compounds. Ribas-Agustí et al.[12] demonstrated that phenolic bioaccessibility of apples increased after applying 1.8 and 7.3 kJ kg⁻¹ PEF treatments. On the other hand, carotenoids bioaccessibility was also enhanced in derived products obtained from PEF-treated tomatoes.[28,29] Therefore, PEF application can also be a potential tool to facilitate bioactive compounds release during digestion and promote their bioaccessibility.[12,28,29]

The implementation of PEF as a pre-processing treatment to enhance bioactive compounds bioaccessibility from solid matrices is underexplored. This information would enable obtaining products with an improved nutritive value. With this perspective, the aim of this study was to investigate the feasibility of PEF as a pre-treatment in order to enhance the bioaccessible content of carotenoids and polyphenols in carrots.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol, acetone and methyl tert-butyl ether were acquired from Fisher Scientific Scharlau Chemie (Loughborough, UK), sodium chloride was purchased from POCH S.A. (Sowińskiego, Poland). Ultrapure water was obtained with a Milli-Q system (Millipore Ibérica, Madrid, Spain). Magnesium chloride hexahydrate, ammonium carbonate, acetonitrile, hexane and ethanol (HPLC grade), acetic acid, ammonium acetate were purchased from Scharlab (Sentmenat, Spain). Calcium chloride dihydrate was obtained from Merck (Darmstadt, Germany). Sodium hydrogen carbonate and potassium dihydrogen phosphate were purchased from VWR (Llinars del Vallès, Spain). Butyl hydroxytoluene (BHT) was acquired from Scharlau Chemie S.A. (Barcelona, Spain). Potassium chloride was obtained from Panreac (Castellar del Vallès, Spain). All digestive enzymes (porcine α -amylase, porcine pepsin, porcine bile extract and porcine pancreatin) were purchased from Sigma-Aldrich (Darmstadt, Germany).

Caffeic acid, ferulic acid, p-coumaric acid and 5-O-caffeoylquinic acid commercial patterns were purchased from Sigma-Aldrich (St. Louis, MO, USA); and quercetin-3-O-glucoside from Extrasynthese (Genay, France). Phytoene and β -carotene standards were obtained from Carote-Nature (Ostermundigen, Switzerland), α -carotene was purchased from Supelco-Merck (Darmstadt, Germany) and lutein from Acros Organics (New Jersey, USA).

2.2. Carrot samples

Carrots (*Daucus carota* cv. Nantes) with calibre 25/35 mm and length of 17 ± 2 cm were purchased in a local supermarket in Lleida (Spain). They were stored at 4 °C until treatment within a week. Carrots were washed with tap water and dried carefully with a paper cloth before treatment application.

2.3. Pulsed electric fields (PEF) treatments

PEF treatments were conducted in a batch PEF system (Physics International, San Leandro, CA, USA) (Figure 1), which delivers exponential shape pulses of 4 μ s from a capacitor of 0.1 μ F at a frequency of 0.1 Hz. It is equipped with a TG-70 gas control unit and a PT55 pulse generator (Pacific Atlantic Electronics Inc., El Cerrito, CA, USA). The treatment chamber consists of a parallelepiped methacrylate container with two parallel stainless-steel electrodes (20×5 cm) separated by a gap of 5 cm. Carrots (~ 0.1 kg) were placed in parallel to the electrodes and immersed in an aqueous solution (conductivity of 10 μ S cm^{-1}). Then, carrots were treated by applying 5 pulses of 3.5 kV cm^{-1} (0.61 kJ kg^{-1}) and were stored at 4 °C for 24 h. The specific energy input was calculated based on Wiktor et al.[22] and medium temperature did not exceed 20.0 °C after PEF application.

The treatment selection was based on the results of previous studies, aimed at maximizing the accumulation of phenolic compounds in carrot[31] and improving carotenoid extractability (data not published) without altering its quality attributes.

2.4. Texture analysis

In order to perform compression tests a texture analyser (TA-XT2 Stable Micro Systems, England), equipped with a loading cell (25 kg-f), a cylindrical flat-head aluminium probe (25 mm diameter, Stable Micro Systems, England) and a heavy-duty platform were used. The probe was set to 10 ± 2 mm above the platform and each carrot sample was placed at the centre. The compression test started with a constant probe speed of 60 mm min^{-1} for a distance of 2 mm into the sample, and then the probe returned to the starting position and repeated the compression test on the same sample for a second time. The maximum peak force detected during the first compression cycle represented as the hardness of the carrot sample, which can be defined sensorially as the force required to compress a food between molars. Two other textural parameters were also considered: adhesiveness (negative area after the first compression cycle that measure the work necessary to pull the compressing probe away from carrot, which relate to the ability of carrot samples to adhere to teeth when chewed) and cohesiveness (the ratio of positive force during the second compression cycle to that of the first compression cycle, indicating the strength of internal bonds making up the body of the food).[32]

Figure 1. Scheme of PEF device.

2.5. Microscopy

Carrot outer and inner parenchyma samples were fixed using glutaraldehyde (2.5 % in phosphate buffer 0.1 M), they were then washed thrice for 10 minutes with phosphate buffer and submerged in osmium tetroxide for 2 hours. Next, samples were immersed into sodium acetate 0.1 M, uranyl acetate 0.5 % and sodium acetate again. After that, samples were dehydrated with acetonitrile (30 – 100 %) solutions, infiltrated and polymerized (Historesin Embedding Kit 812) for 3 days at 60 °C. Samples were cut into 70 – 80 nm and 1 µm sections using a ultramicrotome (Ultracut Leica EM UC6). In order to obtain transmission electronic microscopy (TEM) images, samples were contrasted with Reynolds lead citrate.

2.6. In vitro digestion

The in vitro digestion procedure was adapted according to the standardized COST Infogest protocol,[33] in which electrolyte and enzymatic solutions to simulate the oral, gastric and duodenal phases of human digestion is described. The oral phase was initiated by blending 10 g of carrots (1 cm³ cubes) for 1 minute (Taurus aromatic 150, 150 W) and transferred to a glass bottle. Then, 10 mL of simulated salivary fluid (pH 7 and 37 °C) with α-amylase[33] were added to stir the mix for 1 minute at 37 °C. The gastric phase started adding 20 mL of simulated gastric fluid (pH 3 and 37 °C) and pepsin[33]. After 2 h of incubation at 37 °C with agitation, the duodenal phase was initiated by inserting a cellulose-membrane dialysis bag (molecular weight cut-off 12,000 Da, Sigma-Aldrich) containing simulated intestinal fluid (pH 7 and 37 °C).³³ At this stage, the dialysis bag is used to mimic the role of the intestinal epithelium and separate the compounds that have been released from the undigested product (bioaccessible fraction of phenolic compounds).[12,32] After a period of 30 minutes to reach pH 7, a solution containing

simulated intestinal fluid (pH 7 and 37 °C), bile extract and pancreatin was added to the chyme and the mixture was left to incubate for further 2 h. At the end of digestion, the dialysis bags were rinsed with distilled water until clean and its content was collected. The remaining digesta, which contained carotenoid compounds, was centrifuged (Beckman Coulter, Avanti J-26 XP, California, United States) at 5000 g for 15 minutes at 4 °C [33–35] and supernatant was also collected. Supernatant was divided in two equal aliquots. One was vacuum filtrated (Whatman No. 1), to obtain the released fraction. The other aliquot was microfiltrated across cellulose filter (pore size 1 – 3 µm; 70 mm diameter; Anova S.A., Barcelona, Spain) and the resulting solution corresponded to the micellar fraction. Resulting digested fractions were freeze-dried and stored at -40 °C until analysis of carotenoid content and bioaccessibility.

Digestions were performed in darkness, in absence of oxygen (bottles were flushed with nitrogen gas) in an orbital incubator (Digital incubator OPAQ I10-OE, Ovan, Badalona, Spain) at 37 °C and 120 rpm. Electrolyte concentrations and enzyme activities were prepared following the indications provided by Minekus et al..[33] Blank samples consisting in water instead of carrot, were made in identical conditions.

2.7. Carotenoids extraction

2.7.1. Non-digested samples

Carotenoids were extracted according to the method described by Sadler et al.[37] with slight modifications. Homogenized carrot tissue (1 g) was stirred for 20 minutes with 50 mL of hexane : acetone : ethanol (50 : 25 : 25) solution containing 1 g L⁻¹ BHT. 15 mL of NaCl [10 % (w/v)] solution was added and the samples were stirred for additional 10 minutes. Samples were left to stand for ≥ 3 minutes, and the upper organic phase was analysed by high-performance liquid chromatography (HPLC) according to the method described subsequently. All extractions were performed in duplicate, and samples were protected from light throughout extraction and analysis to avoid carotenoid degradation and isomerization.

Table 1. Chromatographic conditions for identifying carotenoid compounds by HPLC.

Time (minutes)	Methanol/ ammonium acetate 0.1 M (%)	H ₂ O (%)	Methyl tert-butyl ether (%)	Methanol (%)
0	95	5	0	0
10	100	0	0	0
12	95	0	5	0
17	86	0	14	0
22	75	0	25	0
29	95	0	5	0
30	100	0	0	0
40	100	0	0	0
45	0	0	0	100

50	0	0	0	100
55	95	5	0	0
60	95	5	0	0

2.7.2. Digested samples

To extract carotenoids from the digested released and micellar fractions, 5 mL of the extraction solution was added to 0.2 g of freeze-dried digesta. After that, samples were vortexed for 20 s and 1 mL of NaCl solution [10 % (w/v)] was added. Samples were vortexed for another 20 s and centrifuged (Beckman Coulter, Avanti J-26 XP, California, United States) at 4000 g for 5 minutes.[38] An aliquot of the upper organic phase was analysed by HPLC according to the method described subsequently. All extractions were performed in duplicate, and samples were protected from light throughout extraction and analysis to avoid carotenoid degradation and isomerization.

2.8. Identification and quantification of individual carotenoids

Carotenoids were quantified by HPLC, following a procedure validated by Cortés et al..[39] An aliquot of 20 µL of the extracted samples was injected into the 2695 HPLC system, which was equipped with a 600 Controller, a 486 Absorbance Detector, a thermostatic column compartment, and a 717 Plus Auto Sampler with cooling system (Waters, Milford, MA). Carotenoids were separated using a reverse-phase C18 Spherisorb ODS2 (5 µm) stainless steel column (4.6 mm x 250 mm). The mobile phase consisted of methanol/ammonium acetate 0.1 M, milli-Q water, methyl tert-butyl ether and methanol (Table 1). The flow rate was fixed at 1 mL min⁻¹ and the total run time was 60 minutes. The column was set at 30 °C, while sample amber vials on the auto sampler were preserved at 4 °C. The carotenoids were identified by UV–vis spectral data and their retention times.[38,39] Quantification of carotenoids was carried out by integration of the peak areas and comparison to calibration curves. Results were expressed on a fresh weight basis (mg/100 g FW).

2.9. Extraction of phenolic compounds

Phenolic compounds were extracted from freeze-dried undigested or digested carrot samples (0.2 g). Methanol (80:20 v/v) (1.5 mL) was added, samples were vortexed for 1 minute, then centrifuged (16209 g, 15 minutes, 4 °C) (Beckman Coulter, Avanti J-26 XP, California, United States), and the clear supernatant was microfiltered using PVDF filters (0.2 µm) (Scharlab, Barcelona, Spain) prior to injection to the chromatographic system.

2.10. Identification and quantification of individual phenolic compounds

Phenolic compounds and their generated metabolites were determined in methanolic extracts obtained from freeze-dried undigested or digested carrot samples. AcQuity Ultra-Performance™ liquid chromatography (UPLC) coupled to a triple quadrupole detector (TQD) mass spectrometer

(Waters, Milford) was used. The analytical column was an AcQuity BEH C18 column (100 mm $\times$ 2.1 mm i.d., 1.7 μ m,) equipped with a VanGuard™ Pre-Column AcQuity BEH C18 (2.1 $\times$ 5 mm, 1.7 μ m), also from Waters. During the analysis, the column was kept at 30 °C, and the flow Table 2. Chromatographic conditions for identifying phenolic compounds by UPLC.

Time (minutes)	Acetonitrile (%)
0	5
5	10
10	12.4
18	28
21	85
23	100
25.5	100
27	5
30	5

rate was 0.3 mL min⁻¹. Mobile phases were acetic acid (0.2 %) and acetonitrile, and elution gradients are shown in Table 2. Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface (ESI). Ionization was achieved using the electrospray interface operating in the negative mode [M-H]⁻ and the data were acquired through selected reaction monitoring (SRM). Two SRM transitions were selected, the most sensitive one was used for quantification, and the second for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1 software. Results were expressed on a dry weight basis (mg kg⁻¹ DW).

2.11. Definitions and bioaccessibility calculation

Carotenoids bioaccessibility was referred to released carotenoids from the food matrix and their incorporation into micelles after in vitro digestion. The released fraction was calculated as carotenes transferred from the food matrix to supernatant obtained by centrifugation and vacuum filtration after in vitro digestion. The micellar incorporation was estimated as carotenes recovered in the micellar phase isolated by microfiltration.

On the other hand, phenolic compounds bioaccessibility was calculated referred to their concentration in the dialysed fraction of digested samples.

$$\text{Bioaccessibility (\%)} = \frac{\text{CC}_{\text{digested}}}{\text{CC}_{\text{non-digested}}} \times 100 \quad (1)$$

where CC_{digested} corresponds to the overall concentration of each compound in the bioaccessible fraction (micellar for carotenoids and dialysed for phenols) and CC_{non-digested} is the concentration in undigested samples.

The bioaccessibility of each individual compound was determined using Eq. (1) and results were expressed as percentage.

2.12. Statistical analysis

Statistical analyses were carried out using the SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA) and IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL). Each treatment condition was independently replicated thrice using batches of two carrots per replica, and every replica was digested and analysed twice. Results were reported as the mean $\pm$ standard deviation. One-way ANOVA was performed to identify significant differences between untreated and PEF-treated samples. The statistical significance level was set up at $p < 0.05$.

3. Results and discussion

3.1. Effect of PEF on carrot microstructure

Figure 2 shows the outer and inner parenchyma of the untreated carrot. It can be observed that in both zones the cells maintained the integrity of the membranes (tonoplast and plasmalemma), cell walls and middle lamellas as well as the cellular cements. Cells were uniformly stained because the cellulose fibrils were tightly packed together (Figures 2a and b). Using the TEM technique, the observed cell walls were bonded to each other by a firm middle lamella (black arrows in Figures 2c and d) with a uniform packing of the fibrils. Cell protoplast, which comprises the cell membrane or plasmalemma and the cell content, was kept close to the cell wall and could not be easily differentiated in some areas (Figures 2a and b).

Carotenoids were typically located close to the cell walls, in the peripheral cytoplasm. The carotenoids compounds present in carrot studied tissues showed two different subcellular associations (Figures 2a and b). On the one hand, carotenoids were found, near the cell wall, inside the chromoplasts, adopting a globular structure (white arrows in Figure 2b). On the other hand, they appeared forming crystal clusters both inside the cell and on the cell wall (black arrow in Figure 2a). These clusters have also been detected in mango and carrot tissue[41] and in persimmon.[42,43]

PEF treatment caused structural modifications in both, outer and inner parenchyma of carrot (Figure 3). PEF caused the breakdown and degradation of cell walls and membranes (Figure 3a and b). In some areas, the cell walls appeared thin and weakly stained (Figures 3c and d). The cell walls were more faintly stained because the cellulose fibrils were degraded and presented less bundling than in the untreated carrot. Dissolution of the cellulose “cements” favoured the walls of adjoining cells to separate (black arrows in Figure 3b). Moreover, in both, outer and inner parenchyma, the middle lamella appeared dissolved and degraded in some areas (black arrows in Figures 3c and d). Despite the PEF treatment, some cell membranes remained intact, but they

draw away from the cell wall and towards the middle of the cell (Figure 3a and b). All these structural modifications could improve solutes transport through parenchyma, which favoured bioactive compounds bioaccessibility (Tables 3 and 4).

Carotenoid components were mainly located in the cytoplasm, further away from the cell walls than in the untreated carrot (white arrows in Figures 3a and b). Cell wall and membranes degradation produced by the PEF treatment favoured the diffusion of the carotenoid components. However, PEF treatment did not seem to alter the subcellular structures where the carotenoids were initially located; it was possible to observe carotenoids inside the chromoplast and forming crystal clusters in the parenchyma of the treated carrot.

Figure 2. (a) Light micrograph of outer parenchyma of untreated carrots, (b) light micrograph of inner parenchyma of untreated carrots, (c) TEM images of outer parenchyma of untreated carrots, (d) TEM images of inner parenchyma of untreated carrots.

Figure 3. (a) Light micrograph of outer parenchyma of PEF-treated carrots (5 pulses of 3.5 kV/cm), (b) light micrograph of inner parenchyma of PEF-treated carrots (5 pulses of 3.5 kV/cm),

(c) TEM images of outer parenchyma of PEF-treated carrots (5 pulses of 3.5 kV/cm), (d) TEM images of inner parenchyma of PEF-treated carrots (5 pulses of 3.5 kV/cm).

3.2. Impact of PEF on carrot texture

Hardness and adhesiveness of whole carrots were significantly affected by PEF treatment, whereas cohesiveness remained unaltered (Figure 4). On average, the hardness of untreated carrots (321.3 ± 12.5 N) significantly decreased when applying PEF (271.9 ± 21.1 N). These results are in agreement with previous works which found that the application of moderate electric fields of 0.1 to 5 kV cm⁻¹ can induce severe tissue damage through membrane breakdown.⁴⁴ Leong et al. ³² and Wiktor et al. [45] attributed the firmness decrease to changes in the internal structure of carrots, which facilitated the compacting and rearrangement of tissues during compression tests. Considerable softening was also observed in PEF-treated fruit, such as tomatoes, blueberries and apples.[45–47] This fact is supported by obtained microstructure images (Figures 2 and 3). Untreated carrot tissues showed strong intercellular adhesion that will lead to cell breakage during compression test. In contrast, cell separation in PEF-treated tissue is probably enhanced due to middle lamella degradation. Additionally, changes in cell walls could also cause differences in the water absorbed into the polysaccharides network which affect other textural and sensory perception properties.[49] Electroporabilisation and cytoplasmic content leakage could also increase adhesiveness of PEF-treated carrots since Aguiló-Aguayo et al. [50] reported that cellular content moved from vacuoles to cytoplasm and outside the cell. Therefore, release of this intracellular juice rich in sugars may cause the higher adhesion to the texture analyser probe.

Figure 4. Hardness, cohesiveness and adhesiveness (mean $\pm$ SD) of PEF-treated (5 pulses of 3.5 kV cm⁻¹) and untreated carrots. Asterisks indicate significant differences ($p < 0.05$) between treated and untreated carrots.

3.3. Effect of PEF on carrot carotenoids content

Carotenoid content of PEF-treated and untreated carrots is shown in Table 3. Main carotenoids present in carrots are α -carotene and β -carotene, with a lesser presence of lutein and phytoene (Table 3), which is in agreement with what was found in other previous studies.[8,51,52] Carotenoid content of PEF-treated carrots before digestion was similar to that of those untreated. Some authors have reported that electroporabilisation could enhance the liberation of carotenoids[53] depending on the applied conditions. For instance, Wiktor et al. [22] reported that carrot cylinders treated by PEF (15 – 100 pulses of 3 kV cm⁻¹) decreased their total carotenoid content after treatments, which was attributed to reactive oxygen species (ROS) generation and β -carotene chain oxidation. Differences with our study are likely due to time elapsed between treatment and extraction (24 h), sample preparation and specific energy applied, which was highly superior (3 – 30 kJ kg⁻¹). Our results may suggest that treatment was not enough to improve carotenoid extraction before digestion. However, PEF did have a significant impact on the release of carrot carotenoids and their incorporation into micelles during digestion.

3.4. Effect of PEF on carotenoids release after in vitro digestion and their bioaccessibility

PEF had a significant impact on carrot carotenoids release during digestion, which affected their bioaccessibility (Table 3). Total carotenoids and β -carotene from PEF-treated carrots were better released during in vitro digestion, showing 42.9 % and 64.2 % higher contents than those observed in the digesta obtained from untreated carrots. Besides, the total carotenoid content in the micellar fraction of PEF-treated carrots (5.77 mg/100 g FW) was higher than that obtained in those untreated (3.38 mg/100 g FW). A-carotene content was also greater (66.7 %) than that obtained in untreated carrots.

Results indicate that individual carotenoids were differently released from the carrot matrix. In untreated carrots, the percentages of released carotenoids, related to the content in undigested carrot tissues, were in descending order: 22.4 % for phytoene, 16.7 % for lutein, 11.2 % for β -carotene and 9.1 % for α -carotene. However, this order was altered when PEF was applied. Results show that the release of all carotenoids was increased to a similar percentage, between 20 % and 26.8 %. Hence, β -carotene content from PEF-treated carrots in the released fraction (6.98 mg/100 g FW) was significantly higher than that observed in untreated carrots (4.26 mg/100 g FW). Even if carotenoids were better released from food matrix due to structural modifications caused by PEF (Figures 2 and 3), micellar contents were only significantly higher in the case of α -carotene. This suggests that PEF processing did not substantially improve their incorporation

into micelles. A combination of PEF application and fat addition would be useful in order to enhance carotenoids bioaccessibility.

Table 3. Carotenoid content and bioaccessibility (mean $\pm$ SD) in the non-digested, released and micellar fractions from PEF-treated and untreated carrots.

Carotenoid compounds	Treatment	Non-digested fraction (mg/100 g FW)	Released fraction (mg/100 g FW)	Micellar fraction (mg/100 g FW)	Bioaccessibility (%)		
Phytoene	Untreated	1.83 $\pm$ 0.31	0.41 $\pm$ 0.02	0.33 $\pm$ 0.01	17.24 $\pm$ 2.31		
	PEF-treated	1.57 $\pm$ 0.24	0.42 $\pm$ 0.06	0.33 $\pm$ 0.06	19.96 $\pm$ 0.76		
α -carotene	Untreated	15.83 $\pm$ 1.64	1.44 $\pm$ 0.22	0.72 $\pm$ 0.44	6.98 $\pm$ 2.78		
	PEF-treated	12.19 $\pm$ 2.66	2.72 $\pm$ 1.03	1.20 $\pm$ 0.11	***	9.85 $\pm$ 0.92	**
β -carotene	Untreated	37.60 $\pm$ 5.20	4.26 $\pm$ 1.29	2.33 $\pm$ 0.90	7.63 $\pm$ 2.55		
	PEF-treated	35.42 $\pm$ 4.82	6.98 $\pm$ 2.47	* 4.20 $\pm$ 0.32		11.99 $\pm$ 0.90	*
Lutein	Untreated	0.06 $\pm$ 0.01	0.011 $\pm$ 0.008	0.015 $\pm$ 0.001	23.56 $\pm$ 0.53		
	PEF-treated	0.08 $\pm$ 0.03	0.020 $\pm$ 0.004	0.018 $\pm$ 0.007	23.99 $\pm$ 5.84		
Total carotenoids	Untreated	50.82 $\pm$ 7.65	6.83 $\pm$ 1.08	3.38 $\pm$ 0.29	6.61 $\pm$ 0.59		
	PEF-treated	48.54 $\pm$ 8.31	10.14 $\pm$ 1.70	* 5.77 $\pm$ 0.53	**	11.89 $\pm$ 1.06	**

Asterisks indicate significant difference with respect to untreated carrot (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Carotenoid bioaccessibility from untreated carrots varied between individual compounds: lutein (23.6 %) > phytoene (17.2 %) > α - (7 %) and β -carotene (7.6 %). PEF treatment significantly increased total carotenoids bioaccessibility (11.9 %) compared to that obtained in untreated carrots (6.6 %) and those of some individual compounds. Phytoene and lutein were the most bioaccessible compounds in both PEF-treated and untreated carrots (Table 3). After PEF application, bioaccessibility of α -carotene and β -carotene was increased by 59.1 % and 58.0 %, respectively.

Our results are in close agreement with the literature showing that individual carotenoids are differently absorbed depending on their hydrophobicity, molecular structure and deposition form. Generally, the lower the hydrophobicity, the greater their bioaccessibility. Nevertheless, phytoene bioaccessibility is mainly enhanced by its flexible molecular structure[4,7,54,55] and its

deposition form in plastoglobules.[56,57] Phytoene bioaccessibility has been scarcely studied in raw matrices although some results are available in derived products. For instance, this compound was the most bioaccessible carotenoid in carrot juice[52] and in a low-fat tomato puree obtained from PEF-treated tomatoes.[29]

To the best of our knowledge, this is the first study evaluating the bioaccessibility of carotenoids when PEF treatments are applied to whole carrots. Carotenoid bioaccessibility is strongly influenced by factors related to matrix. Specifically, carrots are composed of fibrous and compact cell walls (thickness in a range of 424-954 nm), with a high content in pectin.[58] The increase in carotenoids bioaccessibility from PEF-treated carrots may suggest that electroporeabilisation modifies structural characteristics of the tissular matrix through pores formation and rearrangements in cells distribution, eventually allowing a better release of carotenoids due to changes in their intracellular location (Figures 2 and 3).[53] Besides, during digestion, pectin may also interact with bile salts or digestive enzymes involved in the formation of micelles, which comprises carotenoids bioaccessibility.[58,59] In this line, PEF could also activate some pectic enzymes [e.g. pectin methylesterase (PME) and polygalacturonase (PG)] related to tissue softening, which would be helpful for a better transference of carotenoids to micelles.

3.5. Effect of PEF on phenolic compounds content

Carrot phenolic profile is mainly composed by hydroxycinnamic acids, being 5-caffeoylquinic acid, coumaroylquinic acid, 5-feruloylquinic acid and ferulic acid caffeoyl glucoside the most abundant (Table 4). Caffeic acid derivatives and especially 5-caffeoylquinic acid, showed high sensitivity to PEF treatment. Hence, their content diminished between 18.1 % and 72.3 %. On the other hand, no detrimental effect was observed in most ferulic acid derivatives, excepting ferulic acid glucoside, ferulic acid coumaroyl glucoside and ferulic acid caffeoyl glucoside, which decreased their content by 66.3 %, 25.5 % and 36.2 %, respectively. On the contrary, some phenolic compounds clearly increased after PEF: coumaric acid (163.2 %), caffeoylferuloylquinic acid (422.6 %), caffeic acid arabinoside glucoside (94.5 %), ferulic acid (116 %), 3-feruloylquinic acid (27.8 %) and feruloylquinic acid derivative (30.6 %).

Application of PEF causes electroporeabilisation of cell membranes, leading to microstructural modifications in carrot matrix (Figures 2 and 3), which seem to be directly related to changes in phenolic content. On the one hand, these rearrangements in tissues may entail greater contact between

Table 4. Phenolic compounds bioaccessibility and content (mean $\pm$ SD) in the non-digested and dialysed fractions from PEF-treated carrots.

Phenolic compound	Treatment	Non-digested fraction (mg/ kg DW)		Dialysed fraction (mg/ kg DW)		Bioaccessibility (%)	
Coumaric acid	Untreated	0.26 ± 0.03		2.62 ± 0.25		100	
	PEF-treated	0.68 ± 0.07	***	2.22 ± 0.06	**	100	
Coumaroylquinic acid	Untreated	16.49 ± 2.78		11.65 ± 1.15		70.62 ± 15.82	
	PEF-treated	8.80 ± 1.52	***	4.84 ± 0.24	**	55.08 ± 9.34	
Coumaric acids and derivatives	Untreated	16.75 ± 2.80		14.26 ± 1.36		85.16 ± 18.60	
	PEF-treated	9.47 ± 1.56	***	7.07 ± 0.27	**	74.59 ± 11.72	
Caffeic acid	Untreated	0.33 ± 0.04		nd ¹		0	
	PEF-treated	0.27 ± 0.04	*	nd ¹		0	
Caffeic acid arab/xiloside	Untreated	0.25 ± 0.03		0.04 ± 0.01		14.85 ± 1.57	
	PEF-treated	0.19 ± 0.03	*	0.03 ± 0.01	*	16.57 ± 3.75	
Caffeoylshikimic acid	Untreated	0.06 ± 0.01		0.04 ± 0.01		68.68 ± 18.56	
	PEF-treated	0.04 ± 0.01	*	0.04 ± 0.01		100	*
3-caffeoylquinic acid	Untreated	0.2 ± 0.04		0.07 ± 0.01		35.95 ± 7.98	
	PEF-treated	0.11 ± 0.02	***	0.02 ± 0.01	**	17.40 ± 5.70	***
4-caffeoylquinic acid	Untreated	0.94 ± 0.15		0.38 ± 0.06		40.12 ± 9.3	
	PEF-treated	0.46 ± 0.07	***	0.09 ± 0.01	**	20.13 ± 5.18	***
5-caffeoylquinic acid	Untreated	204.37 ± 23.28		7.65 ± 0.86		3.75 ± 0.79	
	PEF-treated	57.58 ± 8.94	***	1.57 ± 0.13	**	2.72 ± 0.55	*
Dicaffeoylquinic acid	Untreated	0.63 ± 0.17		nd ¹		0	
	PEF-treated	0.17 ± 0.05	***	nd ¹		0	
Caffeoylferuoyl-quinic acid	Untreated	0.08 ± 0.03		nd ¹		0	
	PEF-treated	0.43 ± 0.03	***	0.11 ± 0.01	**	25.99 ± 2.16	**
Caffeic acid arabinoside glucoside	Untreated	0.07 ± 0.01		0.05 ± 0.01		69.14 ± 14.51	
	PEF-treated	0.14 ± 0.01	***	0.05 ± 0.01		36.40 ± 11.10	**
Caffeic acid Glu Acetyl glucoside	Untreated	3.17 ± 0.41		1.12 ± 0.05		35.50 ± 5.52	
	PEF-treated	3.51 ± 0.34		1.03 ± 0.06	*	29.29 ± 3.94	*
Caffeic acid derivative	Untreated	0.07 ± 0.01		nd ¹		0	
	PEF-treated	0.05 ± 0.01	*	nd ¹		0	
Caffeic acids and its derivatives	Untreated	210.98 ± 25.29		9.36 ± 0.92		4.43 ± 0.89	
	PEF-treated	62.94 ± 9.42	***	2.94 ± 0.13	**	4.67 ± 0.83	
Ferulic acid	Untreated	0.48 ± 0.06		0.48 ± 0.02		96.46 ± 7.29	
	PEF-treated	1.03 ± 0.09	***	0.63 ± 0.04	**	60.63 ± 7.36	***
Isoferulic acid	Untreated	0.14 ± 0.02		0.09 ± 0.01		61.64 ± 14.61	
	PEF-treated	0.12 ± 0.03		0.17 ± 0.01	***	100	***
3-feruloylquinic acid	Untreated	0.15 ± 0.02		0.33 ± 0.01		100	
	PEF-treated	0.19 ± 0.03	*	0.32 ± 0.02		100	
4-feruloylquinic acid	Untreated	1.22 ± 0.10		0.97 ± 0.04		47.38 ± 5.38	
	PEF-treated	1.27 ± 0.24		0.92 ± 0.06		43.09 ± 9.04	
5-feruloylquinic acid	Untreated	9.43 ± 0.79		4.47 ± 0.19		79.37 ± 9.29	
	PEF-treated	10.58 ± 1.77		4.56 ± 0.14		72.32 ± 18.97	
Ferulic acid glucoside	Untreated	0.30 ± 0.04		0.08 ± 0.01		25.57 ± 5.75	
	PEF-treated	0.10 ± 0.01	***	0.05 ± 0.01	***	52.21 ± 7.84	***
Ferulic acid derivative	Untreated	1.04 ± 0.08		0.35 ± 0.02		33.28 ± 3.31	
	PEF-treated	1.00 ± 0.13		0.33 ± 0.03		32.90 ± 5.65	
Ferulic acid coumaroyl glucoside	Untreated	1.33 ± 0.27		0.60 ± 0.03		45.10 ± 9.02	
	PEF-treated	0.99 ± 0.18	*	0.49 ± 0.04	***	49.28 ± 10.55	
Ferulic acid caffeoyl glucoside	Untreated	7.41 ± 0.57		1.59 ± 0.1		21.50 ± 1.82	
	PEF-treated	4.73 ± 0.61	***	1.30 ± 0.13	***	27.52 ± 4.84	*
	Untreated	3.95 ± 0.41		1.61 ± 0.18		40.79 ± 7.32	

Feruloylquinic acid derivative	PEF-treated	4.30 ± 0.60		1.28 ± 0.16	**	29.80 ± 7.16	*
Feruloylquinic acid derivative (2)	Untreated	0.85 ± 0.09		0.23 ± 0.01		27.23 ± 4.19	
	PEF-treated	1.10 ± 0.12	**	0.27 ± 0.01	***	24.63 ± 3.67	
Ferulic acid and its derivatives	Untreated	26.29 ± 1.72		10.80 ± 0.30		41.08 ± 2.92	
	PEF-treated	25.41 ± 3.59		10.31 ± 0.42	*	40.59 ± 7.22	
Quercetin-3-O-Galactoside	Untreated	0.05 ± 0.01		nd ¹		0	
	PEF-treated	0.04 ± 0.01		0.02 ± 0.01	**	48.27 ± 13.67	***
Total phenolic compounds	Untreated	254.07 ± 29.19		34.42 ± 2.37		13.55 ± 2.21	
	PEF-treated	97.87 ± 13.86	***	20.34 ± 0.32	**	20.79 ± 3.31	***

Asterisks indicate significant difference between phenolic content and bioaccessibility from untreated and PEF-treated (5 pulses of 3.5 kV cm⁻¹) carrot (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). 1 nd: Non-detected.

some phenolic compounds and oxidative enzymes [polyphenol oxidase (PPO) or peroxidases (POD)] which would explain the decrease in caffeic acid and its derivatives with an easily oxidizable ortho-diphenol group, absent in ferulic and p-coumaric acids.[60] In this line, the decrease in phenol glycosides and the increase in aglycones may suggest that other enzymes, e.g. hydrolases, may also be involved. A strong reduction in the amount of 5-caffeoylquinic acid was also reported by Ribas-Agustí et al.[47] after submitting apples to 1.8 and 7.3 kJ kg⁻¹, which was not observed after 0.01 kJ kg⁻¹ treatments. This suggests that higher PEF intensities could generate an excessive level of ROS that could degrade some hydroxycinnamic acids. On the other hand, the increase could be attributed to a better extraction due to membrane breakdown.[61] Besides, this structural damage could trigger a plant defence response against abiotic stress, which would activate phenylalanine ammonia lyase (PAL) and the accumulation of phenolic compounds.[62]

3.7. Effect of PEF on phenolic compounds fate after in vitro digestion and bioaccessibility

PEF had a significant influence on the phenolic content of carrots, as well as on their release from the food matrix during in vitro digestion and, consequently, on their bioaccessibility (Table 4). Regarding the sum of coumaric and caffeic acids, their content was, respectively, 50.5 % and 68.6 % lower than that found in the dialysed fraction of untreated carrots. Nevertheless, the dialysed content of ferulic acid derivatives remained similar to that obtained in those untreated. Besides, some individual compounds showed an increase: isoferulic acid (97.7 %), ferulic acid (29.2 %) and feruloylquinic acid derivative (18.1 %). In addition, caffeoylferuloylquinic acid and quercetin-3-O-galactoside were dialysed whereas they were not detected in dialysed fraction from untreated carrots.

A higher content in dialysed fraction does not necessarily entail a greater bioaccessibility, since it must also be calculated according to the amount found in the non-digested fraction. The bioaccessibility of some phenolic compounds in carrots dramatically decreased after PEF application. The most affected compounds were some caffeic acid derivatives, whose content in

the dialyzed fraction declined by 17.5 % - 51.6 %. Besides, ferulic acid, 4-feruloylquinic acid and feruloylquinic acid derivatives also showed diminished bioaccessibility, with values ranging between 8.9 % and 40.3 % below those determined for untreated carrots. Nevertheless, the overall phenolic bioaccessibility showed a considerable increase when PEF was applied. Total phenols from PEF-treated carrots had a bioaccessibility of 20.8 % whereas those of untreated carrots showed a bioaccessibility of 13.6 %. In addition, the bioaccessibility of some individual compounds noticeably increased in comparison to their content in untreated carrots: caffeoylshikimic acid (68.9 %), isoferulic acid (62.2 %), ferulic acid glucoside (104.2 %) and ferulic acid caffeoyl glucoside (28 %). Besides, some compounds became bioaccessible after PEF, such as caffeoylferuloylquinic acid (26 %) and quercetin-3-O-galactoside (48.3 %).

Phenolic compounds bioaccessibility is highly influenced by their chemical structure, concentration in food matrix and interactions to other constituents.[12] Given that the most abundant compounds found in undigested carrots did not match with the highest bioaccessibility rates, it may be suggested that the native content in the food matrix is a secondary factor to consider when optimizing treatments to enhance bioaccessibility of carrot phenolic compounds. On the other hand, those compounds whose content was positively influenced by PEF in undigested fractions had also a higher dialysed content. Therefore, the increment in both fractions could be triggered by changes in their chemical structure and/or microstructural modifications of matrix that lead their better extraction.

Some compounds characterized by a low molecular weight showed greater bioaccessibility in untreated carrots (e.g. ferulic acid). Probably, PEF treatment promoted their release from cells in gastric phase and would be more exposed to be entrapped by other compounds. Hence, the formation of complexes with large molecular weight or their association to dietary fibre is favoured, [63] avoiding their crossing through the dialysis membrane.

4. Conclusions

This study provides a first approach towards the use of PEF as a pre-treatment to obtain carrots with health-promoting properties. Whereas carotenoid content remained unaltered after applying PEF, important changes in phenolic content occur depending on their chemical structure. Caffeic acid decreased between 18 - 72 %, ferulic acid derivatives were generally maintained, and other compounds increased, such as coumaric acid (163 %). Total bioaccessibility was enhanced by 11.9 and 20.8 % for carotenoid and phenolic compounds, respectively. However, individual content and bioaccessibility of carotenoids and phenolic compounds is differently affected depending on their chemical class. These changes in bioaccessibility could also be related to structural modifications caused by PEF. Reported degradation of cell wall and membranes could favour the release of carotenoids and phenolic compounds during digestion leading to some of

them to be more available to be absorbed or, on the contrary, entrapped by other macromolecules. Decreases in hardness may negatively affect sensory quality of carrots. However, PEF could be suitable to obtain fresh-cut products saving production costs, since less force would be needed to cut them while bioaccessibility is enhanced.

Extensive studies have exploited the application of PEF to modify cell membrane permeability for extracting intracellular compounds or modify the textural properties of whole commodities. However, to the best of our knowledge, its implementation as a treatment to enhance bioactive compounds bioaccessibility has been scarcely explored so far. Therefore, assessing the relationship between structural changes caused by PEF and bioactive compounds bioaccessibility is valuable to develop new strategies to obtain food products with high health-related properties. Future studies should be focused on studying the feasibility of applying PEF to enhance bioactive compounds bioaccessibility in different carrot derived products. Likewise, it is necessary to delve into the causes that produce changes in bioaccessibility to successfully apply PEF processing to other vegetable products.

Conflicts of interest

There are no conflicts to declare.

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