Application of pulsed electric fields as a strategy for enhancing the content and bioaccessibility of carotenoids and phenolic compounds in carrots and their derived products

Gloria Mª López Gámez

Universitat de Lleida

TESI DOCTORAL

Application of pulsed electric fields as a strategy for enhancing the content and bioaccessibility of carotenoids and phenolic compounds in carrots and their derived products

Gloria Mª López Gámez

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Directors

Dr. Robert Soliva Fortuny

Dr. Pedro Elez Martínez

Tutor/a

Dr. Robert Soliva Fortuny

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ABSTRACT

The growing demand for minimally processed products with health-promoting properties has triggered an increased interest in developing strategies to obtain products with greater nutritional value. In this sense, the application of pulsed electric fields (PEF) is investigated as an alternative to enhance the antioxidant content of plant-based food products by triggering a stress defence response. Besides, structural changes induced by PEF could favour the release of bioactive compounds during digestion, hence improving their bioaccessibility. The objective of this Doctoral Thesis was to evaluate the effects of PEF on the content and bioaccessibility of carotenoid and phenolic compounds from carrots and their derived products.

On the one hand, the effect of PEF on phenolic and carotenoid content of carrots was investigated. Likewise, their cell viability and quality attributes were assessed. After the selection of PEF treatment conditions, some indicators of stress induction such as enzyme activity [pectinmethylesterase (PME), polygalacturonase (PG), polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia lyase (PAL)] or respiration activity were evaluated during post-treatment time. Both phenolic and carotenoid contents were increased after PEF. The highest phenolic contents were obtained in those carrots treated by 5 pulses of 3.5 kV cm^{-1} (39.5 %) and 30 pulses of 0.8 kV cm^{-1} (40.1 %) and stored 24 h at 4 ºC. The largest increases in carotenoids were reached just after applying 5 pulses of 3.5 kV cm^{-1} (83.8 %). At such conditions, quality attributes were not significantly affected, although weight loss (2.5 %) and cell viability loss (17 %) significantly occurred. Individual compounds were differently affected by PEF intensity and post-treatment time. Hence, phytoene (59.1 – 95.5 %) and β-carotene (36.5 – 91.8 %) increased just after 2 or 3.5 kV cm^{-1}, whereas lutein decreased (15.1 – 96.4 %) after 0.8 or 2 kV cm^{-1}. Likewise, *p*-hydroxybenzoic (94.7 %), chlorogenic acid (74.9 %) and ferulic acid (52.2 %) increased 24 h following the application of 5 pulses of 3.5 kV cm^{-1}, and decreased subsequently. In concomitance, larger CO_2 production, the presence of acetaldehyde and ethanol, the enhancement of PAL activity and the alteration of enzyme activities were observed in PEF-treated carrots during post-treatment time.

On the other hand, the impact on microstructure, phenolic and carotenoid bioaccessibility was investigated on PEF-treated carrots, their derived products (juice, puree and oil-added puree) and in a directly PEF-treated oil-added puree. The effect of thermal treatment (T) in such derived products was also studied. Bioaccessibility of each individual compound was influenced by the characteristics of the product matrix, the nature and structure of the compound, as well as the applied processing treatments. Degradation of cell wall, decrease in particle size and oil addition favoured carotenoid bioaccessibility in the following way: PEF- and T-treated oil-added purees (20.4 %) > T-treated oil-added purees obtained from PEF-treated carrots (7.8 %) > T-treated purees obtained from PEF-treated carrots (3.3 %) > T-treated juices obtained from PEF-treated carrots (0.6 %). Lutein and phytoene had higher bioaccessibility than α-carotene and β-carotene. Bioaccessibility of phenolic compounds was mainly dependent on their chemical structure and matrix, whereas oil presence and decrease in particle size did not necessarily improved their bioaccessibility. The highest bioaccessibility was reported in PEF-treated oil-added purees as well as in purees from PEF-treated carrots (100 %). Ferulic and coumaric acid derivatives were more bioaccessible than caffeic acid derivatives.

The results obtained in this work evidence the feasibility of PEF to obtain carrot and derived products with enhanced carotenoid and phenolic bioaccessibility. Therefore, these results provide an innovative approach for developing healthier carrot derived products.

RESUMEN

La creciente demanda de productos mínimamente procesados con propiedades beneficiosas para la salud ha provocado que aumente el interés por desarrollar estrategias para obtener productos con mayor valor nutricional. En este sentido, se investiga la aplicación de pulsos eléctricos (PE) como alternativa para potenciar el contenido de antioxidantes de los productos alimenticios de origen vegetal al desencadenar una respuesta de defensa al estrés. Además, los cambios estructurales inducidos por los PE podrían favorecer su liberación durante la digestión, y, por tanto, mejorar su bioaccesibilidad. El objetivo de esta Tesis Doctoral fue evaluar los efectos de los PE sobre el contenido y bioaccesibilidad de carotenoides y compuestos fenólicos de la zanahoria y sus productos derivados.

Por una parte, se investigó el efecto de los PE sobre el contenido fenólico y carotenoide en zanahorias. Asimismo, se evaluaron su viabilidad celular y atributos de calidad. Tras la selección de las condiciones de tratamiento de PE, se evaluaron algunos indicadores de estrés como la actividad enzimática [pectinmetilesterasa (PME), poligalacturonasa (PG), polifenol oxidasa (PPO), peroxidasa (POD), fenilalanina amonio liasa (PAL)] o la actividad respiratoria durante el tiempo de postratamiento. Tanto el contenido de compuestos fenólicos como de carotenoides aumentaron tras aplicar PE. Los máximos incrementos fenólicos se obtuvieron en aquellas zanahorias tratadas con 5 pulsos de 3.5 kV cm^{-1} (39.5 %) y 30 pulsos de 0.8 kV cm^{-1} (40.1 %) y almacenadas 24 h a 4 ºC. Los mayores aumentos de carotenoides se alcanzaron justo tras aplicar 5 pulsos de 3.5 kV cm^{-1} (83.8 %). En tales condiciones los atributos de calidad no se vieron afectados significativamente, aunque se produjeron significativas pérdidas de peso (2.5 %) y de viabilidad celular (17 %). Los compuestos individuales se vieron afectados de manera diferente por la intensidad de los PE y el tiempo postratamiento. Por tanto, el fitoeno (59.1 – 95.5%) y el β-caroteno (36.5 – 91.8 %) aumentaron justo después de 2 o 3.5 kV cm^{-1}, mientras que la luteína disminuyó (15.1 – 96.4 %) después de 0.8 o 2 kV cm^{-1}. Asimismo, los ácidos *p*-hidroxibenzoico (94.7 %), clorogénico (74.9 %) y ferúlico (52.2 %) aumentaron a las 24 h tras aplicar 5 pulsos de 3.5 kV cm^{-1}, mientras que el contenido disminuyó posteriormente. En concomitancia, se observó una mayor producción de CO_2, la presencia de acetaldehído y etanol, el aumento de la actividad PAL y la alteración de la actividad enzimática en las zanahorias tratadas con PE durante el tiempo postratamiento.

Por otro lado, se investigó el impacto en la microestructura, la bioaccesibilidad fenólica y carotenoide de zanahorias tratadas con PE, sus productos derivados (zumo, puré y puré con aceite) y en un puré con aceite tratado directamente con PE. El efecto del tratamiento térmico (T) en dichos productos derivados también se estudió. La bioaccesibilidad estuvo influenciada por las características de la matriz del producto, la naturaleza y estructura del compuesto individual, así como los tratamientos de procesado aplicados. La degradación de la pared celular, la disminución del tamaño de partícula y la adición de aceite favorecieron la bioaccesibilidad de los carotenoides de la siguiente forma: purés con aceite añadido tratados con PE y T (20.4 %) > purés con aceite añadido tratados con T y obtenidos de zanahorias tratadas con PE (7.8 %) > purés sin aceite tratados con T y obtenidos de zanahorias tratadas con PE (3.3 %) > zumo tratado con T y obtenido de zanahorias tratadas con PE (0.6 %). La luteína y el fitoeno tuvieron mayor bioaccesibilidad que el α-caroteno y β-caroteno. La bioaccesibilidad de los compuestos fenólicos dependió principalmente de su estructura química y la matriz, mientras que la presencia de aceite y la disminución del tamaño de las partículas no necesariamente mejoraron su bioaccesibilidad. La mayor bioaccesibilidad de compuestos fenólicos se registró en los purés con aceite tratados con PE, así como en los purés sin aceite obtenidos a partir de zanahorias tratadas con PE (100 %). Los

derivados del ácido ferúlico y cumárico fueron más bioaccesibles que los derivados del ácido cafeico.

Los resultados obtenidos en este trabajo evidencian la viabilidad de los PE para obtener zanahorias y productos derivados con mayor bioaccesibilidad de carotenoides y fenoles. Por lo tanto, estos resultados proporcionan un enfoque innovador para desarrollar productos derivados de zanahoria más saludables.

RESUM

La creixent demanda de productes mínimament processats amb propietats beneficioses per a la salut ha fet que augmenti l'interès per desenvolupar estratègies per obtenir productes amb major valor nutricional. En aquest sentit, s'investiga l'aplicació de polsos elèctrics (PE) com alternativa per millorar el contingut d'antioxidants dels productes alimentaris d'origen vegetal en desencadenar una resposta de defensa a estrès. A més, els canvis estructurals induïts pels PE podrien afavorir el l'alliberament de compostos bioactius durant la digestió i millorar la seva bioaccessibilitat. L'objectiu d'aquesta Tesi Doctoral ha estat avaluar els efectes dels PE sobre el contingut i la bioaccessibilitat de carotenoides i components fenòlics de la pastanaga i els seus productes derivats.

D'una banda, es va investigar l'efecte de PE sobre el contingut fenòlic i carotenoide en pastanaga. Així mateix, es va avaluar la seva viabilitat cel·lular i atributs de qualitat. Després de la selecció de les condicions del tractament amb PE, es van avaluar alguns indicadors d'inducció d'estrès com l'activitat enzimàtica [pectinmetilesterasa (PME), poligalacturonasa (PG), polifenol oxidasa (PPO), peroxidasa (POD), fenilalanina amoníac liasa (PAL)] o l'activitat respiratòria durant el temps posterior al tractament. Tant el contingut en compostos fenòlics com el contingut en carotenoides van augmentar després de l'aplicació dels PE. Els màxims increments en compostos fenòlics s'obtingueren en les pastanagues tractades amb 5 polsos de 3.5 kV cm^{-1} (39.5 %) i 30 polsos de 0.8 kV cm^{-1} (40.1 %) emmagatzemades durant 24 h a 4 ºC. Els majors augments de carotenoides es van assolir tot just després d'aplicar 5 polsos de 3.5 kV cm^{-1} (83.8 %). Sota aquestes condicions els atributs de qualitat no es van veure afectats significativament, encara que es produïren pèrdues de pes (2.5 %) i de viabilitat cel·lular (17 %). Els compostos individuals es van veure afectats de manera diferent per la intensitat del tractament dels PE i el temps post-tractament. Per tant, fitoè (59.1 – 95.5 %) i β-carotè (36.5 – 91.8 %) augmentaren just després de l'aplicació d'intensitats de camp de 2 o 3.5 kV cm^{-1}, mentre que la luteïna va disminuir (15.1 – 96.4 %) després de l'aplicació de 0.8 o 2 kV cm^{-1}. Així mateix, els continguts en àcids *p*-hidroxibenzoic (94.7 %), clorogènic (74.9 %) y ferúlic (52.2 %) van augmentar després de 24 h d'aplicar 5 polsos de 3.5 kV cm^{-1}, per minvar posteriorment. A més, es va observar una major producció de CO_2, la presència d'acetaldehid i etanol, l'augment de l'activitat PAL i l'alteració de l'activitat enzimàtica en pastanagues tractades amb PE durant el temps posterior al tractament.

Per una altra part, es va investigar l'impacte sobre la microestructura, la bioaccessibilitat de compostos fenòlics i carotenoides en pastanagues tractades amb PE, sobre productes processats a partir d'aquestes (suc, puré i puré amb oli) i sobre un puré amb oli tractat directament amb PE. També es va estudiar l'efecte del tractament tèrmic (T) en aquests productes derivats. La bioaccessibilitat de cada compost es va veure influenciada per les característiques de la matriu, la naturalesa i estructura del compost en qüestió, i les condicions de processament. La degradació de la paret cel·lular, la disminució de la mida de la partícula i l'adició d'oli va afavorir la bioaccessibilitat dels carotenoides en se següent ordre: purés amb oli afegit tractats amb PE i T (20.4 %) > purés amb oli afegit tractats amb T i obtingut de pastanaga tractat amb PE (7.8 %) > purés tractats amb T i obtingut de pastanaga tractat amb PE (3.3 %)> suc tractat amb T i obtingut de pastanaga tractat amb PE (0.6 %). La luteïna i el fitoè van presentar una major bioaccessibilitat que α-carotè i β-carotè. La bioaccesibilitat dels fenols depengué principalment de la seva estructura química i de la matriu alimentària, mentre que la presència d'oli i la disminució d la mida de les partícules no necessàriament comportà una millora en la seva bioaccessibilitat dels compostos fenòlics. La bioaccessibilitat més alta es va informar en purés amb oli afegit tractats

amb PE, així com en purés de pastanagues tractades amb PE (100 %). Els derivats de l'àcid ferúlic i cumaric foren més bioaccessibles que els derivats de l'àcid cafeic.

Els resultats obtinguts en aquest treball evidencien la viabilitat dels polsos elèctrics per obtenir pastanagues i productes derivats amb major bioaccessibilitat del seu contingut en carotenoides i compostos fenòlics. En conclusió, aquests resultats proporcionen un enfocament innovador per desenvolupar productes derivats de pastanaga més saludables.

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INTRODUCTION

INTRODUCTION

1. Carrot and derived products

Carrot (*Daucus carota* L.) is an economically important horticultural crop worldwide that belongs to *Apiaceae* family. Some varieties are easily differentiated because of their colour. Those most representative are orange carrots, characterized by their high β-carotene content, although purple (high anthocyanin content) and yellow (high lutein content) carrots are also popular. Despite their low energy density, it is considered a primary vegetable in countries such as China, Russia or United States, which produce nearly 50 % of the global production (Arscott and Tanumihardjo, 2010). According to the Ministry of Agriculture and Fisheries, Food and the Environment of Spain (MAPA, 2019), Cádiz, Segovia, Valladolid, Alicante and Sevilla produce about 98 % of the Spanish carrot, about 384,295 tonnes.

Carrots are usually consumed as fresh product, although it is commercialized in fresh-cut form (sliced, diced, grated), canned, dehydrated, as juice or beverage (Sharma et al., 2012). This crop has gained popularity in recent decades due to awareness of its nutritional value. Likewise, new products in the market such as organic carrots or packs containing yellow, purple and orange carrots have increased the consumption of fresh carrots in United States, that reach the highest since 1997 (Lucier and Parr, 2020).

Carrots are composed by water (88 %), protein (1 %), carbohydrate (7 %), fat (0.2 %), and fibre (3 %) (USDA, 2019), although their nutrient content vary with cultivar, season, environmental conditions and maturity (Arscott and Tanumihardjo, 2010). Their major carbohydrates are sucrose, glucose and fructose. The insoluble fibres, cellulose and hemicellulose, constitute the greatest portion (50 % – 92 %) of the total dietary fibre, whereas the rest correspond to soluble fibres such as fermentable hemicellulose and pectin (Arscott and Tanumihardjo, 2010). Consumption of carrots significantly contributes to reach the recommended daily intake of dietary vitamin A thanks to their great content in α-carotene and β-carotene. Likewise, carrots are also a good source of phenolic compounds, vitamins and polyacetylenes. However, this Thesis will focus on their carotenoids and phenolic compounds.

2. Carotenoid compounds in carrots

Carotenoids are lipophilic pigments consisting of a polyene chain with 11 conjugated double bonds and a β ring at each end of the molecule. Chemically, carotenoids can be divided into xantophylls, which contain one or more oxygenated groups in their structure (e.g. lutein and

zeaxanthin), and carotenes, that are unoxygenated carotenoids (e.g. lycopene and β-carotene) (Nagarajan et al., 2017). Carotenoids can be found as *cis* or *trans* isomers due to their structure rich in conjugated doubled bonds. Long-chain carotenoids are much more prone to oxidization and isomerization, which could occur during processing and storage (Nagarajan et al., 2017).

In plants, carotenoids are usually stored in chromoplasts, where they are also biosynthesized. Their biosynthesis pathway consists of five main stages in which specific enzymes and active phosphate groups are involved: Formation of isopentyl diphosphate (IPP); formation of phytoene from IPP; desaturation of phytoene to lycopene; cyclisation; and xanthophyll formation (**Figure I.1**).

IPP is the precursor for carotenoids formation derived from mevalonic acid or pyruvate and D-glyceraldehyde-3 phosphate. Thereafter, geranylgeranyl pyrophosphate (GGPP) is formed by an elongation reaction, and phytoene is obtained by condensation of two GGPP. Phytoene can be further desaturated to phytofluene, then to ζ-carotene and lycopene. One or both ends of the lycopene molecule can then undergo cyclisation to form α or β ionone rings, which lead to form α-, β-, ɣ-, δ-, or ε-carotene. These carotenes can then be hydroxylated to form xanthophylls such as lutein or zeaxanthin (Bohn, 2008).

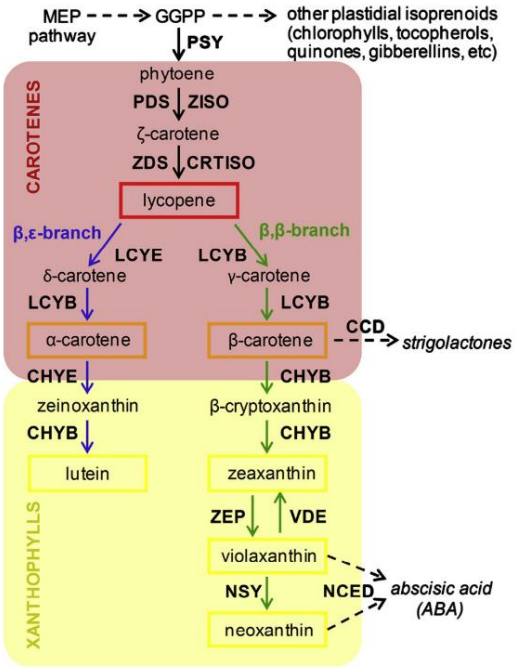


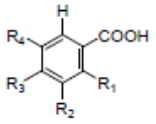
Figure I.1. Pathway for carotenoid biosynthesis in plants. Methylerythritol 4- phosphate (MEP); geranylgeranyl diphosphate (GGPP). Dashed arrows represent multiple steps. The main carotenoids found in plant tissues are boxed. Enzymes are indicated in bold. Phytoene synthase (PSY); phytoene desaturase (PDS); 15-cis- ζ –carotene isomerase (ZISO); ζ –carotene desaturase

(ZDS); carotenoid (pro-lycopene) isomerase (CRTISO); lycopene β-cyclase (LCYB); lycopene ε-cyclase (LCYE); carotenoid β-hydroxylase (CHYB); carotenoid ε-hydroxylase (CHYE); zeaxanthin epoxidase (ZEP); violaxanthin deepoxidase (VDE); neoxanthin synthase (NSY); carotenoid cleavage dioxygenase (CCD); 9-cis-epoxycarotenoid dioxygenase (NCED) (Rodriguez-Concepcion and Stange, 2013).

The most abundant carotenoid found in carrot is β-carotene, considered an essential micronutrient for its antioxidant activity and its ability to act as a provitamin A. Lutein and α-carotene are also present in a lesser extent (Granado-Lorencio et al., 2007). Lutein is a xanthophyll with polar characteristics that is accumulated in the eye and reduced the risk of macular degeneration (Schweiggert & Carle, 2017), whereas α-carotene is also a pro-vitamin A carotenoid.

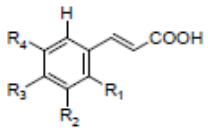
3. Phenolic compounds in carrots

Phenolic compounds are characterized by the presence of one or more aromatic rings, which include at least a hydroxyl group. It is a heterogeneous group that can be classified based on their structure: phenolic acids, flavonoids, coumarins, stilbenes or lignans. Main phenolic compounds found in carrots are hydroxycinnamic and hydroxybenzoic acids (**Figure I.2**), being chlorogenic acid the most abundant (42 – 61 %). Generally, phenolics are stored as free form in vacuoles or bound to cell wall components in ester form (37 %). Thus, they are differently distributed in tissues: peel > phloem > xylem (Arscott and Tanumihardjo, 2010).



Hydroxybenzoic acid

Name	R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH
p-hydroxybenzoic acid	H	H	OH	H
Protocatechuic acid	H	OH	OH	H



Hydroxycinnamic acid

Name	R_1	R_2	R_3	R_4
Caffeic acid	H	OH	OH	H
p-coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H

Figure I.2. Chemical structure of main phenolic compounds in carrots.

Phenolic compounds present in plants are synthetized during their development or after detecting a biotic (e.g. microbial invasion) or abiotic (e.g. wounding, UV) stress. Regarding carrots, they specially accumulate chlorogenic acid and *p*-hydroxybenzoic acids during storage when tissues are wounded (e.g. shredding) (Surjadinata and Cisneros-Zevallos, 2012). Phenolic compounds biosynthesis depends on L-phenylalanine availability since it is the main amino acid used as precursor for the biosynthesis of phenylpropanoids (Becerra-Moreno et al., 2015). L-phenylalanine is biosynthesized by the shikimic acid pathway, which is part of the primary metabolism of plants. Phenylalanine ammonia lyase (PAL) is the branch point enzyme between primary (shikimic acid pathway) and secondary metabolism (phenylpropanoid pathway) and the key enzyme in phenolic compounds biosynthesis (Dixon and Paiva, 1995). Severa1 simple phenylpropanoids (with the carbon skeleton of phenylalanine) are produced from cinnamate via a series of hydroxylation, methylation, and dehydration reactions; these include *p*-coumaric, caffeic, ferulic, synaptic acids and simple coumarins. Free acids are usually conjugated to sugars, cell wall carbohydrates or organic acids (e.g. chlorogenic acid) instead of accumulating in free form (Dixon and Paiva, 1995) (**Figure I.3**).

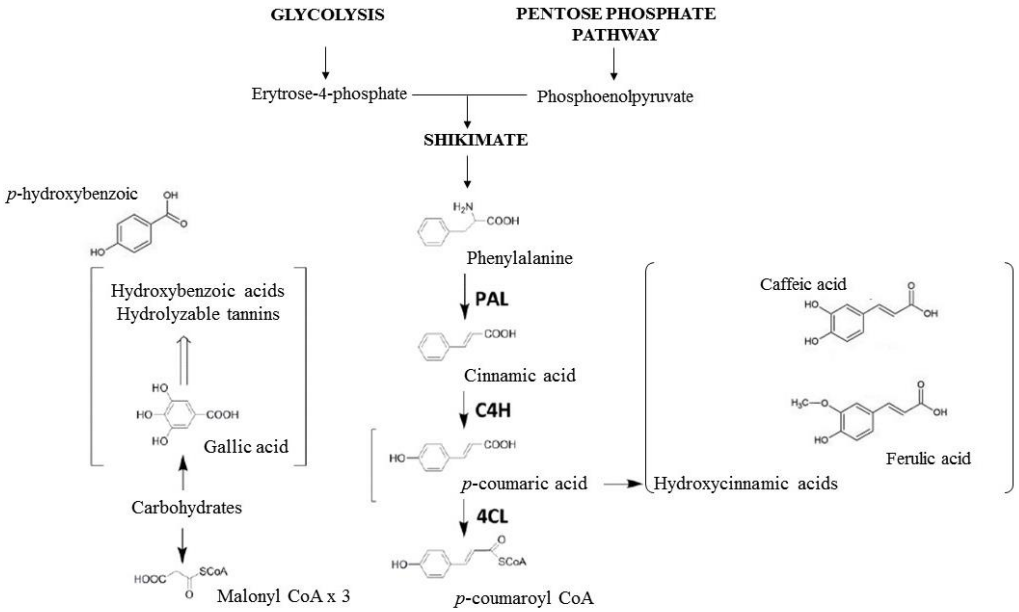


Figure I.3. Biosynthesis pathway scheme of the most common phenolic compounds found in carrots. Phenylalanine ammonia lyase (PAL), cinammate 4 hydrolase (C4H) and 4-coumaroyl CoA ligase (4CL). Adapted from Balaša (2014).

4. Health-related effects of carotenoids and phenolic compounds

Bioactive compounds such as carotenoids and phenolic compounds are not synthesized by human metabolism, hence their dietary consumption is essential to health. Fruit and vegetables are considered as products rich in such substances. Thus, their consumption has been related to lower incidence of cancer, diabetes, some cardiovascular and degenerative diseases (Bohn, 2014; Rao and Rao, 2007). In addition, phenolic compounds such as chlorogenic acid or provitamin A carotenoids like α-carotene and β-carotene act as antioxidants scavenging reactive oxygen species (ROS) (Heo et al., 2007; Maiani et al., 2009) both in plant and human body. In addition, lutein intake is essential for the visual system, preventing macular degeneration and from photooxidative damage (Schweiggert & Carle, 2017). Nevertheless, both phenolic and carotenoid compounds need to be released from the matrix in which they are embedded in order to exert their positive effects in the organism.

5. Bioaccessibility

Bioaccessibility is defined as the amount of a compound that is released from the food matrix into the gastrointestinal tract and is available for absorption, therefore, it is roughly more relevant than their content in a food matrix (Rodríguez-Roque et al., 2014). *In vitro* models have

been widely used for determining bioaccessibility of bioactive compounds, given that they allow simulate *in vivo* conditions (pH changes, electrolytes presence and enzyme actions) while being cost-effective, rapid and reproducible. Furthermore, it has been checked that *in vitro* results correlated well with those obtained *in vivo* (Brodkorb et al., 2019). A static *in vitro* digestion model proposed by Minekus et al., (2014) has been incorporated in most bioaccessibility studies.

In general, bioaccessibility depends on bioactive compound content and chemical structure, matrix properties and interactions during digestion. Food processing or the addition of adjuvants (e.g. milk or oil) have the potential to modify these characteristics, and therefore, improve bioactive compounds bioaccessibility. In the next sections, the factors affecting carotenoids and phenolic compounds bioaccessibility will be discussed.

5.1. Factors affecting carotenoids bioaccessibility

Carotenoids are lipophilic compounds that require their release from the food matrix, their solubilization and incorporation into micelles for absorption during digestion. However, within these events, there are diverse factors that may interfere with their bioaccessibility: food matrix and its structure, their concentration, deposition and distribution in chromoplasts, their chemical structure or their linkages to other constituents (dietary fibre, proteins, carbohydrates, among others). Some of them can be positively or negatively affected by processing technologies, cooking methods, the addition of adjuvants (oil, milk, …) or ultimately the combination of these factors.

Previous studies have proved that the break or weaken of natural barriers (cell wall, membrane and chromoplasts) is essential for releasing carotenoids and facilitate their solubilization into the micellar phase, thus improving carotenoids bioaccessibility (Hornero-Méndez & Mínguez-Mosquera, 2007; Palmero et al., 2013; Svelander, 2011). Hence, numerous research works have related a smaller particle size with carotenoid bioaccessibility (Knockaert et al., 2012a; Lemmens et al., 2010; Moelants et al., 2012). Therefore, food processing has become a valuable tool to this purpose as reported by numerous authors (Cilla et al., 2018). Thermal processing (pasteurization and sterilization) has been conventionally applied in order to obtain microbiologically safe products while some studies demonstrated that bioaccessibility may be also improved. However, high temperatures may lead to degradation or isomerization of carotenoids while causing detrimental effects on sensory and nutritional quality attributes. Non-thermal processing technologies such as pulsed electric fields (PEF) have been proposed as an alternative, as will be further discussed.

Individual carotenoids are differently absorbed depending on intrinsic characteristics such as their molecular structure and deposition form. Generally, those carotenoids characterized by a linear shape and high number of double bonds have more possibilities of aggregation (Meléndez-Martínez et al., 2014), which explains the lower bioaccessibility of *trans* isomers. In addition, those compounds with a more flexible structure are usually easily absorbed (e.g. phytoene) (Granado-Lorencio et al., 2007; Sy, Gleize, Dangles, Landrier, Vayrat & Borel, 2012). Furthermore, Tyssandier et al., (2001) suggested that less hydrophobic compounds (e.g. xanthophylls) would be easily transferred to micelles due to their location in surface of lipid droplets, which is supported by several studies (Mapelli-Brahm et al., 2017; Schweiggert et al., 2012; Sy, Gleize, Dangles, Landrier, Veyrat and Borel, 2012). Nevertheless, carotenoid bioaccessibility is not only dependent on the food matrix or chemical structure**.** It has been reported that the same product may contain carotenoids that, even presenting a similar structure, differ in their absorption. This is the case of ζ-carotene and lycopene, which, in tomato products, are highly and poorly bioaccessible compounds, respectively; this difference in absorption could be related to their deposition form as reported Panozzo et al., (2013) in tomato varieties or Palmero et al., (2013) in tomato and carrot varieties. Carotenoids are synthesized and stored in diverse types of chromoplasts and deposited also differently (e.g. solid crystalloid, plastoglobuli) depending on the food product. Their physical state could prevent their absorption during digestion (crystalloid aggregates) such as in carrots and tomatoes or could enable an efficient release and assimilation (globular-tubular form), like in butter squash or sweet potato (Jeffery et al., 2012; Jeffery et al., 2012; Schweiggert et al., 2012).

Additionally, cell properties (membrane thickness, size, organization) and linkages with other compounds also play an important role. Jeffery et al., (2012) established that fibrous cell wall, compact cell organization and small cell size may reduce bioaccessibility as well as the presence of large amounts of dietary fibre, which impairs micelles formation, thereby blocking carotenoids absorption in small intestine (Palafox-Carlos et al., 2011). This effect is clearly observed in raw products, but also some types of processing are prone to modify the rheological properties of liquid matrices.

Regarding the effect of adjuvant addition, several studies have demonstrated that oil incorporation during processing or digestion is essential to enhance carotenoids bioaccessibility, because it promotes their micellarization. It has been reported that the lipids with a high unsaturation degree and a long chain length of fatty acids (e.g. olive oil) are more beneficial to bioaccessibility than those of short or middle length (e.g. coconut oil). Besides, both the type and quantity of added oil is crucial to enhance bioaccessibility, which is highly related to lipid hydrolysis mechanism (Huo et al., 2007). Monoacylglycerols and free fatty acids contribute to

the formation of mixed micelles that encapsulate carotenoids when lipid load is low. However, when lipid load is higher, the hydrolysis of triglycerides is incomplete, which leads to form a lipid phase that entraps carotenoids avoiding their micellarization.

5.2. Factors affecting phenolic bioaccessibility

Phenolic compounds are mostly linked to carbohydrates or organic acids, although some of them are also stored in vacuoles or present in cytosol where they are synthesized (Bohn, 2014). Phenolic bioaccessibility is affected by several factors such as food matrix, chemical structure, interactions with other compounds and food processing. As occurs with carotenoids, in order to be absorbed, phenolic compounds must be first released from the food matrix. Afterwards, they are either assimilated in the small intestine or further fermented in colon if they are linked to dietary fibre. Therefore, disruption of cell walls and cellular compartments as well as decrease in particle size are essential for phenolic release and absorption, which can be achieved through the application of processing technologies. Furthermore, phenolic compounds are easily released from food matrices poor in dietary fibre, such as juices and beverages (Palafox-Carlos et al., 2011).

Processing generally reduces particle size, which has been associated with an enhanced phenolic bioaccessibility (Ribas-Agustí et al., 2018). However, it also enables the creation of new interactions between compounds characterized by the presence of hydrophobic aromatic rings and hydroxyl groups and other macromolecules such as polysaccharides (starch, cellulose and pectin), proteins and lipids (Jakobek, 2015), which would negatively affect bioaccessibility. Liquid products have lower viscosity, proteins and carbohydrates, which may be favourable to avoid interactions during digestion. Nevertheless, phenols would be more exposed to be degraded or transformed than in solid foods (Bohn, 2014).

According to Alminger et al., (2014) several changes in the structure (hydroxylation, methylation, glycosylation) of these compounds occur during digestion, as well as the formation of other derivatives. Likewise, it is important to highlight that a large proportion of polyphenols (e.g. ferulic or caffeic acid) are associated with dietary fibre, which difficult their absorption (Bohn, 2014). Hence, they cannot be extracted with common solvents (e.g. methanol) in undigested foods, but they can be released during digestion due to pH conditions (González-Aguilar et al., 2017).

To the best of our knowledge, the information about the effect of dietary lipids on phenolic bioaccessibility is limited. Although most phenolic compounds are hydrophilic, more

apolar compounds (e.g. curcumin) can be positively affected by the presence of lipids, as mixed micelles can stabilize or protect them (Oliveira et al., 2018; Ortega et al., 2009).

6. Pulsed electric fields (PEF)

PEF is a non-thermal processing technology that consists of delivering pulses (ms or μs) of high voltage energy characterized by intensities between 0.1 – 40 kV cm^{-1} (Soliva-Fortuny et al., 2009) to food products. A PEF system is commonly composed by a high voltage source and a pulse generator, a treatment chamber containing the food product and monitoring devices (**Figure I.4**). The generation of pulsed electric fields requires slow charging and a fast discharging of energy. The charging voltage required to generate pulses of sufficient electric field strength is dependent on the electrode distance. For two parallel plate electrodes the electric field strength (E) is given by Eq. (1):

$$E = \frac{U}{d} \quad (1)$$

where U is the voltage (kV) and d (m) the gap between the electrodes.

Likewise, PEF treatments are characterized by different processing parameters (electric field strength, pulse shape, width and frequency, total treatment time, among others), which must be optimized depending on the purpose that is pursued.

The application of PEF causes the formation of irreversible or reversible pores in cell membrane, a phenomenon known as electropermeabilization (**Figure I.4**). This results from applying an external electrical field that induces an increase in the transmembrane potential due to the accumulation of opposite charges on both sides of the membrane. This fact induces membrane thinning up to the point a critical value is exceeded and pores are formed (Toepfl, Heinz, & Knorr, 2005). Such critical value depends on the applied parameters as well as on the characteristics of the plant material (e.g. cell size and geometry, electrical conductivity, particle size) (Leong et al., 2018a). It has been reported that 1 – 2 kV cm^{-1} application is necessary to achieve critical values in plant cells (Toepfl et al., 2005). Treatments characterized by low intensity induce small pores in comparison to the membrane area, which can be resealed when the external field strength is removed. The application of higher electric field strengths and/or treatment times will result in the formation of large pores, thus leading to irreversible membrane disruption (Angersbach et al., 2000).

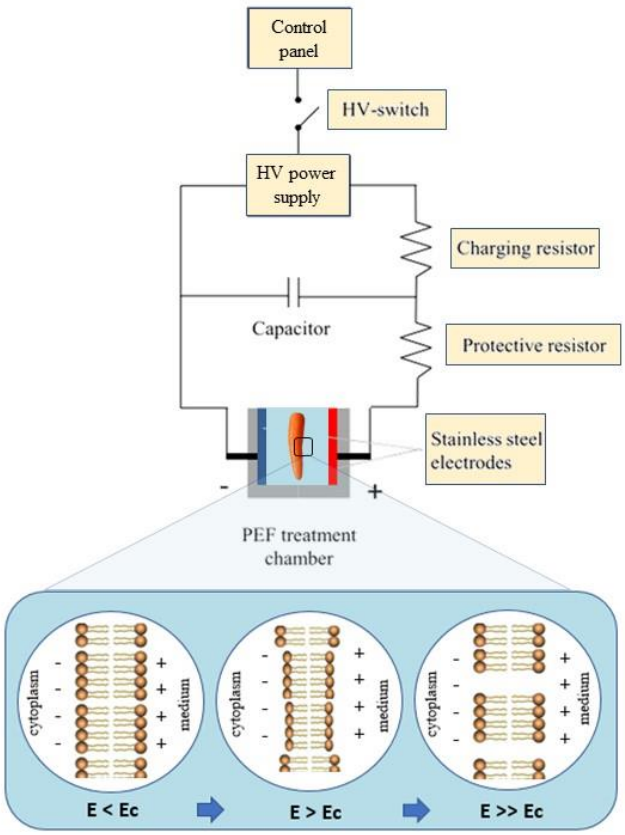


Figure I.4. Scheme of a PEF system [adapted from Martín-Belloso & Soliva-Fortuny (2011)] and mechanism of PEF induced permeabilization.

Therefore, different applications based on the electropermeabilization principle have been developed during the last years. High-intensity (15 – 40 kV cm^{-1}, 40 – 1000 kJ kg^{-1}) treatments have been extensively applied to inactivate microorganisms and enzymes in liquid products. On the other hand, low and moderate intensities (0.1 – 5 kV cm^{-1}, 0.5 – 20 kJ kg^{-1}) have been applied to solid matrices to facilitate the extraction of intracellular compounds or reduce time and energy in mass transference processes such as drying, osmotic dehydration or freezing (Leong and Oey, 2014; Tylewicz et al., 2017). Besides, the application of PEF as a tool to induce stress defence response in plant products or cell cultures is currently under exploration, which would be a good alternative to produce valuable secondary metabolites (Toepfl, Mathys, Heinz, & Knorr, 2006).

6.1. PEF as source of abiotic stress in plant products

Cell membrane permeability changes caused by PEF, leads to think that the cause behind the increase in bioactive compounds content is their enhanced extractability. However, biological membrane disruption is similar to that caused by abiotic stresses such as wounding or pathogen infections. Therefore, the detection of this mechanical damage can also trigger a plant defence

response that leads to accumulate secondary metabolites (e.g. carotenoids or phenolic compounds) to avoid oxidative stress.

Plant metabolism is characterized by their flexibility to adapt to a changing environment. Nevertheless, defence response would be different depending on the duration and intensity of such stress. Excessive intensities would induce irreparable damage in cells, which would prevent the possibility to develop an efficient adaptative response. In this case, cell membrane rupture would likely promote bioactive compounds extractability. On the other hand, low intensities would allow plant cells to start a repairment process by synthetizing antioxidants (e.g. carotenoids or phenolic compounds) or activating other enzymatic mechanisms [e.g. polyphenol oxidase (PPO), peroxidase (POD), superoxide dismutase (SOD)] to overcome cellular damage. Once the hazard is controlled, plant would maintain the production rate or, if the requirements would be too demanding to maintain them throughout time, production level would decay, in some cases reaching cellular death by exhaustion (Balaša, 2014). The mechanisms by which non-thermal technologies induce the biosynthesis of bioactive compounds in plants are still poorly understood, however, some hypotheses have been proposed.

Sabri et al., (1996) described a mechanism about the induction of stress in plant cells by PEF application (**Figure I.5A**). Authors suggest that the cell membrane disruption caused by electropermeabilization would activate the same defence mechanism as a pathogen infection. After its detection, a membrane channel would introduce Ca^{2+} into the cells, an important signalling molecule in stress conditions. Cell disruption caused by PEF would facilitate the Ca^{2+} influx without needing the membrane channel. Then, the increment of intracellular Ca^{2+} would activate a protein kinase that would trigger the synthesis of ROS, required to induce the synthesis of secondary metabolites.

Additionally, Cuéllar-Villarreal et al., (2016) pointed out the importance of differentiating the immediate and late responses. Those events that take place right after the application of the stress are considered within immediate response. For instance, cell membrane disruption and pore formation will lead to a higher extractability of bioactive compounds. In addition, changes in respiration rate and ROS generation are also induced after applying such stress. These signals will cause the overexpression of genes involved in biosynthesis pathways of bioactive compounds. Late response is considered to occur during storage and several hours after applying the stress, which includes changes in enzyme activities or the accumulation of bioactive compounds.

Finally, Jacobo-Velázquez et al., (2017) proposed a common mechanism for non-thermal technologies [PEF, ultrasounds (US) and high-pressure processing (HPP)] showing similarities with wound induced response in plants (**Figure I.5B**). Authors proposed that as an immediate response, ATP would be released from damaged cells and detected by those that are surrounding them, thus eliciting ROS, jasmonic acid (JA) and ethylene generation. These are signalling molecules that would activate transcription factors and the biosynthesis and accumulation of secondary metabolites during storage (late response). Furthermore, Gómez Galindo et al., (2009) demonstrated that PEF-specific response to stress was slightly different from that caused by wounding. Authors evaluated metabolites generated after both stresses (immediately and after 24 h). After PEF application, cells obtained energy through degrading starch and accumulating hexoses to recover membranes properties, which is a typical response when osmoregulation is necessary.

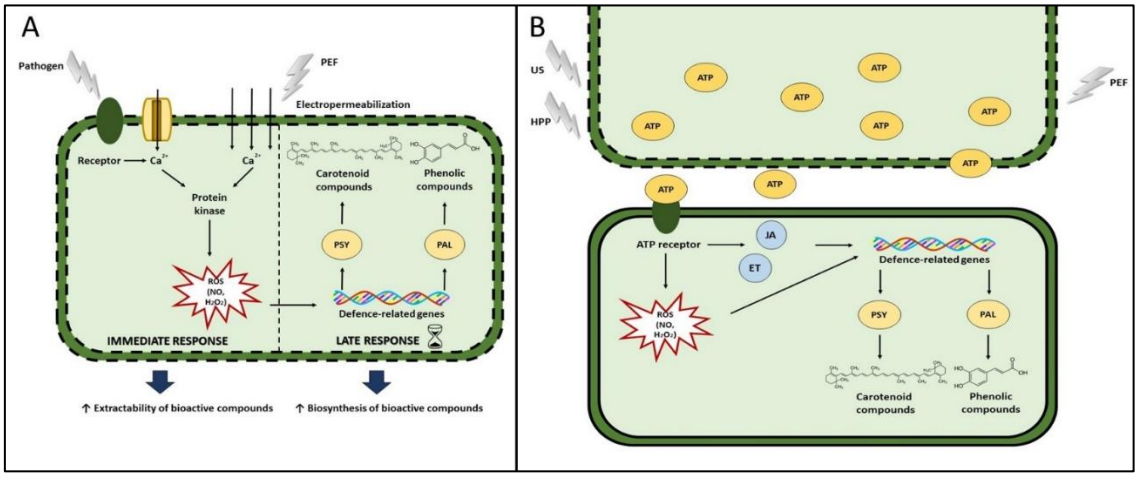


Figure I.5. (A) Hypothetical mechanisms throughout pulsed electric fields (PEF) would trigger a stress defence response and cause accumulation of secondary metabolites [adapted from Sabri et al., (1996)]; (B) PEF, ultrasounds (US) and high pressure processing (HPP) common action mechanism [adapted from Jacobo-Velázquez et al., (2017)]. Phytoene synthase (PSY); phenylalanine ammonia lyase (PAL); adenosine triphosphate (ATP); reactive oxygen species (ROS).

6.2. Impact of PEF on carotenoid and phenolic compounds

Most studies in which PEF are applied to plant products, attribute the increase in bioactive compounds to a greater extraction. Jin et al., (2017) related the increment in phenolic content of PEF-treated blueberries (100 pulses/s of 2 kV cm^{-1} for 2, 4 and 6 min) with their softening and their better extractability. Even though authors provide texture analysis, it would have been

desirable to support these results with physiological data concerning respiration rate or enzyme activities to discard the induction of a stress response.

In some cases, the increase in bioactive compounds content has been related to the induction of a stress response. However, the presence of physiological data (e.g. respiration or enzyme activity) supporting this hypothesis is rarely reported. For instance, Vallverdú-Queralt et al., (2013b) characterized the content of carotenoids and phenolic compounds of PEF-treated tomatoes (0.4 – 2 kV cm^{-1} and 5 – 30 pulses) stored for 24 h. The maximum phenolic enhancement was obtained after applying 30 pulses of 1.2 kV cm^{-1}; caffeic and ferulic acids increased by 140 % and 110 %, respectively. Regarding carotenoids, the highest content was attained after submitting carrots to 5 pulses of 1.2 kV cm^{-1}, when 9-, 13- and 15-*cis*-lycopene were increased by 94 %, 140 % and 33 %, respectively. Nevertheless, the application of 2 kV cm^{-1} (5, 18 and 30 pulses) caused a decrease in some phenolic and carotenoid compounds, suggesting that those treatments may cause lethal damage to cells (Vallverdú-Queralt et al., 2013b). Soliva-fortuny et al., (2017) determined that total phenolics and flavan-3-ols of PEF-treated apples (0.008 kJ kg^{-1}) peaked 24 h after treatment. These authors attributed the increase in carotenoids and phenolic content to a PEF-induced stress response, although analysis concerning firmness, respiration rate, or bioactive content immediately after treatments would have been desirable to differentiate changes caused by an enhanced extractability or promoted biosynthesis.

Different approaches are needed to understand which are the main causes behind changes in bioactive content. Determination of bioactive content just after treatments and throughout post-treatment time, structure modifications (microstructural studies, firmness analysis, cell viability or ion leakage assays), changes in enzyme activities, ROS production, respiration rate, volatile organic compounds generation or gene expression studies are some strategies that would be helpful to elucidate the main causes underpinning these changes in bioactive contents.

González-Casado et al., (2018a) applied PEF (0.4 – 2 kV cm^{-1} and 5 – 30 pulses) to tomato fruit, that were stored for 24 h. Authors determined an increase in respiration rate and production of acetaldehyde and ethylene in PEF-treated tomatoes, which supported the hypothesis of PEF as stress inductor. Nevertheless, softening observed in PEF-treated tomatoes when increasing the electrical intensity would indicate that a better extraction could explain a percentage of the carotenoids increase.

Balaša, (2014) studied the effect of PEF and post-treatment time on phenolic content and the key enzyme in their biosynthesis (PAL). They reported an immediate increase in phenolic content (32 – 65 %) of PEF-treated apples (0.3 – 1.2 kV cm^{-1}), which was attributed to a stress

response. Conversely, decreases in phenolic content were observed after applying a higher electric field strength (4 kV cm^{-1}), which probably caused cell viability loss and degradation of antioxidants. It was also reported that PEF-treated (2 kV cm^{-1}; 4.1 J kg^{-1}) apple cell cultures increased their phenolic content by 20 % and their PAL activity by 54 % after 9 h of applying treatment.

The effect of PEF (0.01, 1.8 and 7.3 kJ kg^{-1}) on quality attributes and phenolic content of apples was also investigated by Ribas-Agustí et al., (2019a). The application of 0.01 kJ kg^{-1} induced a 25 – 26% increase in total phenolics and 43 – 35% in total flavan-3-ols just after and 24 h after treatment. At such intensity authors attributed the immediate phenolic increase to a stress defence response given that firmness was maintained. However, a significant reduction was reported after the application of higher treatment intensities (1.8 and 7.3 kJ kg^{-1}) for both total phenolic content (32 – 43 %) and total flavan-3-ols (19 – 51 %), even though firmness dramatically decreased. Therefore, matrix changes did not enhance phenolic extractability, but their oxidation by oxidative enzymes was likely favoured.

The application of moderate intensity PEF to metabolically active tissues such as fruit and vegetables makes possible to induce the generation of bioactive compounds. On the other hand, scarce information about their application to derived products is available in literature. PEF application would not be useful to trigger bioactive compounds biosynthesis in this type of product. However, PEF treatments could improve the extractability of valuable compounds or even enhance their bioaccessibility.

6.3. Effects of PEF on carotenoid bioaccessibility

Limited information is available concerning the effect of PEF on carotenoids bioaccessibility. Some authors have studied the feasibility to use PEF on whole matrices as a pre-treatment to obtain from them a derived product with enhanced bioactive content and bioaccessibility. For instance, González-Casado et al., (2018b) investigated carotenoids bioaccessibility from low fat tomato purees obtained from PEF-treated tomatoes (0.4 – 2 kV cm^{-1}; 5, 18, 30 pulses; 0.02 – 2.31 kJ kg^{-1}). They obtained whole tomatoes with higher carotenoid contents, which was attributed to two main causes: 1) the triggering of carotenogenic pathways by induction of a stress response and 2) a better extractability caused by structural modifications. From these fruits, they produced low fat purees (5 % olive oil) with an enhanced carotenoid content and higher bioaccessibility of some compounds (**Table I.1**). Nevertheless, some treatments decreased their bioaccessibility, which was explained by a competitive inhibition between carotenoids during micellar incorporation provoked by the elevated content initially

found in matrix. In the same line, Jayathunge et al., (2017) observed an increased bioaccessibility of *trans*-lycopene (6.2 %) and *cis*-lycopene (31 %) immediately and 24 h, respectively, after applying PEF (4 μs of 1 kV cm^{-1}) to whole tomatoes. Thereafter, juices were obtained from those PEF-treated tomatoes and were treated by thermal (95 ºC for 20 min) and non-thermal technologies [US; high intensity pulsed electric fields (HIPEF); US + HIPEF]. US and thermal treatments decreased *trans*-lycopene bioaccessibility, but that of *cis*-lycopene was enhanced. Authors attributed the decrease to the formation of fibre networks that hinder their release during digestion given that *trans*-isomers are easily entrapped than *cis* isoform.

Cis isomers are usually better absorbed than *trans* isomers. Hence, the application of PEF could enhance bioaccessibility since it favours isomerization. Vallverdú-Queralt et al., (2013a) reported that the application of PEF to whole tomatoes improved 15-*cis*-lycopene content (63 – 65 %) in those juices obtained from such tomatoes. These changes were attributed to the activation of phytoene synthase and ζ-carotene desaturase. Similar enhancements in bioaccessibility have been reported in US-treated tomato juices and HPP-treated tomato purees (Knockaert, Pulissery, Colle, et al., 2012; Zhang et al., 2019).

Unfortunately, there is still limited information regarding the application of PEF to whole commodities to improve bioaccessibility of derived products. However, PEF has been combined with heating to understand how these technologies and natural barriers affect carotenoids bioaccessibility. Bot et al., (2018) studied the effect of PEF (7.6 MJ kg^{-1}; 40 – 45 ºC), heating (7.6 MJ kg^{-1}; 85 – 90 ºC) or its combination in tomato fractions (tissue, cell clusters, single cells and chromoplasts). The highest bioaccessibility of β-carotene and all-*trans*-lycopene was observed in chromoplast fraction, which was attributed to the absence of cell wall polysaccharides that impair the action of digestive enzymes. However, bioaccessibility decreased after applying PEF, which was associated to formation of carotenoid-protein complexes (Faulks and Southon, 2005), given that PEF can induce modifications in protein conformation (Perez and Pilosof, 2004). In tomato tissues, all-*trans*-lycopene bioaccessibility decreased after applying heating, which is in accordance to results reported by Jayathunge et al., (2017), whereas β-carotene was not affected, probably related to their different chemical structure (Palmero et al., 2014). In cell clusters or single cells, bioaccessibility was not affected by any treatments, which seems to indicate that the effect of PEF strongly depends on structural barriers and their complexity.

During digestion, pectin characteristics affect the formation of micelles by binding bile salts or interacting with lipase (Cervantes-Paz, Ornelas Paz, Pérez-Martínez, Reyes-Hernández et al., 2016). These properties can be modified by applying processing technologies such as HPP or US (Colle, Van Buggenhout, Van Loey, & Hendrickx, 2010; Panozzo et al., 2013). Buniowska et

al., (2017) investigated the effect of applying same specific energies (32 and 256 kJ kg^{-1}) of PEF and US to a fruit juice sweetened with *Stevia rebaudiana*. They found that total carotenoids were better absorbed after applying both PEF treatments and US (32 kJ kg^{-1}) and attributed these changes to modifications in juice rheological properties that favoured the accessibility of digestive enzymes. Liu et al., (2019) deepened the relationship between pectin characteristics and carotenoids bioaccessibility and found a correlation in which lower methoxylation degree and emulsifying activity of pectin caused a higher bioaccessibility, as high methoxylated pectin was expected to form a closed structure around lipid droplets which restricts lipase access and prevent lipid digestion. However, as far as we know, the relationship among pectin, bioacessibility and PEF processing have not been yet studied.

To the best of our knowledge, few studies about the effect of PEF on carotenoid bioaccessibility of food products with added oil has been reported. However, the application of other non-thermal technologies that also affect cell permeability (e.g. US, HPP) have demonstrated that it can be enhanced as long as cells are disrupted and a fibre network is not formed during processing (Anese et al., 2015; Moelants et al., 2012).

6.4. Effects of PEF on phenolic bioaccessibility

Information about the effect of PEF on phenolic bioaccessibility is rather limited (**Table I.2).** Some authors have investigated phenolic bioaccessibility after applying non-thermal technologies to whole fruit and vegetables. Ribas-Agustí et al., (2019b) evaluated phenolic bioaccessibility of PEF-treated whole apples (0.01, 1.8 and 7.3 kJ kg^{-1}). Generally, more intense treatments caused greater bioaccessibility likely due to cell disruption and induced structural changes, given that an inverse correlation between some individual compounds (5-caffeoylquinic acid, epicatechin and phloretin xyloglucoside) bioaccessibility and apple toughness was found. Some of them were easily released due to microstructural changes caused by electropermeabilization, but others were independent from matrix structure (*p*-coumaroylquinic acid, phloretin glucoside and quercetin derivatives). In addition, phenolic profile and content in non-digested apples varied after storage and PEF treatments, which was also important for bioaccessibility.

Some research works have been performed to study the effect of non-thermal technologies on phenols bioaccessibility of liquid matrices. Buniowska et al., (2017) evaluated phenolic bioaccessibility of a fruit juice composed by mango, papaya and sweetened with *Stevia rebaudiana* after submitting them to PEF or US treatments (32 and 256 kJ kg^{-1}). Juices submitted to PEF (256 kJ kg^{-1}) and US (32 and 256 kJ kg^{-1}) showed higher bioaccessibility than untreated

juices, which was attributed to a better release from the food matrix. In addition, Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, (2015) evaluated the bioaccessibility of phenolic compounds from a fruit beverage after being submitted to HPH (400 MPa for 5 min), PEF (35 kV cm^{-1} for 1800 μs) or thermal treatment (90 ºC for 1 min). Total phenolic bioaccessibility was enhanced after applying any of the compared processing technologies. Nevertheless, individual compounds were differently affected depending on their chemical structure. PEF and HPH improved bioaccessibility of several phenolic substances, whereas the lowest increases were obtained after thermal treatments. Authors proposed that processing caused changes in the phenol structure (hydroxylation, methylation, glycosylation, among others) thus enabling their absorption. Also, their degradation or conjugation may occur during digestion, causing a decrease in bioaccessibility.

As far as we know, limited information is available regarding the effects of oil in the bioaccessibility of phenolic compounds in derived plant-based products. However, some studies have been carried out about the influence of non-thermal processing and milk addition on phenolic bioaccessibility. Obtained results depend on the specific compounds of each fruit juice matrix. Some compounds are prone to interact with milk proteins, leading to form complexes and insoluble aggregates that, consequently, decrease bioaccessibility (He et al., 2016; Quan et al., 2020). Similar results were reported by Rodríguez-Roque, Anco, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, (2015), who reported that the highest phenolic bioaccessibility was obtained in juices containing whole milk, as lipids seemed to interfere with the formation of protein-phenol complexes.

7. Final remarks

Further studies should be focused on developing innovative strategies to obtain food products with enhanced health related properties that contribute to meeting consumer demands. Extensive studies have exploited the application of PEF to modify cell membrane permeability for extracting intracellular compounds, enhance dehydration rate, or modify the textural properties of whole commodities. However, to the best of our knowledge, its implementation as a treatment to enhance bioactive compounds content or their bioaccessibility has been scarcely explored so far. Therefore, delve into relationship between structural changes caused by PEF and bioactive compounds bioaccessibility would be valuable to develop new strategies to provide food products with high health-related properties. Carrot is an economically important crop, considered as a good source of carotenoids and phenolic compounds and highly consumed as a minimally processed product. Therefore, obtaining carrots or derivates with highly bioaccessible compounds is of great interest to population health and even to food industries.

Table I.1. Studies evaluating the effect of pulsed electric fields (PEF) and their combination with non-thermal or thermal treatments on structure, content and bioaccessibility of carotenoid compounds in different vegetal matrices.

Food matrix	Processing conditions		Structure	Content before digestion	Bioaccessibility increase	Bioaccessibility decrease	References
Carrot	PEF (0.9 and 191 kJ kg^{-1}) in water PEF (0.9 and 191 kJ kg^{-1}) in 300 ppm $CaCl_2$		PEF (191 kJ kg^{-1}in water or in 300 ppm $CaCl_2$): Decrease hardness PEF (191 kJ kg^{-1}in $CaCl_2$ + B): Increase hardness	No changes in β-carotene content	No changes	No changes	(Leong, Du, & Oey, 2018)
Tomato puree (5 % olive oil)	PEF (0.02 – 2.31 kJ kg^{-1}) applied to whole tomato		↓Firmness (0.06 – 2.31 kJ kg^{-1})	↑Total (0.06 – 2.31 kJ kg^{-1}) ↑ β-carotene (0.06 – 0.38 kJ kg^{-1}; 1.38 – 2.31 kJ kg^{-1}) ↑Lycopene (0.14 – 2.31 kJ kg^{-1}) ↑Lutein (0.14, 0.5 2.31 kJ kg^{-1}) ↑Phytofluene, phytoene, δ-carotene (all treatments) ↑ɣ-carotene (0.09, 0.14, 2.31 kJ kg^{-1})	Total (0.38 kJ kg^{-1}) β-carotene (0.38 kJ kg^{-1}) Lycopene (0.09-0.38 kJ kg^{-1}; 1.38-2.31 kJ kg^{-1}) Lutein (0.09-0.38 kJ kg^{-1}) ɣ-carotene (0.09-0.38; 0.83-2.31 kJ kg^{-1})	Total (0.02 and 0.5 kJ kg^{-1}) β-carotene (0.02 and 0.06 kJ kg^{-1}) Lycopene (0.02 and 0.06 kJ kg^{-1}) Lutein (0.06 and 0.5 kJ kg^{-1}) Phytofluene (0.02, 0.06, 0.14, 0.5, 0.83, 1.38 and 2.31 kJ kg^{-1}) Phytoene δ-carotene (0.02-0.06 kJ kg^{-1}; 0.5-2.31 kJ kg^{-1})	(González-Casado et al., 2018a)
Tomato fractions	PEF (7.6 MJ kg^{-1}; 40-45 ºC) PEF + Heat (7.6 MJ kg^{-1}; 85-90 ºC)	Tissue	Cell detachment (PEF + Heat)	No changes	No increases	all-*trans*-lycopene (Heat; PEF + Heat)	(Bot et al., 2018)
		Cell clusters	Cell membranes damaged	↓β-carotene (PEF; Heat)		No changes	
		Single cells	Cell membranes damaged	↓β-carotene (PEF; Heat)		No changes	
		Chromoplasts	No differences	↓β-carotene (PEF; Heat; PEF + Heat) and all-*trans*-lycopene (PEF; PEF + Heat)		all-*trans*-lycopene and β-carotene (PEF; PEF + Heat)	

Table I.1. Continuation.

Food matrix	Processing conditions	Structure	Content before digestion	Bioaccessibility increase	Bioaccessibility decrease	References
Tomato	PEF (1 kV cm^{-1}; 0.1 Hz; 0, 4, 80 or 320 μs treatment duration) Storage: 0 h, 24 h or 48 h	Irregular cell wall structure by increasing holding time treatment	↑Total lycopene (all treatments) ↑All-*trans*-lycopene (80 and 320 μs) ↑*Cis*-lycopene (all treatments excepting 4 μs at 0 h)	Total lycopene (4 μs at 24 h) All-*trans*-lycopene (4 μs at 0 h) *Cis*-lycopene (all treatments at 24 h and 320 μs at 0 h)	Total lycopene (80 μs; 320 μs at 24 and 48 h) All-*trans*-lycopene (80 and 320 μs at 0 and 24 h and 320 μs at 48 h) *Cis*-lycopene (all treatments at 48 h and 4 μs at 0 h)	(Jayathunge et al., 2017)
Tomato juice	PEF (1 kV cm^{-1}; 0.1 Hz; 4 μs) Blanching (B) (90 ºC/2 min) PEF+B PEF + B + HIPEF (35 kV cm^{-1}; 1500 μs; 4 μs width pulses; 100 Hz) PEF + B + US (20 kHz, 20 % amplitude, 7 min) PEF + B + US + HIPEF	No information about structure	↑Total lycopene (all treatments) ↑All-*trans*-lycopene (PEF, PEF+B+US, PEF+B+HIPEF) ↑*Cis*-lycopene (PEF, PEF+B+HIPEF) ↓*Cis*-lycopene (PEF+B+US, PEF+B+US+HIPEF)	*Cis*-lycopene (all treatments) *Trans*-lycopene (PEF+B+HIPEF, PEF+B+US+HIPEF)	*Trans*-lycopene (B, B+US)	(Jayathunge et al., 2017)
Mango and papaya juice sweetened with *Stevia rebaudiana*	PEF (32 and 256 kJ kg^{-1})	No information about structure	PEF (32 kJ kg^{-1}): ↑ Total carotenoids No changes in total phenolic content	PEF (32 and 256 kJ kg^{-1}): Total carotenoids PEF (256 kJ kg^{-1}): Total phenolic content	No decreases	(Buniowska et al., 2017)

Table I.2. Studies evaluating the effect of pulsed electric fields (PEF) on structure, content and bioaccessibility of phenolic compounds in different vegetal matrices.

Food matrix	Processing conditions	Structure	Content before digestion	Bioaccessibility increase	Bioaccessibility decrease	References
Mango and papaya juice sweetened with *Stevia rebaudiana*	PEF (32 and 256 kJ kg^{-1})	No information about structure	PEF (32 kJ kg^{-1}): ↑Total phenolic content	PEF (256 kJ kg^{-1}): Total phenolic content Total anthocyanins	No decreases	(Buniowska et al., 2017)
Fruit juice-based beverage mixed with water	PEF (35 kV cm^{-1} electric field strength in bipolar mode, 4 μs pulse width, 200 Hz pulse frequency and 1800 μs total treatment	No information about structure	↑ Caffeic acid, ferulic acid, ↓Total phenolic content chlorogenic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, hesperidin, quercetin, rutin	Caffeic acid, *p*-coumaric, hesperidin, quercetin, rutin	Total phenolic content, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid	(Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015)
Fruit juice-based beverage mixed with milk	PEF (35 kV cm^{-1} electric field strength in bipolar mode, 4 μs pulse width, 200 Hz pulse frequency and 1800 μs total treatment)	No information about structure	↑Total phenolic content, caffeic acid, chlorogenic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, hesperidin, naringenin, quercetin ↓ ferulic acid, rutin	Total phenolic content, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, hesperidin, quercetin, rutin	No decreases	(Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015)
Fruit juice-based beverage mixed with soymilk	PEF (35 kV cm^{-1} electric field strength in bipolar mode, 4 μs pulse width, 200 Hz pulse frequency and 1800 μs total treatment time)	No information about structure	↑Total phenolic content, caffeic acid, chlorogenic acid, *p*-coumaric, *p*-hydroxybenzoic acid, hesperidin, naringenin, quercetin, rutin ↓ ferulic acid	Total phenolic content, quercetin, rutin	*p*-coumaric acid	(Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015)

Table I. 2. Continuation.

Food matrix	Processing conditions		Structure	Content before digestion	Bioaccessibility increase	Bioaccessibility decreases	References
Apple	PEF and storage for 0 h and 24 h	0.01 kJ kg^{-1}	Unaltered toughness	0 h: ↓5-caffeoylquinic acid 24 h: ↑5-caffeoylquinic acid, total phenolic content	No increases	0 h: 5-caffeoylquinic acid Total phenolic content	(Ribas-Agustí et al., 2019b)
		1.8 kJ kg^{-1}	↓ Toughness	0 h and 24 h: ↓5-caffeoylquinic acid, 4-caffeoylquinic acid, *p*-coumaroylquinic acid, phloretin xyloglucoside, total phenolic compounds 24 h: ↓Epicatechin	0 h and 24 h: Phloretin xyloglucoside 24 h: Total phenolic content Epicatechin 5-caffeoylquinic acid Phloretin glycoside	24 h: Quercetin glycoside Quercetin xyloside Quercetin galactoside Quercetin arabinoside	
		7.3 kJ kg^{-1}	↓ Toughness	0 h and 24 h: ↓5-caffeoylquinic acid, 4-caffeoylquinic acid, *p*-coumaroylquinic acid, phloretin glucoside, phloretin xyloglucoside, total phenolic compounds 0 h: ↓Epicatechin	24 h: Total phenolic content	0 h: 4-caffeoylquinic acid	

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OBJECTIVES

OBJECTIVES

This Doctoral Thesis was aimed at evaluating the effects of applying pulsed electric fields (PEF) on carotenoids and phenolic compounds content and bioaccessibility in carrots and their derived products. The secondary objectives proposed to achieve such aim were:

- To evaluate the impact of PEF processing and post-treatment time on carotenoid and phenolic profiles as well as their effects on cell permeability and quality attributes of whole carrots.

- To study the main causes behind the increase in bioactive compounds content of PEF-treated carrots by evaluating their enzymatic activity, respiration rate and phenolic and carotenoid content during storage.

- To assess the influence of PEF treatments on the bioaccessibility of carotenoids and phenolic compounds from whole carrots and their derived products as well as their effect on the product matrix structure.

- To evaluate the feasibility of directly applying PEF to selected derived products in order to enhance the bioaccessibility of carotenoids and phenolic compounds and delve into the causes underlying such change.

MATERIAL AND METHODS

MATERIAL AND METHODS

1. Chemicals and reagents

HPLC grade methanol, acetone, methyl tert-butyl ether and analytical reagent grade sodium carbonate were acquired from Fisher Scientific Scharlau Chemie (Loughborough, UK). HPLC grade hexane, ethanol, acetonitrile, ammonium carbonate, magnesium chloride hexahydrate, acetic acid and ammonium acetate were purchased from Scharlab (Sentmenat, Spain). Ultrapure water was obtained with a Milli-Q system (Millipore Ibérica, Madrid, Spain). 2, 3, 5-triphenyltetrazolium chloride, citrus pectin, bromothymol blue, bovine serum albumin, catechol, polygalacturonic acid, D-galacturonic acid, 2-mercaptoethanol and L-phenylalanine were acquired from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent, EDTA, formic, acetic and ortho-phosphoric acids was acquired from Scharlau S.L. (Barcelona, Spain) and sodium chloride was purchased from POCH S.A. (Sowińskiego, Poland). Ethyl acetate, diethyl ether, sodium hydroxide and 2-cyanoacetamide were purchased from Acros Organics (New Jersey, USA), *p*-phenylendiamine was purchased from Merck (Hohenbrunn, Germany), polyvinylpyrrolidone was acquired from Fischer Scientific (Geel, Belgium), hydrochloric acid was purchased from Panreac Química S.A. (Barcelona, Spain), hydrogen peroxide was acquired from ChemLab (Zedelgem, Belgium) and Coomassie brilliant blue G250 was purchased from Fluka Chemika (Buchs, Switzerland). Butyl hydroxytoluene (BHT) was acquired from Scharlau Chemie S.A. (Barcelona, Spain). Calcium chloride dihydrate was obtained from Merck (Darmstadt, Germany). Sodium hydrogen carbonate and potassium dihydrogen phosphate were acquired from VWR (Llinars del Vallès, Spain). Potassium chloride was obtained from Panreac (Castellar del Vallès, Spain). Digestive enzymes (porcine α-amylase, porcine pepsin, porcine bile extract, porcine pancreatin and porcine lipase) were acquired from Sigma-Aldrich (Darmstadt, Germany).

Caffeic acid, ferulic acid, protocatechuic, *p*-hydroxybenzoic, gallic, chlorogenic, *p*-coumaric acids and 5-O-caffeoylquinic acid commercial patterns were obtained 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. Raw material

2.1. Carrots

Carrots (*Daucus carota* cv. Nantes) were purchased in a local supermarket (Lleida, Spain). They were selected based on their uniform size and shape (caliber 25/35 mm and length 17 ± 2 cm), avoiding those with visual defects. Carrots were stored at 4 ºC, within a week until processing. Prior to treatments, carrots were washed with tap water and the excess was removed with a paper cloth.

Once PEF treatments were performed, whole carrots were stored at 4 ºC during post-treatment time corresponding to each experiment. For the experiments that did not involve *in vitro* digestion, carrot top ends (2.5 ± 0.5 cm) were removed, and the rest was cut in 1 cm-thickness slices and frozen at -40 ºC until extraction. Just before extraction procedures they were crushed (Moulinex, 700 W) to overcome the possible heterogeneity within differently located tissues in chamber. On the other hand, to perform *in vitro* digestion experiments, carrots were not frozen before obtaining derived products nor before digestions.

2.2. Carrot derived products

Three different carrot derived products were obtained: juices, purees and oil-added purees. Purees were prepared by blending 500 g of carrot slices (1 cm- thickness) with water (1:1) (w/w) in a food processor (Taurus Mycook) in crushing function at full power in two 10-seconds intervals. To prepare oil-added carrot purees, extra virgin olive oil (Borges Branded Foods, S.L.U., Tàrrega, Lleida) was added (5 % w/w) to purees and they were stirred for 15 min at 8000 rpm with an Ultra-Turrax IKA equipped with a 3-blade stirring rod at room temperature.

On the other hand, 500 g of carrots were used for preparing carrot juices. They were obtained using a cold blender (Imetec Succovivo SJ1000) accoupled with a filter of 0.4 mm.

3. Non-thermal and thermal treatments

3.1. Pulsed electric fields (PEF) treatments

PEF treatments were carried out in a batch lab scale system (Physics International, San Leandro, CA, USA), equipped with a TG-70 gas control unit and a PT55 pulse generator (Pacific Atlantic Electronics Inc., El Cerrito, CA, USA). The system supplied pulses of 4 μs with

exponentially decaying waveform from a capacitor of 0.1 μF at a fixed frequency of 0.1 Hz. The treatment chamber, a parallelepiped methacrylate container, equipped with two stainless steel electrodes (20 × 5 cm) with a gap between them of 5 cm was filled with an aqueous solution (0.01 S m^{-1}) and carrots (~ 0.1 kg) were individually placed in parallel to electrodes in a ratio 1:3 (w: v) carrots: aqueous solution. The range of treatments applied were based on Toepfl et al., (2005) and Soliva-Fortuny et al., (2009), who established that the optimum electric field strength for electropermeabilized plant tissues was in the range of 1 and 2 kV cm^{-1}. The specific energy input (Ws), expressed in kJ kg^{-1}, was calculated according to Eq. (1) (Wiktor et al., 2015)., and equivalences are displayed in **Table M.1.**

$$Ws = \frac{V^2\ C\ n}{2\ m} \quad (1)$$

where V [V], C [F], n, and m [kg] are the voltage, capacitance of the energy storage capacitor, number of pulses and mass of the sample in the treatment chamber, respectively. The temperature of the aqueous solution after PEF application was measured to assure it did not increase.

Table M.1. PEF treatment conditions and specific energy inputs.

Electric field strength (kV cm^{-1})	**Number of pulses**	**Specific energy input (kJ kg^{-1})**
0	0	Untreated
0.8	5	0.14
	12	0.38
	30	0.87
2.0	5	0.22
	12	0.50
	30	1.19
3.5	5	0.61
	12	1.92
	30	3.93

3.2. Thermal treatments

Carrot derived products (200 g) were packed in re-sealable polyethylene bags (20 × 15 cm) and heated in a water bath for 10 min at 70 °C in order to reduce pectinolytic and oxidative enzyme activity (Balogh, Smout, Nguyen, Van Loey, & Hendrickx, 2004; Houben, Jamsazzadeh Kermani, Van Buggenhout, Van Loey, & Hendrickx, 2014; Soysal & Söylemez, 2005). Temperature of products was monitored during treatment to assure that they did not exceed 70 ºC. Thereafter, purees were cooled under a constant flow of cold water for 3 min.

4. Physicochemical characterization of carrots and derived products

4.1. Colour

A Minolta colorimeter (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) was used to characterize external, cortical and vascular tissues of carrots as well as derived products colour. The apparatus was set up for a D65 illuminant and 10° observer angle with an aperture of 10 mm. A white standard plate (Y=94.00, x=0.3158, y=0.3322) was used for calibration. The CIEL*a*b* parameters (lightness, L*; green-red chromaticity, a*; and blue-yellow chromaticity, b*) were obtained to calculate: chroma (C*) [Eq. (2)], hue angle (h) [Eq. (3)], a*/b* ratio, browning index (BI) [Eq. (4) and (5)] and total colour difference (ΔE) [Eq. (6)] and to assess carrot colour modification.

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (2)$$

$$h = \arctan \frac{b^*}{a^*} \quad (3)$$

$$BI = \frac{100\,(X - 0.31)}{0.17} \quad (4)$$

$$\text{where } X = \frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*} \quad (5)$$

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{0.5} \quad (6)$$

where L*, a* and b* refer to data collected after treatments and L^*_0, a^*_0 and b^*_0 refer to those of untreated carrots or derived products. Three measurements of each specific tissue were taken, and each measurement represents the average of three readings. These were made by changing the position of the colorimeter in each measure. Regarding derived products, colour was measured thrice using an optical glass cuvette.

4.2. Firmness

Penetration analysis and texture profile analysis (TPA) were performed with a TA-XT2 texture analyser (Stable Micro Systems Ltd., Surrey, England) equipped with a loading cell (25 kg-f).

4.2.1. Penetration analysis

Firmness of cortical tissue and vascular cylinder of carrots were determined in three carrot disks (15 mm height and 32 ± 3 mm diameter) of each replica. These disks were cut out of top end (1/3 of total length) of the carrot. The texture analyser was equipped with a 4-mm-diameter cylinder steel probe, which penetrated 10 mm the carrot tissue at a constant rate of 5 mm/s. The motion of the blade was perpendicular to the surface of carrot disks. Hardness (N s) was determined as area under the curve between the graph of y (force) and x (time) (Ribas-Agustí et al., 2019a).

4.2.2. Texture profile analysis (TPA)

A cylindrical flat-head aluminum probe (25 mm diameter, Stable Micro Systems) 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 center. 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. For each sample treatment combination, 10 carrot samples were analysed using texture analyser to assess their texture profile. 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) (Leong, Du, & Oey, 2018).

4.3. pH

Previous to pH measurements in whole carrots, they were ground in a blender to obtain a homogeneous sample, which was used for determining the pH values. It was directly measured in derived products. A pH-meter (Crison 2001, Crison Instruments S.A., Alella, Barcelona, Spain) was used. Measures were performed twice in each replicate.

4.4. Total soluble solids (TSS)

The same homogeneous sample previously described in paragraph 4.3 was used for measuring total soluble solids in whole carrots. In derived products it was directly measured. TSS was determined by refractometry (RX-1000, Atago, Tokyo, Japan) and results were expressed as % of total soluble solids. Measures were performed twice in each replicate.

4.5. Viscosity

Viscosity measurements (mPa s) were performed in derived products (10 mL) by using a vibro-viscometer (SV-10, A&D Company, Tokyo, Japan) vibrating at 30 Hz, with constant amplitude and working at room temperature. Measures were performed thrice in each replicate.

5. Cell permeability

5.1. Media conductivity

The electrical conductivity of the aqueous solution contained in the treatment chamber was measured before and after PEF treatments using a conductometer Testo 240 (Lenzkirch, Germany) (Ersus & Barrett, 2010a; Faridnia, Burritt, Bremer, & Oey, 2015). The solution was replaced after each treatment and measures were performed twice.

5.2. Weight loss

Carrots weight loss was evaluated after PEF treatments. Gravimetric methods were used [AND electronic Balance FX-2000 (AND Company Limited, Tokyo, Japan)] and measures were performed twice. Weight loss was calculated in reference to untreated carrots with the Eq. (7)

$$\text{Weight loss (\%)} = \frac{W_0 - W}{W_0} \times 100 \quad (7)$$

where W_0 is referred to weight of untreated carrots and W is referred to weight of PEF-treated carrots. Lag time between treatment and weight measurement just after treatment averaged 20 min.

6. Cell viability

Tetrazolium salt staining was used to evaluate the effect of PEF on cell/tissue viability within carrot slices and to determine the proportion of viable cells, as previously described by Faridnia, Burritt, Bremer, & Oey, (2015). The principle of tetrazolium salt staining is based on the formation of an insoluble red formazan from the reduction of the salt by oxidoreductase enzymes. The amount of formazan is directly proportional to the number of living cells as they contain oxidoreductases (Berridge, Herst, & Tan, 2005). A 0.5% (w/v) tetrazolium salt solution was prepared with Milli-Q water on the same day as the PEF treatment. Immediately after PEF treatment, each carrot was sliced (1.5 mm thickness and 32 ± 3 mm diameter). Three slices of each carrot were placed in a petri dish and fully immersed in the tetrazolium solution. Petri dishes were then covered in tin foil to protect the samples from light and left for 24 h at 18–20 °C. After 24 h slices were rinsed with water, blotted dry with a paper cloth and photographed. Image analysis using colour threshold method [ImageJ software, (Abràmoff, Hospitals, Magalhães, & Abràmoff, 2007)] was applied to measure the red and unstained areas on each slice, indicative of living and dead cells, respectively. Cell viability (%) of each slice was calculated using the following Eq. (8):

$$Cell\ viability\ (\%) = \frac{Stained\ area}{Total\ area} \times 100 \quad (8)$$

Measures were conducted in three slices for each treatment replicate.

7. Respiratory activity

A static system was used to determine the respiratory activity and volatile organic compound production of the carrots. Changes in headspace composition were measured over 36 h using a gas analyser (490 Micro GC, Agilent Technologies, Santa Clara, USA). Just after PEF treatment, carrots (ca. 0.12 ± 0.02 kg) were individually placed in hermetic containers of 2.25 L

and gas sample (1.7 mL) was withdrawn from the headspace through an adhesive rubber septum with a syringe. Respiration as carbon dioxide production was expressed as mg kg^{-1} s^{-1} [Eq. (9)] according to Tappi et al. (2014) and the production of ethylene, ethanol and acetaldehyde was expressed as ng kg^{-1} s^{-1} or pg kg^{-1} s^{-1}.

$$RRCO_2 = \frac{mm_{co2} \cdot V_{head} \cdot \frac{\%CO_{2\,head}}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283} \quad (9)$$

where mm_{CO2} refer to gas molar mass (g mol^{-1}), V_{head} represents the container headspace volume (dm^3), and % CO_{2head} refer to gas percentages in the container headspace at time t (h); m is the sample mass (kg); and R is the gas constant (8.314472 dm^3 kPa K^{-1} mol^{-1}). Measures were performed twice in each replicate.

8. Enzyme activities

8.1. Pectinmethylesterase (PME) activity

The PME extraction and activity determination were performed as described by Hagerman and Austin (1986), adapted to 96-well microplates. All the solutions must be previously adjusted to pH 7.5. The reaction mixture consisted of 30 µL of PME extract, 70 µL of distilled water, 180 µL of citrus pectin 0.5 % (w/v) solution and 30 µL of bromothymol blue 0.01 % (w/v). PME activity was determined by monitoring the colour change during 3 min at 620 nm in a microplate spectrophotometer (Thermo Scientific Multiskan GO, Vantaa, Finland). Galacturonic acid was used to make a standard curve and calculate PME activity, which was expressed as nanomoles of galacturonic acid produced per minute and per milligram of protein (U mg^{-1} protein). All extractions and determinations were performed in duplicate.

8.2. Polygalacturonase (PG) activity

PG extraction was based on the procedure described by Pressey (1988) and PG activity determination was carried out as described by Houben et al. (2014), by measuring the formation of reducing groups from a polygalacturonic acid substrate at 35 °C. The absorbance was determined spectrophotometrically in a quartz microplate (Thermo Scientific Multiskan GO, Vantaa, Finland) at 276 nm and 22 °C. A standard curve of monogalacturonic acid allowed to calculate PG activity. PG activity was expressed as nanomoles of reducing groups from

polygalacturonic acid per minute and per milligram of protein (U mg^{-1} protein). All extractions and determinations were performed in duplicate.

8.3. Polyphenol oxidase (PPO) activity

PPO extraction was based on the procedure described by Alegria et al. (2016) with slight modifications. In this case, PPO was extracted from carrot tissues (4 g) adding 15 mL of cold phosphate buffer (0.1 M; pH 6.5) and 0.4 g of polyvinylpyrrolidone. Then, samples were vortexed for 1 min and centrifuged at 20000 g for 15 min at 4 ºC. The resulting supernatant was filtered across Whatman No. 1 filter. During the whole procedure, samples were maintained in an ice-bath to prevent protein denaturation.

PPO activity was assayed spectrophotometrically measuring the catechol oxidation rate at 420 nm for 2 min (Thermo Scientific Multiskan GO, Vantaa, Finland). The reaction mixture was adapted to 96-well microplate, which contained 10 µL of enzymatic extract and 290 µL of catechol (0.05 M) prepared in extraction buffer just before the analysis. Results were expressed as nanomoles of enzymatic extract that causes an increase of one unit of absorbance at 420 nm on a protein basis (U mg^{-1} protein). All extractions and determinations were performed in duplicate.

8.4. Peroxidase (POD) activity

POD extraction was carried out following the same procedure as previously described for PPO extraction. Determination was performed placing 10 µL of enzyme extract into 96-well microplate. Then, 260 µL of extraction buffer, 20 µL of *p*-phenylenediamine 1 % (w/v) and 10 µL of H_2O_2 1.5 % (v/v) were added. Spectrophotometric readings at 485 nm were registered every 10 s during 10 min of incubation in a spectrophotometer (Thermo Scientific Multiskan GO, Vantaa, Finland). POD activity was expressed as nanomoles of enzymatic extract causing an increase of one unit of absorbance at 485 nm on a protein basis (U mg^{-1} protein). All extractions and determinations were performed in duplicate.

8.5. Phenylalanine ammonia-lyase (PAL) activity

PAL extraction was performed as described by Alegria et al. (2016) with slight modifications. After homogenizing carrot tissue (4 g) with polyvinylpolypyrrolidone and sodium borate buffer 100 mM (pH 8.7), samples were vortexed for 1 min and centrifuged at 20000 g for 15 min at 4 ºC. Then, supernatants were filtered across Whatman No. 1 filter. Extractions were also made using cold buffers and an ice-bath to prevent protein denaturation.

PAL activity determination was adapted to 96-well quartz microplate. L-phenylalanine substrate solution (100 µL), extraction buffer (100 µL) and PAL extract (50 µL) were mixed. Then, spectrophotometric readings at 290 nm were registered every 10 min during 1 h of incubation at 37 ºC (Thermo Scientific Multiskan GO, Vantaa, Finland) in a quartz microplate. PAL activity was expressed as nanomoles of *trans*-cinnamic acid per minute on a protein content basis (U mg^{-1} protein). All extractions and determinations were performed in duplicate.

9. Protein content

Protein content in crude enzyme extracts was measured according to Bradford (1976) using bovine serum albumin as a standard. Bradford reagent (270 µL) was mixed with enzyme extract (30 µL) and added to 96-well microplate. Absorbance was measured spectrophotometrically at 595 nm (Thermo Scientific Multiskan GO, Vantaa, Finland). All extractions and determinations were performed in duplicate.

10. Microstructure

10.1. Optical microscopy

To obtain microstructure images from carrot outer and inner parenchyma samples, they were fixed using glutaraldehyde (2.5 % in phosphate buffer 0.1 M). Then, they were washed thrice for 10 min 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 ºC. Samples were cut into 70 – 80 nm and 1 µm sections using a Ultracut Leica EM UC6.

Microstructure of carrot derived products was investigated throughout using a light microscope (BX41, Olympus, Göttingen, Germany) equipped with UIS2 optical system. 10 µL drops were mounted on glass slides without staining and were microscopically observed. A general inspection of the samples was made, and representative photos with 10x lens were taken. All images were processed using the instrument software (Olympus CellSense, Barcelona, Spain).

10.2. Transmission electronic microscopy (TEM)

The same procedure described in paragraph 10.1 was performed for preparing carrot outer and inner parenchyma samples. In order to obtain images for TEM, sections of 1 μm were contrasted with Reynolds lead citrate.

10.3. Particle size distribution

A Mastersizer 3000™ (Malvern Instruments Ltd., Worcestershire, UK) was used to measure particle size distribution of juices and purees. Results were expressed in terms of volume and surface diameter, D [4, 3] and D [3, 2], respectively. The refractive index of water was 1.33 and the particle calculation was set for irregular particles. Analysis was performed thrice in each replicate.

11. *In vitro* digestion

The *in vitro* digestion procedure was performed according to the standardized COST Infogest protocol (Minekus et al., 2014), in which electrolyte and enzymatic solutions to simulate the phases of human digestion is described. Digestions were performed in darkness, in absence of oxygen (bottles were flushed with nitrogen gas) in an orbital incubator (Ovan, Badalona, Spain) at 37 °C and 120 rpm. Electrolyte concentrations and enzyme activities were prepared following the indications provided by Minekus et al. (2014) (**Table M.2**) and blank samples consisting in water instead of carrot products, were made in identical conditions.

Oral phase. The oral phase was initiated by blending 10 g of carrots (1 cm^3 cubes) for 1 min (Taurus aromàtic 150 W) and transferred to a glass bottle. Then, 10 mL of simulated salivary fluid (pH 7 and 37 ºC) with porcine α-amylase (Minekus et al., 2014) were added to stir the mix for 1 min at 37 ºC. Oral phase was omitted in derived products digestions due to the very short residence times in the oral cavity (Minekus et al., 2014).

Gastric phase. The gastric phase started adding 20 mL of simulated gastric fluid (pH 3 and 37 ºC) and porcine pepsin to carrot (10 g) or derived product (20 g). This mixture was incubated at 37 °C for 2 h in agitation.

Intestinal phase. The duodenal phase was initiated by inserting a cellulose-membrane dialysis bag (molecular weight cut-off 12,000 Da, Sigma-Aldrich), which contained simulated intestinal fluid (pH 7 and 37 ºC). This dialysis bag simulates the intestinal epithelium and it harbor

phenolic compounds released from matrix (bioaccessible fraction) (Minekus et al., 2014; Ribas-Agustí et al., 2019b). After 30 min of incubation, pH was adjusted to 7 and a solution containing simulated intestinal fluid (pH 7 and 37 ºC), porcine bile extract, porcine pancreatin (and porcine lipase in case of oil-added purees) was added and the mixture was incubated 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 carotenoids compounds, was centrifuged at 5000 g for 15 min at 4 °C (Brodkorb et al., 2019; Eriksen, Luu, Dragsted, & Arrigoni, 2017; Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011) and supernatant was also collected. Resulting digested fractions were both freeze-dried and stored at -40 ºC until analysis of bioaccessibility. *In vitro* digestions of each replicate were performed in duplicate.

Table M.2. Electrolyte concentrations for *in vitro* digestions.

Solutions	Simulated salivary fluid (SSF) V = 400 mL	Simulated gastric fluid (SGF) V = 800 mL	Simulated intestinal fluid (SIF) V = 1200 mL
0.5 M KCl	15.1 mL	13.8 mL	20.4 mL
0.5 M KH_2PO_4	3.7 mL	1.8 mL	2.4 mL
1 M $NaHCO_3$	6.8 mL	25 mL	127.5 mL
2 M NaCl	-	23.6 mL	28.8 mL
0.15 M $MgCl_2(H_2O)_6$	0.5 mL	0.8 mL	3.3 mL
0.5 M $(NH_4)_2CO_3$	0.06 mL	1.0 mL	-

11.1. Bioaccessibility calculation

The bioaccessibility of each individual compound was determined using Eq. (10) and results were expressed as percentage.

$$\text{Bioaccessibility (\%)} = \frac{\text{CCdigested}}{\text{CCnon-digested}} \times 100 \quad (10)$$

where $CC_{digested}$ corresponds to the overall concentration of each compound in the absorbable fraction and $CC_{non\text{-}digested}$ is the concentration in non-digested samples.

Carotenoid bioaccessibility was calculated referred to the concentration found in the digested micellar fraction, whereas phenolic compounds bioaccessibility was calculated in reference to their concentration in the dialyzed digested fraction.

12. Extraction of carotenoid compounds

Carotenoids were extracted according to the method described by Sadler, Davis, & Dezman (1990), with slight modifications. Homogenized carrot tissue (1 g), juice (1 g) or puree (2 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 HPLC according to the method described subsequently.

To extract carotenoids from the digested 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 at 4000 g for 5 min (Svelander, 2011). 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.

12.1. Identification and quantification of carotenoid compounds by High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD)

Carotenoids were quantified by HPLC, following a procedure validated by Cortés, Esteve, Frígola, & Torregrosa (2004). An aliquot of 20 µL of the extracted samples was injected into the 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 C_{18} 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. The flow rate was fixed at 1 mL min^{-1} and the total run time was 60 min (**Table M.3**). The column was set at 30 ºC, while sample amber vials on the auto sampler were preserved at 4 ºC. The carotenoids were identified by UV–vis spectral data and their retention times (Cortés et al., 2004; Mouly, Gaydou, & Corsetti, 1999). Quantification of carotenoids was carried out in duplicate by integration of the peak areas and comparison to calibration curves.

Table M.3. Chromatographic conditions for carotenoids identification.

Time (min)	Methanol/ ammonium acetate 0.1 M (%)	H_2O (%)	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

13. Extraction and determination of phenolic compounds

Three types of extraction were performed depending on the objective of the study and determination technique. The first one was carried out to extract free phenolic compounds and determine spectrophotometrically the total phenolic content relative to untreated carrots. The second type was used to determine free individual phenolic compounds by UPLC-MS/MS in non-digested and digested carrots or derived products. Finally, the third extraction was performed to determine individual free and bound phenolic compounds in carrots by HPLC-DAD.

13.1. Methanolic extraction type I

Phenolic compounds were extracted following the methodology proposed by Ribas-Agustí, Cáceres, Gratacós-Cubarsí, Sárraga, & Castellari, (2012) with slight modifications. Carrot tissue (5 g) was homogenized with an 80 % (v/v) methanol solution (25 mL) using an Ultra-Turrax T25 (IKA® WERKE, Germany). Homogenates were centrifuged at 21612 g at 4–6 °C for 20 min. Supernatants were collected and 25 mL of methanol solution was added to the precipitate. In order to ensure a complete extraction, precipitates were vortexed for resuspension and

immersed in an ultrasonic bath for 5 min. Samples were centrifuged under the same conditions explained above. Both supernatants were collected, make up to 50 mL in a volumetric flask and stored at -40 °C (for up to ten days until spectrophotometric analysis).

13.1.1. Total phenolic compounds determination

The total phenolic content was determined according to the Folin–Ciocalteu procedure adapted to 96-wells microplates. Methanolic extracts (30 µL) were placed into a microplate. Then, 150 µL of 10% (v/v) Folin-Ciocalteu's reagent and 120 µL of Na_2CO_3 7.5 % (w/v) were added. After an incubation period of 90 min at room temperature in darkness, the absorbance was measured at 765 nm using a microplate reader (Thermo Scientific Multiskan GO, Vantaa, Finland). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight relative to those of untreated carrots. Phenolic content was extracted twice per replica and spectrophotometrically determined twice.

13.2. Methanolic extraction type II

Phenolic compounds were extracted from freeze-dried non-digested or digested carrot samples (0.2 g). Methanol (80:20 v/v) was added, 1.5 mL for carrot tissues (non-digested and digested) and purees (non-digested) or 1 mL for juices (non-digested and digested) and purees (digested). Samples were vortexed for 1 minute, then centrifuged (16209 g for 15 min at 4 ºC) , and the clear supernatant was microfiltered using polyvinylidene difluoride (PVDF) filters (0.2 µm, ø 13mm) (Scharlab, Barcelona, Spain) prior to injection to the chromatographic system.

13.2.1. Identification and quantification of phenolic compounds by Ultra-Performance™ Liquid Chromatography (UPLC-MS/MS)

AcQuity Ultra-Performance™ liquid chromatography (UPLC) coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford) was used to determine phenolic compounds in methanolic extracts obtained from freeze-dried carrot non-digested and digested samples. The analytical column was an AcQuity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C_{18} (2.1 x 5 mm, 1.7 µm) (Waters, Milford). The column was kept at 30 °C, and the flow rate was 0.3 mL min^{-1}. Mobile phases were acetic acid (0.2 %) and acetonitrile (**Table M.4**). 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). The dwell time established for each transition was 30 ms and MassLynx 4.1 software was used for data acquisition. Results were expressed in a dry weight basis as mg of phenolic compound per kg. Phenolic content was extracted and determined twice per replica.

Table M.4. Chromatographic conditions for phenolic compounds identification by UPLC.

Time (min)	Acetic acid (0.2 % v/v) (%)	Acetonitrile (%)
0	95	5
5	90	10
10	87.6	12.4
18	72	28
21	15	85
23	0	100
25.5	0	100
27	95	5
30	95	5

13.3. Extraction by acid and alkaline hydrolysis

Free and bound phenolic compounds were extracted following the methodology proposed by Mattila and Kumpulainen (2002) with slight modifications, in which acid and alkaline hydrolysis were performed. The modification consisted in adding a centrifugation step (8784 g for 5 min) before filtrating samples. Pulverized freeze-dried carrot (1 g) was weighed into a 50-mL graduated plastic test tube and homogenized with 7 mL of methanol: acetic acid [10%(v/v)] (85:15) containing BHT (2 g L^{-1}) solution using an Ultra-Turrax T25 (IKA® WERKE, Germany). Homogenates were ultrasonicated for 30 minutes, made up to 10 mL with distilled water and mixed up. Samples were then centrifuged at 8784 g for 5 minutes and 1 mL of supernatant was collected and filtered through a polytetrafluoroethylene (PTFE) membrane filter (0.45 µm, ø 13mm, Labbox Labware S.L., Barcelona) for the HPLC analysis of free phenolic acids.

After taking the sample for analyzing free phenolic acids, an acid hydrolysis was performed by adding 12 mL of distilled water and 5 mL of 10 M NaOH into the test tube, and its contents were flushed with nitrogen to eliminate oxygen and stirred at 120 rpm in an orbital shaker (Ovan, Badalona, Spain) overnight at room temperature (about 16 h).

The solution was then adjusted to pH 2, and phenolic acids were extracted three times with 15 mL of a mixture of cold diethyl ether (DE) and ethyl acetate (EA) (1:1) by manually shaking and centrifuging. DE/EA layers were combined, evaporated to dryness, and dissolved into 1 mL of methanol. Samples were filtered through a membrane filter (see above) before injection in the HPLC system. Then, an acid hydrolysis was performed by adding 2.5 mL of concentrated HCl into the test tube and incubating the tube in a water bath (85 °C) for 30 min. Sample was allowed to cool, and the pH was adjusted to 2. The DE/EA extraction performed was similar to that for alkaline hydrolysis and evaporated extract was then dissolved into 1 mL of methanol, filtered through a membrane filter (see above), and analysed by HPLC-DAD. After quantification, the results from alkali and acid hydrolysates were calculated to represent total phenolic acids (mg phenolic compound/100 g of dry weight).

13.3.1. Identification and quantification of phenolic compounds by HPLC-DAD

Phenolic compounds were separated following the procedure described by Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2011) (**Table M.5**) using a reverse-phase C_{18} Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm x 250 mm) at room temperature. The HPLC system comprised of a 600 Controller, a 486 Absorbance Detector programmed to scan from 200 to 350 nm, a thermostatic column compartment, and a 717 Plus Auto Sampler with cooling system (Waters, Milford, MA). Flow rate of the mobile phase was 1 mL min^{-1} and the injection volume were 20 μL. Individual phenols were identified based on their UV-vis spectral data and retention times which were compared to their reference standards. Quantification of phenolic compounds was carried out by integration of the peak areas. Data were compared to calibration curves of each phenolic compound and results were expressed on a dry weight basis. Phenolic content was extracted and determined twice per replica.

Table M.5. Chromatographic conditions for phenolic compounds identification by HPLC-DAD.

Time (min)	Formic acid/water (2.5 % v/v) (%)	Formic acid/methanol (2.5 % v/v) (%)
0	95	5
15	87	13
20	85	15
28	85	15

32	70	30
35	55	45
40	10	90
45	10	90
50	95	5

14.Statistical analysis

Statistical analyses were carried out using the JMP Pro v.13 software (SAS Institute, Cary, NC, USA), IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL) or SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA). Results were reported as the mean ± standard deviation. Results were subjected to an analysis of variance (ANOVA) followed by Tukey *post hoc* test or to pairwise comparisons of means (Student's test) in order to establish statistical differences among mean values. The relationship between variables was determined using the Pearson (r) coefficient. Rho of Spearman (r_s) was used to establish a correlation when there was outliers presence. The statistical significance level was set up at $p < 0.05$.

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PUBLICATIONS

Chapter 1. Enhancing phenolic content in carrots by pulsed electric fields during post-treatment time: Effects on cell viability and quality attributes

Gloria López-Gámez, Pedro Elez-Martínez, Olga Martín-Belloso, Robert Soliva-Fortuny

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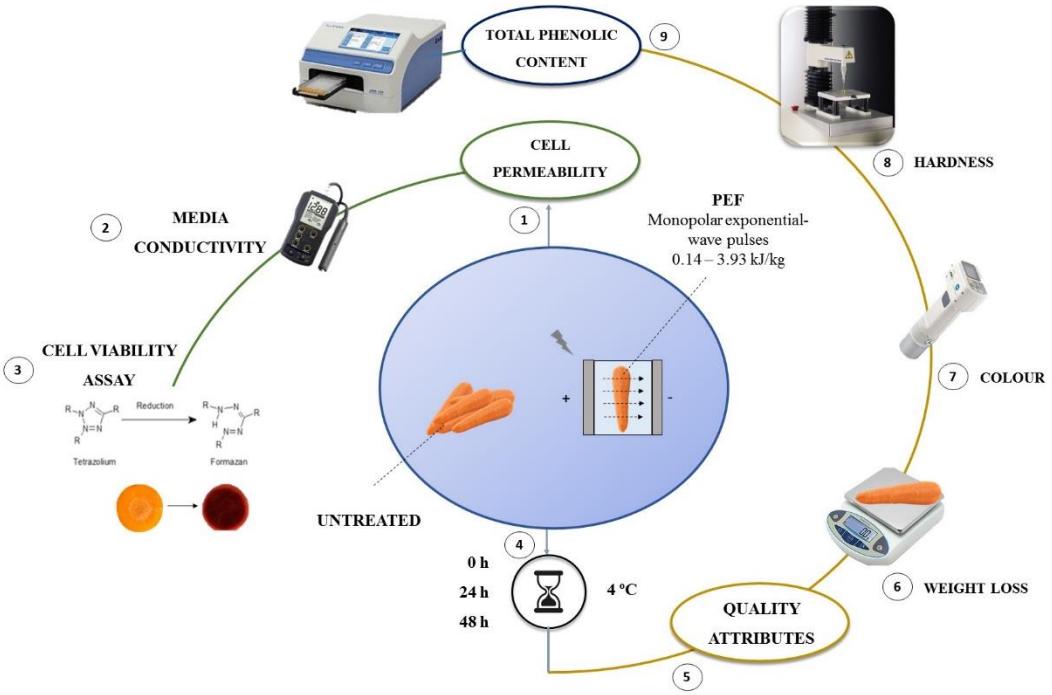
Abstract

The impact of pulsed electric fields (PEF) and post-treatment time on the phenolic content and quality attributes of carrots was studied. Additionally, their influence on cellular permeability and viability was analysed. Carrots were subjected to different electric field strengths (0.8, 2 and 3.5 kV cm^{-1}) and number of pulses (5, 12 and 30). The largest increases in phenolic content were produced 24 h after applying 30 pulses of 0.8 kV cm^{-1} (40.1 %) and 5 pulses of 3.5 kV cm^{-1} (39.5 %). At such conditions, the colour was not affected but softening occurred after applying the highest electric field strength. Moreover, the increase in the specific energy input correlated with the decrease in cell viability. Carrot weight loss over time, media conductivity increase, and cell viability decrease are related to the destabilization of cell membranes, which would entail a physiological response to stress, leading to a higher content in phenolic compounds.

Keywords: Pulsed electric field; polyphenols; quality attributes; cell viability; carrot

GRAPHICAL ABSTRACT



PEF: Pulsed Electric Fields

1. Introduction

Carrot is an economically important crop whose consumption is becoming increasingly popular due to its nutritional value. Carrots are known as a good source of bioactive compounds such as carotenoids, phenolic compounds, vitamin C and fibre, among others (Arscott & Tanumihardjo, 2010). Most information about their phytochemicals is related to carotenoids and their antioxidant properties, whereas their phenolic content has been less studied. Hydroxycinnamic acids, such as chlorogenic acid (CHA), 3,5-dicaffeoylquinic acid (3, 5-diCQA), and 4,5-dicaffeoylquinic acid (4, 5-diCQA) are the main polyphenols accumulated in carrots (Becerra-Moreno et al., 2015), resulting from the induction of the phenylpropanoid pathway, as a plant defence response to stress (Zhao, Davis, & Verpoorte, 2005). Plant growth is affected by environmental factors such as extreme temperatures, drought, nutrients imbalance, wounding (abiotic stresses) or infections by pathogenic organisms (biotic stresses). Nevertheless, plants have developed adaptation mechanisms, e.g. the synthesis of secondary metabolites such as polyphenols. Heredia & Cisneros-Zevallos (2009) reported that carrots showed a higher antioxidant activity compared to other fruits and vegetables in response to wounding. In that sense, it was concluded that more phenolic compounds were accumulated in treatments that involved the most intensive damage. Some studies have reported that polyphenol biosynthesis and accumulation in plant tissues may be induced through the application of postharvest abiotic stresses such as wounding (Jacobo-Velázquez, González-Agüero, & Cisneros-Zevallos, 2015) or UV-light radiation (Aguiló-Aguayo, Gangopadhyay, Lyng, Brunton, & Rai, 2017; Alegria et al., 2012), among others.

Recently, the application of non-thermal processing technologies such as pulsed electric fields (PEF) has been proposed as an innovative stress promoter to accumulate bioactive compounds (Jacobo-Velázquez et al., 2017). PEF consists on delivering short high-voltage bursts of electrical energy to a food placed between two electrodes. PEF application induces a transmembrane potential difference across the cell membrane. A phenomenon known as electropermeabilization occurs when this potential reaches a critical value, which results in increasing the cell permeability (Knorr & Angersbach, 1998) and the breakage of the membrane in a reversible or irreversible way. As a response, several metabolic and structural changes are triggered in plant cells, which may have an impact on quality attributes and bioactive compounds content. PEF treatments have exhibited the potential of maintaining the physicochemical quality of liquid food products inactivating microorganisms and enzymes without significantly impacting their properties (Martín-Belloso & Elez-Martínez, 2005b), enhancing intracellular metabolite extraction or improving the drying efficiency (Soliva-Fortuny et al., 2009). However, the application of PEF as an abiotic stressor for the biosynthesis of secondary metabolites in fresh

produce is a research area still under development. PEF parameters must be optimized to avoid undesirable effects on sensorial or nutritional characteristics. In this sense, it is important to understand how PEF affects the viability of plant cells and to evaluate its uniformity across tissues. The effects of PEF on quality attributes have been independently studied of those caused on bioactive compounds. It has been reported that PEF may induce softening of carrot tissues and reduce their cutting resistance (Lebovka, Praporscic, & Vorobiev, 2004; Leong & Oey, 2014; Wiktor et al., 2018) as well as modify their colour (Wiktor et al., 2015). There are only few reports assessing non-thermal processing effect on bioactive compounds in carrots [e.g. ultrasounds (Cuéllar-Villarreal et al., 2016a; Nowacka & Wedzik, 2016) or UV radiation (Formica-Oliveira, Martínez-Hernández, Díaz-López, Artés, & Artés-Hernández, 2017)]. An enhancement in the extractability of carotenoids from carrot slices after PEF was achieved after applying 1.85 kV cm^{-1} and 10, 50 and 100 pulses (Wiktor et al., 2015). There are also studies in other whole products: potato (Gómez Galindo et al., 2009), apple (Soliva-Fortuny, Vendrell-Pacheco, Martín-Belloso, & Elez-Martínez, 2017), and tomato (Vallverdú-Queralt, Oms-Oliu, et al., 2013), although effect of PEF on quality attributes were only studied in blueberry (Jin et al., 2017) and tomato (González-Casado et al., 2018b). As far as we know, there is no available information about the effect of PEF and post-treatment time on the phenolic content and on the quality attributes of whole carrots. The identification of the optimal conditions that enhance phenolic content without altering the quality of carrots provides an opportunity to obtain derived products with health-promoting properties and meet the consumer demand for natural and functional products.

The central idea behind this study was the application of PEF treatments as a strategy to enhance the phenolic content of carrots. In addition, their effects on quality attributes and PEF impact on cell viability and permeability were determined.

2. Material and methods

2.1. Chemicals and reagents

HPLC grade methanol and analytical reagent grade sodium carbonate were acquired from Fisher Scientific Scharlau Chemie (Loughborough, UK), 2, 3, 5-triphenyltetrazolium chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA), Folin-Ciocalteu reagent was acquired from Scharlau S.L. (Barcelona, Spain) and sodium chloride was purchased from POCH S.A. (Sowińskiego, Poland).

2.2. Carrots sample

Carrots (*Daucus carota* cv. Nantes) were obtained in a local supermarket in Lleida (Spain) and were stored at 4 ºC for less than a week until treatment. Whole carrots with caliber 25/35 mm and length of 17 ± 2 cm were washed with tap water and the excess was carefully removed with a paper cloth before application of PEF treatments.

2.3. Pulsed electric fields (PEF) treatments

PEF treatments (**Table 1.1**) were conducted in a batch PEF system (Physics International, San Leandro, CA, USA). The equipment delivers pulses with exponentially decaying waveform pulses of 4 μs from a capacitor of 0.1 μF 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 pulse generator had a charge voltage of +5 to +7kV DC, a trigger pulse of +250 V and an output voltage of +50 kV. The treatment chamber consisted of a parallelepiped methacrylate container with two parallel stainless steel electrodes (20 × 5 cm) separated by a gap of 5 cm. Carrots were placed in parallel to the electrodes and immersed in an aqueous solution (conductivity of 10 μS cm^{-1}). Different electric field strengths (0.8, 2 and 3.5 kV cm^{-1}) and number of pulses (5, 12 and 30 pulses) were applied in accordance to Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso (2009). The specific energy input (Ws), expressed in kJ kg^{-1}, delivered with each treatment was calculated according to Eq. (1):

$$Ws = \frac{V^2 Cn}{2m} \quad (1)$$

where V [V], C [F], n, and m [kg] are the voltage, capacitance of the energy storage capacitor, number of pulses and mass of the sample in the treatment chamber, respectively. The temperature of the treatment aqueous solution after PEF application was below 20.0 °C. The mass of the sample was equal to about 0.1 kg and the ratio carrots:aqueous solution was 1:3 (w:v).

Table 1.1. PEF processing treatment conditions.

Electric field strength (kV cm^{-1})	**Number of pulses**	**Specific energy input (kJ kg^{-1})**
0	0	Untreated

0.8	5	0.14
	12	0.38
	30	0.87
2.0	5	0.22
	12	0.50
	30	1.19
3.5	5	0.61
	12	1.92
	30	3.93

2.4. Cell permeability

2.4.1. Electrical conductivity

The electrical conductivity of the aqueous solution contained in the treatment chamber was measured before and immediately after PEF treatments using a conductometer Testo 240 (Lenzkirch, Germany). The solution was replaced after each treatment and measures were performed twice.

2.4.2. Weight

Carrots weight loss was evaluated after PEF treatments. Gravimetric methods were used [AND electronic Balance FX-2000 (AND Company Limited, Tokyo, Japan)] and measures were performed twice. Weight loss was calculated in reference to untreated carrots with the Eq. (2).

$$\text{Weight loss } (\%) = \frac{W_0 - W}{W_0} \times 100 \quad (2)$$

where W_0 is referred to weight of untreated carrots and W is referred to weight of PEF-treated carrots (immediately, 24 h and 48 h). Lag time between treatment and weight measurement just after treatment averaged 20 min.

2.5. Cell viability

Tetrazolium salt staining was used to evaluate the effect of PEF on cell/tissue viability within carrot slices and to determine the proportion of viable cells, as previously described by Faridnia, Burritt, Bremer, & Oey (2015). The principle of tetrazolium salt staining is based on the formation of an insoluble red formazan from the reduction of the salt by oxidoreductase enzymes. The amount of formazan is directly proportional to the number of living cells as they contain oxidoreductases (Berridge et al., 2005). A 0.5% (w/v) tetrazolium salt solution was prepared with Milli-Q water on the same day as the PEF treatment. Immediately after PEF treatment, each carrot was sliced (1.5 mm thickness and 32 ± 3 mm diameter). Three slices of each carrot were placed in a petri dish and fully immersed in the tetrazolium solution. Petri dishes were then covered in tin foil to protect the samples from light and left for 24 h at 18–20 °C. After 24 h slices were rinsed with water, blotted dry with a paper towel and photographed. Image analysis using colour threshold method [ImageJ software, (Abràmoff, Hospitals, Magalhães, & Abràmoff, 2007)] was applied to measure the red and unstained areas on each slice, indicative of living and dead cells, respectively. Cell viability (%) of each slice was calculated using the following Eq. (3):

$$\text{Cell viability (\%)} = \frac{\text{Stained area}}{\text{Total area}} \times 100 \quad (3)$$

Measures were conducted in three slices for each treatment replicate.

2.6. Post-treatment determinations

Carrots were treated with different PEF conditions in order to evaluate their effect on the total phenolic content and over their quality attributes immediately, 24 h or 48 h at 4 ºC after PEF treatments.

2.6.1. Extraction and analysis of phenolic compounds

Phenolic compounds were extracted following the methodology proposed by Ribas-Agustí, Cáceres, Gratacós-Cubarsí, Sárraga, & Castellari (2012) with slight modifications. Carrot tissue (5 g) was homogenized with an 80% (v/v) methanol solution (25 mL) using an Ultra-Turrax T25 (IKA® WERKE, Germany). Homogenates were centrifuged at 21612 *g* at 4-6 °C for 20 min. Supernatants were collected and 25 mL of methanol solution was added to the precipitate. In order to ensure a complete extraction, precipitates were vortexed for resuspension and immersed in an

ultrasonic bath for 5 minutes. Samples were centrifuged under the same conditions explained above. Both supernatants were collected and stored at -40 ºC until analysis (for up to ten days until analysis).

The total phenolic content was determined according to the Folin–Ciocalteu procedure adapted to 96-wells microplates. Methanolic extracts (30 µL) were placed into a microplate. Then, 150 µL of 10% (v/v) Folin-Ciocalteu's reagent and 120 µL of Na_2CO_3 7.5% (w/v) were added. After an incubation period of 90 min at room temperature in darkness, the absorbance was measured at 765 nm using a microplate reader (Thermo Scientific Multiskan GO, Vantaa, Finland). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight relative to those of untreated carrots. Phenolic content was extracted and spectrophometrically determined twice per replica.

2.6.2. Quality attributes

2.6.2.1. Hardness. Hardness of cortical tissue and vascular cylinder of carrots were determined in three carrot slices (15 mm height and 32 ± 3 mm diameter) of each replica. These disks were cut out of top end (1/3 of total length) of the carrot. Hardness was determined with a TA-XT2 texture analyser (Stable Micro Systems Ltd., Surrey, England), equipped with a 4-mm-diameter steel probe, which penetrated 10 mm the carrot tissue at a constant rate of 5 mm s^{-1}. The motion of the blade was perpendicular to the surface of carrot disks. Hardness (N s) was determined as area under the curve between the graph of y (force) and (time) (Ribas-Agustí et al., 2019a)

2.6.2.2. Colour. The CIEL*a*b* parameters (lightness, L*; green-red chromaticity, a*; and blue-yellow chromaticity, b*) were utilized to characterise the external colour of carrots from each treatment using a Minolta colorimeter (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan). The apparatus was set up for a D65 illuminant and 10° observer angle. A white standard plate (Y=94.00, x=0.3158, y=0.3322) was used for calibration. The colour was assessed by measuring the lightness (L*), the a*/b* ratio and total colour difference (ΔE), calculated by Eq. (4).

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{0.5} \quad (4)$$

where L*, a* and b* refer to data collected after treatments and L^*_0, a^*_0 and b^*_0 refer to untreated carrots. Three readings were made in each replica by changing the position of the carrot in each measure.

2.7. Statistical analysis

Statistical analyses were carried out using the JMP Pro v.13 software (SAS Institute, Cary, NC, USA) and IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL). There were three different replicates for each assayed treatment condition and each analysis was conducted at least twice. Results were reported as the mean ± standard deviation. Normality and homocedasticity criteria were evaluated by Shapiro-Wilk and Levene tests, respectively. Results were subjected to an analysis of variance (ANOVA) followed by Tukey *post hoc* test in order to establish statistical differences among mean values. The partial square eta value (η_p^2) was reported to indicate the effect size of each analysed parameter. Repeated measures ANOVA was applied to establish differences among tissues hardness. The relationship between variables was determined using the Pearson (r) coefficient. Rho of Spearman (r_s) was used to establish a correlation between the specific energy input and media conductivity because of the outliers presence. The statistical significance level was set up at $p < 0.05$.

3. Results and discussion

3.1. Effects of PEF on cell permeability

In the present study, electroporation efficiency was evaluated based on changes in media conductivity immediately after treatments (**Table 1.1**) and in carrots' weight throughout 48 h after applying PEF treatments in a range of energies (0 – 3.93 kJ kg^{-1}).

Conductivity of the media in which carrots were immersed during treatments significantly ($p < 0.05$) increased after applying electric field intensities of 2 kV cm^{-1} or 3.5 kV cm^{-1}. The obtained results suggest that the electric field strength ($\eta_p^2 = 0.982$) followed by the number of pulses ($\eta_p^2 = 0.938$) and their interaction ($\eta_p^2 = 0.953$) significantly ($p < 0.001$) affected conductivity values. The specific energy input has also influence in the conductivity increase, as a significant ($p < 0.001$) correlation ($r_s = 0.774$) between those factors was found (**Figure 1.1**). However, the increase in pulse number only caused an increment in media conductivity when 3.5 kV cm^{-1} treatments were applied. Changes in pulse number and electric field strengths are highly related to changes in the specific energy input [Eq. (1)]. The effect of pulse number was clear for

carrot tissues subjected to treatments of 3.5 kV cm^{-1}, as the range of energies applied was broader than those for 0.8 kV cm^{-1} and 2 kV cm^{-1} treatments.

It is well known that the application of PEF to cell tissues results in electropermeabilization, which leads to release intracellular content, mainly ions and charged particles to the surrounding medium, increasing its conductivity and promoting weight loss. However, few studies have reported results using this approach in whole products (Ersus & Barrett, 2010b; Faridnia et al., 2015). In order to electropermeabilize carrots, Bazhal, Lebovka, & Vorobiev (2003) determined an optimal value of 2.5 ± 0.2 kV cm^{-1}. Similar results were reported by Wiktor et al. (2015), who established that 1.85 kV cm^{-1} was not enough for that purpose. Reversibility generally occurs when only a small portion of the membrane has pores (Knorr & Angersbach, 1998) or the total membrane area is bigger than the electropermeabilized zone, which enables cells to repair themselves. Obtained results demonstrate that higher field strengths caused higher cell disruption and suggest that 0.8 kV cm^{-1} treatments were probably insufficient to form pores or cause irreversibility. On the other hand, more intense treatments caused an increase in conductivity because of the release of intracellular content, probably associated to the formation of irreversible pores. This increase in conductivity may be consequence of a better water transference. In this sense, Aguiló-Aguayo et al. (2014) confirmed that cytoplasmic content of PEF-treated carrots was mixed with the extracellular liquid after treatments. Furthermore, the formation of pores caused by PEF enhances the diffusion of low molecular weight compounds such as fructose, glucose and sucrose (Janositz, Noack, & Knorr, 2011) and losses of ions, such as Ca^{2+} or K^{+} (Faridnia et al., 2015), into the medium by passive processes.

Some PEF treatments and post-treatment time caused a significant ($p < 0.05$) decrease in carrots weight. The electric field strength applied was the main PEF processing parameter affecting weight loss ($\eta_p^2 = 0.868$) ($p < 0.001$), followed by the interaction with the number of pulses ($\eta_p^2 = 0.865$) ($p < 0.001$). Furthermore, a strong and direct correlation was found between the specific energy input and weight loss of carrots immediately after treatments ($r = 0.820$; $p < 0.001$). **Figure 1.2** shows that weight decreased throughout 48 h, specifically after the most intense treatments. Weight loss was observed immediately after applying energies higher than 0.5 kJ kg^{-1} ($E \geq 2$ kV cm^{-1}). In addition, differences 24 h after applying 2 kV cm^{-1} and 3.5 kV cm^{-1} treatments were also noticeable. Carrots treated by energies higher than 0.61 kJ kg^{-1} ($E = 3.5$ kV cm^{-1}) showed remarkable decreases in weight, reaching a maximum loss of 9.4 ± 1.3 % at 48 h. To the best of our knowledge, there are scarce studies evaluating weight loss of vegetable products after PEF treatments. The results suggest that the weight loss and the increase in media conductivity were highly correlated ($r = 0.931$; $p < 0.001$), meaning that changes in weight were

mainly caused by the release of intracellular fluids due to electropermeabilization. Weight loss throughout time may be related to the formation of irreversible pores. Some authors reported that minimal processing (e.g. fresh-cut processing) increases the stress and respiration rate (Sandhya, 2010) causing structural changes as redistribution of water in tissues and cellular decompartmentalization, which could entail weight loss increase.

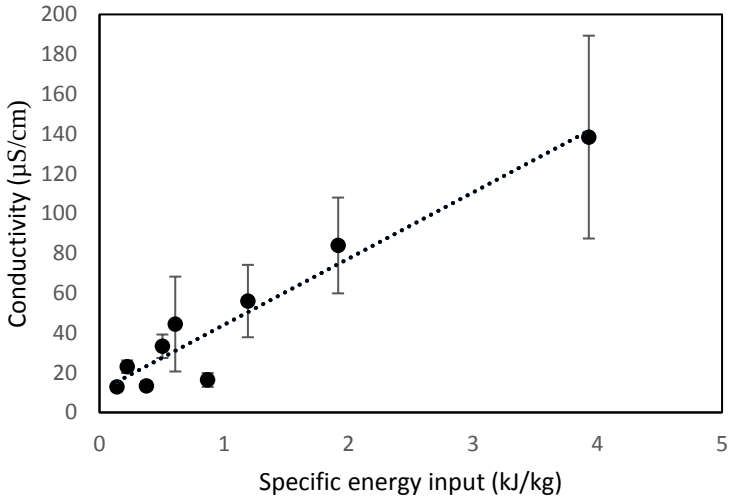


Figure 1.1. Correlation between the electrical conductivity of the treatment solution media and the specific energy input.

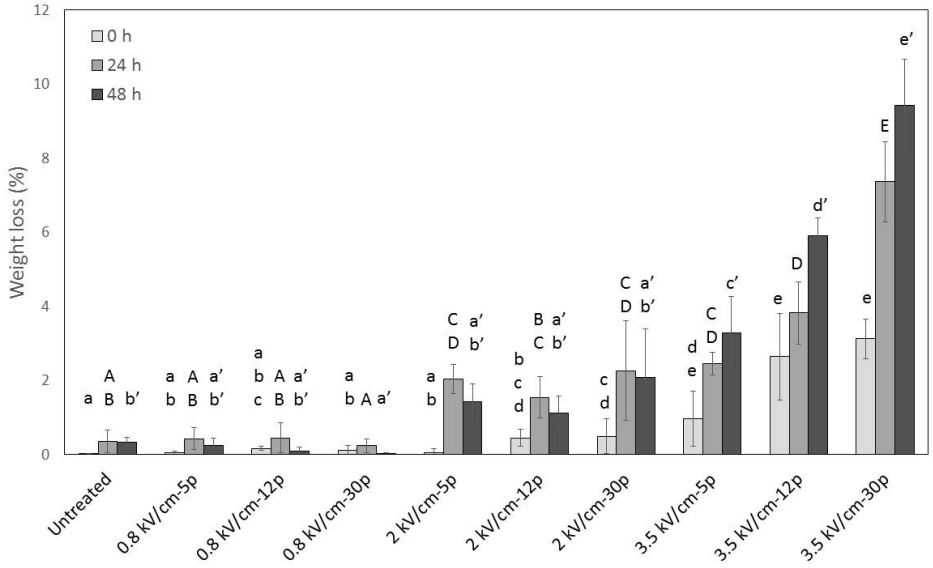


Figure 1.2. Changes on weight of untreated and PEF-treated carrots throughout 48 h after treatments. Different letters indicate significant ($p < 0.05$) differences among treatments at the same post-treatment time.

3.2. Influence of PEF treatment on cell viability

The impact of PEF on cell viability of carrots was evaluated after staining carrot slices with tetrazolium salt. The staining pattern (**Figure 1.3**) was not homogeneous throughout PEF-treated carrot tissues and among treatments. The effect of PEF depends on size, shape, orientation or electric properties of cells as observed by Faridnia, Burritt, Bremer, & Oey (2015) in PEF-treated potatoes. Furthermore, different physiological types of cells are generated after applying PEF: dead cells (membrane integrity and metabolic activity lost), intact cells (intact membrane and metabolic activity) and sublethally injured cells (SICs) (partial loss of membrane integrity and intact metabolic activity). Then, it must be taken into account that SICs and intact cells cannot be differentiated with this type of tinction.

A correlation between the specific energy input and cell viability was found (r = - 0.706; $p < 0.01$). Nevertheless, decreases in cell viability fitted better to increments in the electric field strength (**Table 1.2**). Statistical analysis indicate that electric field strength was the most influential parameter on cell viability ($\eta_p^2 = 0.617$) ($p < 0.001$). Carrots treated with an electric field strength of 0.8 kV cm^{-1} showed mainly live cells with a similar pattern as those untreated, indicating that pores were not formed or their formation was reversible under these conditions. Formed pores must be small in comparison to total area of the cell membrane, therefore carrot cells were able to repair themselves and maintain their integrity. At this electric field strength, cell viability was maintained (91.1 – 84.1 %) regardless the number of pulses applied. These results are in accordance with the insignificant weight loss (**Figure 1.2**) and the stability of media conductivity (**Figure 1.1**) in the same treatment conditions. On the other hand, after 2 kV cm^{-1} and 3.5 kV cm^{-1} treatments, an increase in media conductivity was observed and an immediate weight loss was reported after the most intense. In this sense, after applying 2 kV cm^{-1}, cell viability was significantly lower (81.0 – 73.5%) than that of untreated ones. Dead cells were mainly observed in the cortical parenchyma when 5 and 12 pulses were applied (0.22 and 0.50 kJ kg^{-1}), which may be explained due to different cell properties between tissues such as cell size. Vascular cylinder is composed by smaller cells; therefore, a higher electric field strength should be applied to achieve their electroporation. Interestingly, after applying 30 pulses (1.19 kJ kg^{-1}), the pattern was more similar to those obtained in 3.5 kV cm^{-1} treatments. Despite the qualitative dissimilarities, quantitative data did not show significant differences among 2 kV cm^{-1} treatments with different number of pulses. After applying 3.5 kV cm^{-1}, viability significantly ($p < 0.05$) decreased (87.5 - 79.4%). As shown in **Figure 1.3**, the epidermis was the most affected tissue, which at least had metabolic activity in these conditions. According to Jacobo-Velázquez et al. (2017), damaged cells (in this case, epidermis cells) would release ATP as a signaling molecule

for undamaged cells (internal tissues), that would trigger the activation of stress response leading to higher antioxidant activities to avoid damage.

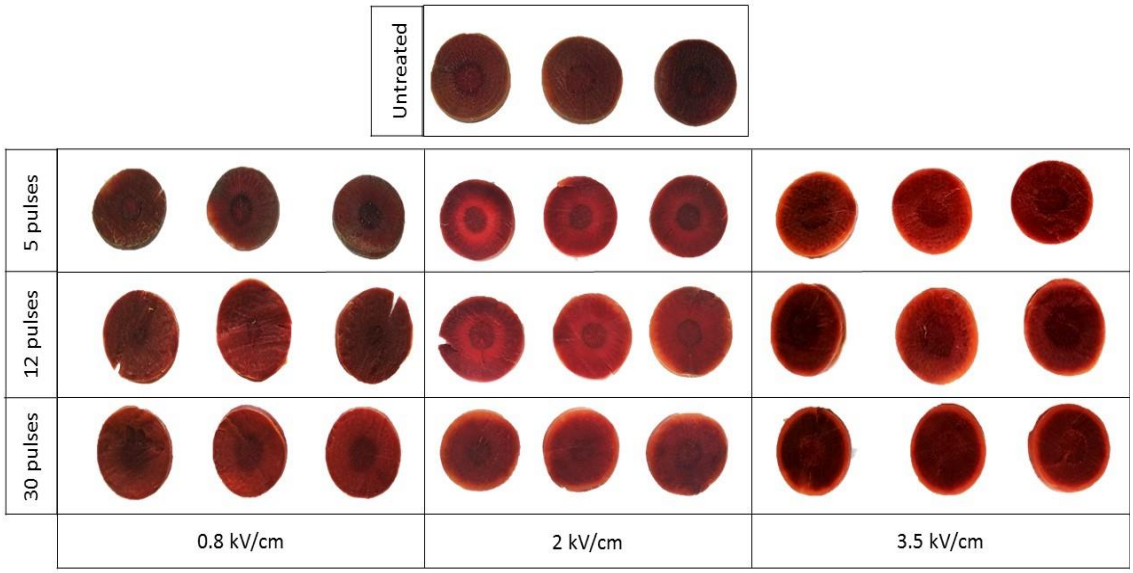


Figure 1.3. Representative photographs of carrots slices (1.5 mm thickness and 32 ± 3 mm diameter) obtained from untreated and PEF-treated carrots stained with tetrazolium salt. Dark and unstained regions indicate viable and dead cells, respectively.

Table 1.2. Changes in cell viability (%) after PEF treatments obtained by image processing.

Electric field strength (kV cm^{-1})	**Number of pulses**	**Cell viability (%)**
0	0	100 a
0.8	5	91 ± 6 b
	12	90 ± 5 bc
	30	84 ± 7 bcd
2.0	5	74 ± 8 d
	12	81 ± 3 bcd
	30	74 ± 7 d
3.5	5	83 ± 4 bcd
	12	75 ± 7 d
	30	73 ± 4 d

Different letters indicate significant ($p < 0.05$) differences among treatments.

3.3. Effects of PEF and time post-treatment on quality attributes

3.3.1. Hardness

Carrots have a complex tissue with different cell sizes and orientations, which may cause a heterogeneous effect of PEF across the whole product. Hence, the influence of PEF treatments on the hardness of carrots was investigated through a penetration test in cortical tissues and vascular cylinder immediately, 24 h and 48 h after treatments.

Hardness was significantly ($p < 0.05$) different between both cortical tissues and vascular cylinder depending on the treatment applied (**Figure 1.4**). Statistical analysis indicated that the main parameter affecting hardness was the electric field strength ($p < 0.01$), followed by the interaction with the number of pulses ($p < 0.05$). However, the electric field strength had bigger impact on the variability of hardness values ($\eta_p^2 = 0.868$).

Differences among tissues treated under the same conditions were observed immediately after treatments. Vascular cylinders were softer (26.50 % – 8.75 %) ($p < 0.05$) than the cortical tissues. However, neither the hardness of the cortical tissue nor that of the vascular cylinder were significantly ($p > 0.05$) affected in comparison to the same tissues in untreated carrots, regardless the number of pulses applied. Differences among tissues observed just after treatments were not maintained after 24 h. At such time, the hardness of the cortical tissue of carrots treated by 30 pulses of 3.5 kV cm^{-1} (3.93 kJ kg^{-1}) was lower (8.3 ± 1.0 N s) compared to untreated carrots (11.4 ± 1.0 N s), but the hardness of the vascular cylinder was similar among treatments. After 48 h of applying 3.5 kV cm^{-1} treatments, hardness decreased in both cortical (8.60 – 9.12 N s) and vascular tissues (7.40 – 8.39 N s) compared to that of untreated ones (cortical tissue: 11.5 ± 0.1 N s; vascular cylinder: 8.6 ± 0.9 N s). On the other hand, cortical hardness of carrots treated by 2 kV cm^{-1} increased after 48 h, in contrast to that obtained 24 h after treatments, whereas vascular tissue remained similar to those untreated.

Regarding the differences among vascular cylinder and cortical tissues within the same treatments, the effect of PEF conditions depends on numerous factors including cell properties (size, conductivity, shape and orientation) and PEF treatments parameters (electric field strength, pulse amplitude, shape, duration and number) (Lebovka & Vorobiev, 2009). Obtained results suggest that changes observed throughout time were mainly due to permeabilization of membranes in the most intense treatments. A decompartmentalization process, a water redistribution across the tissues and a loss of turgor occurs immediately after PEF and throughout post-treatment time (Aguiló-Aguayo et al., 2014). These results are in accordance with the

correlations found among hardness decrease and weight loss, decrease in lightness and increase in media conductivity (Supplementary material). On the other hand, the hardness increase in carrots treated by 2 kV cm^{-1} after 48 h may be a result of lignification, a plant defence mechanism to prevent water loss (Becerra-Moreno et al., 2015).

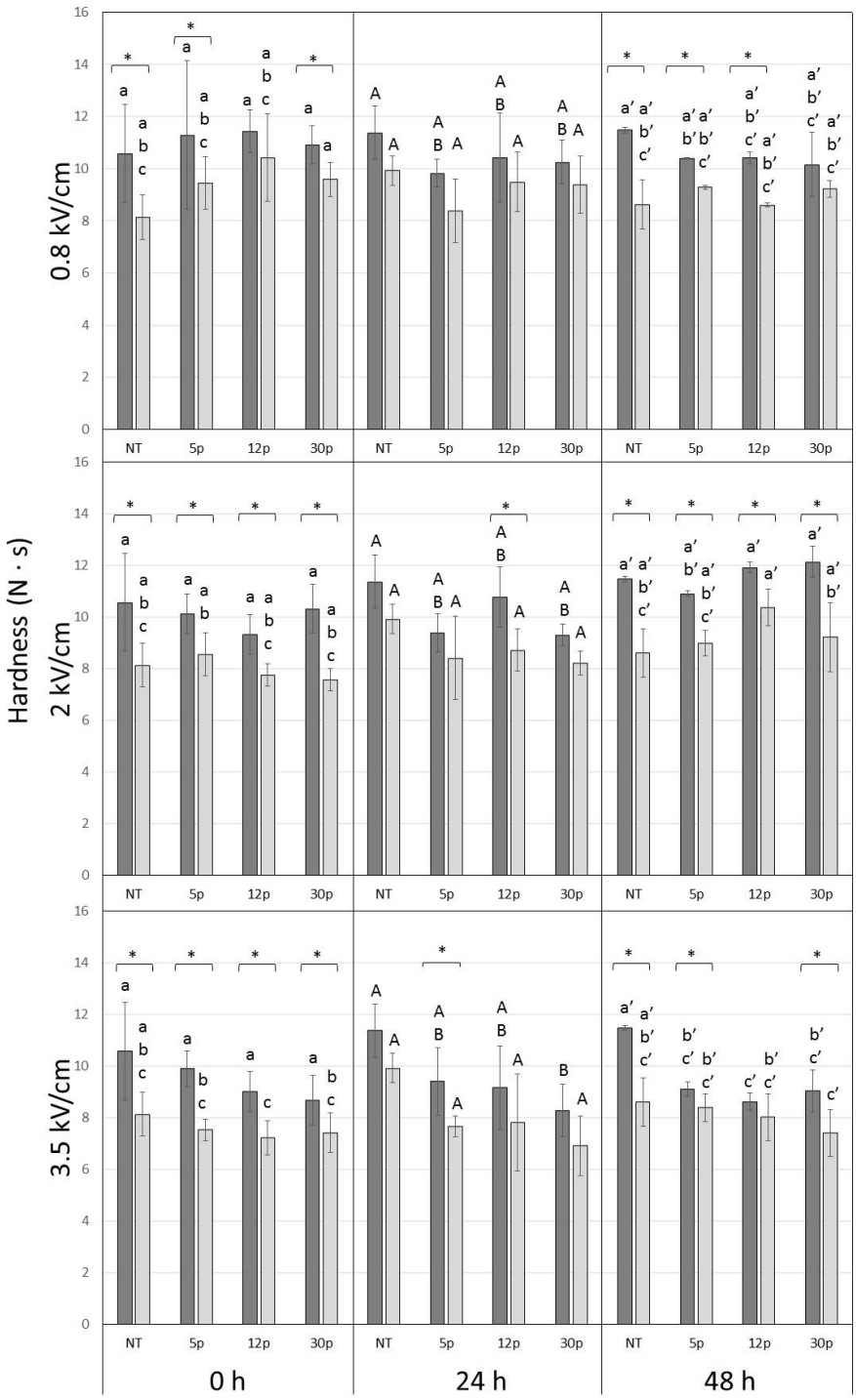


Figure 1.4. Effect of PEF on cortical tissue (■) and vascular cylinder (□) hardness throughout 48 h after treatments. Different letters indicate differences ($p < 0.05$) among carrots treated by

different PEF treatments at the same post-treatment time and non-treated carrots (NT). The asterisk symbol points differences between the hardness of tissues subjected to the same treatment at the same post-treatment time.

3.3.2. Colour

Colour is one of the most important quality parameters to be preserved in fruits and vegetables. Certain changes in colour provides information related to tissue destructuration and consumers' perception. Changes in colour were evaluated throughout time measuring of L*, a*/b* ratio and ΔE.

The application of PEF and time after treatment contributed to significant ($p < 0.05$) changes in colour. The main parameter responsible for this effect was the electric field strength ($\eta_p^2 = 0.848$) ($p < 0.001$), followed by its interaction with the number of pulses ($\eta_p^2 = 0.346$) ($p < 0.05$). The application of higher electric field strengths was associated with a significant ($p < 0.05$) decrease in lightness in comparison to untreated carrots. As **Table 1.3** shows, 24 h after PEF, only carrots subjected to the most intense treatments (12 and 30 pulses of 3.5 kV cm^{-1}) showed significantly decreased L* values (47 ± 4 and 47.0 ± 2.1, respectively) compared to those untreated (53.9 ± 2.4). Moreover, 48 h later, differences were noticeable in all PEF-treated carrots except for the 0.8 kV cm^{-1} conditions, which did not cause significant changes in lightness throughout time. These results are in agreement with previous data suggesting that 0.8 kV cm^{-1} treatments are insufficient to cause irreversible electropermeabilization. Changes in L* values are a consequence of decompartmentalization and cell membrane disruption, which favour the contact between oxidative enzymes, such as peroxidase (POD) and polyphenol oxidase (PPO) and their phenolic substrates, previously located in the vacuoles. A correlation ($p < 0.001$) was found between L* values corresponding to 24 h after PEF and the specific energy input applied ($r_s = - 0.697$). These results suggest that initial decrease in lightness could be a direct consequence of electropermeabilization and cell disruption. However, the latest changes may be due to metabolic alterations induced by structural cell damage. Regarding the a*/b* ratio, it remained stable among treatments and over 48 h, indicating no differences in redness and yellowness, parameters that have been related to carotenoid content in tomato fruit (Arias, Lee, Logendra, & Janes, 2000).

The total colour difference (ΔE) was significantly ($p < 0.05$) affected by PEF treatments and post-treatment time ($\eta_p^2 = 0.499$). The parameters that had bigger impact on ΔE values were the electric field strength ($\eta_p^2 = 0.706$) and number of pulses ($\eta_p^2 = 0.684$) followed by their interaction ($\eta_p^2 = 0.397$). Immediately, and 24 h after treatment, differences were only significant

after applying 12 pulses of 3.5 kV cm^{-1} and 30 pulses of 2 kV cm^{-1}, respectively. However, after 48 h, a noticeable increase in comparison to untreated carrots was observed in carrots treated by 2 kV cm^{-1} and 3.5 kV cm^{-1}. These variations are mainly related to L* value decrease explained above. It is remarkable that ΔE values were higher than 2 regardless the treatment applied and time after PEF. Values > 2 indicates that such colour change could be visible by a consumer with the naked eye (Tiwari, Patras, Brunton, Cullen, & O'Donnell, 2010). Therefore, subjecting carrots to PEF would modify the product appearance. Wiktor et al., (2015) determined similar effects on ΔE values of PEF-treated fresh-cut carrots, although a time effect after treatments on this parameter was not found. This fact might be related to some variations between both studies. Measurements were made 60 min after PEF instead of 48 h in this study, mechanical damage caused by cylinder cutting instead of using the whole vegetable or the orientation of the carrot with respect to the electrodes (data not provided), which may explain the differences regarding the PEF effect on tissues (Faridnia et al., 2015).

Table 1.3. L*, a*/b* values and total colour difference (ΔE) of untreated and PEF-treated carrots immediately, 24 h and 48 h after treatments.

Time post-treatment (h)	Electric field strength (kV cm^{-1})	Number of pulses	L*	a*/b*	ΔE
0	Untreated		52.6 ± 2.1 ab	0.55 ± 0.03 a	2.2 ± 0.4 a
	0.8	5	52.8 ± 1.2 ab	0.60 ± 0.03 a	5.0 ± 2.1 ab
		12	56 ± 3 a	0.57 ± 0.02 a	3.4 ± 2.5 ab
		30	52.6 ± 2.4 ab	0.57 ± 0.03 a	5.7 ± 0.8 ab
	2.0	5	53.9 ± 2.4 ab	0.58 ± 0.05 a	2.7 ± 1.1 ab
		12	48.6 ± 1.4 b	0.58 ± 0.09 a	8.3 ± 2.7 ab
		30	51.5 ± 0.5 ab	0.57 ± 0.01 a	7 ± 4 ab
	3.5	5	51.5 ± 2.1 ab	0.56 ± 0.03 a	4 ± 3 ab
		12	48.9 ± 1.5 b	0.51 ± 0.01 a	10 ± 4 b
		30	48.9 ± 1.3 b	0.53 ±0.03 a	9.1 ± 2.0 ab
24	Untreated		53.9 ± 2.4 a	0.56 ± 0.03 a	3.2 ± 0.9 a
	0.8	5	54.5 ± 0.7 a	0.60 ± 0.04 a	2.7 ± 1.3 ab
		12	53.2 ± 0.4 ab	0.57 ± 0.00 a	3.7 ± 1.4 ab
		30	51.5 ± 1.7 abc	0.55 ± 0.03 a	4.9 ± 1.2 ab
	2.0	5	52.7 ± 1.9 abc	0.58 ± 0.04 a	2.8 ± 0.7 ab

		12	50.2 ± 1.8 abc	0.56 ± 0.03 a	4.6 ± 2.4 ab
		30	50 ± 3 abc	0.57 ± 0.02 a	8.9 ± 1.4 b
	3.5	5	48.7 ± 1.4 abc	0.57 ± 0.03 a	5.7 ± 1.9 ab
		12	47 ± 4 bc	0.55 ± 0.02 a	7 ± 4 ab
		30	47.0 ± 2.1 c	0.57 ± 0.06 a	7 ± 4 ab
	Untreated		55.9 ± 0.3 a	0.61 ± 0.05 a	2.3 ±1.9 a
	0.8	5	53.2 ± 0.3 b	0.58 ± 0.04 a	4.2 ±1.3 ab
		12	54.0 ± 1.8 ab	0.57 ± 0.06 a	5 ± 4 ab
		30	53.8 ± 0.4 ab	0.58 ± 0.11 a	7 ± 5 abc
	2.0	5	48.7 ± 0.5 c	0.60 ± 0.04 a	10.1 ± 0.5 bc
48		12	48.9 ± 0.8 c	0.56 ± 0.03 a	8.4 ± 0.7 abc
		30	45.4 ± 0.5 d	0.60 ± 0.01 a	16 ± 3 c
	3.5	5	49.7 ± 0.2 c	0.56 ± 0.00 a	7.1 ± 0.5 bc
		12	48.6 ± 0.7 c	0.56 ± 0.02 a	8.9 ± 0.8 bc
		30	48.4 ± 0.6 c	0.52 ± 0.02 a	10.0 ± 0.3 bc

Different letters in the same column at the same post-treatment time indicate significant ($p < 0.05$) differences.

3.4. Effects of PEF and time post-treatment on total phenolic compounds content

Results indicate that the application of PEF and post-treatment time significantly ($p < 0.001$) affected phenolic compounds content in carrots (**Figure 1.5**). The analysis of variance showed that both post-treatment time and PEF parameters (electric field strength and number of pulses) had significant ($p < 0.001$) impact on phenolic compounds content. Obtained results demonstrate that post-treatment time ($\eta_p^2 = 0.992$) and the electric field strength ($\eta_p^2 = 0.669$) had bigger impact on the variability of phenolic content. In addition, the interaction between the electric field strength and number of pulses plays a significant ($p < 0.05$) role ($\eta_p^2 = 0.471$).

No significant ($p > 0.05$) changes in phenolic compounds content were reported compared to untreated carrots immediately after PEF application. After 24 h of PEF, some treatments caused a significant ($p < 0.05$) increase in the phenolic content compared to that found in untreated carrots stored under the same conditions. Higher increases in comparison to untreated carrots were given after 5 pulses of 3.5 kV cm^{-1} (0.61 kJ kg^{-1}) (39.5 ± 0.1 %) and 30 pulses of 0.8 kV cm^{-1} (0.87 kJ kg^{-1}) (40.1 ± 0.2 %). The remaining treatments caused a minor increase, with the exception of 12 pulses of 2 kV cm^{-1} (0.5 kJ kg^{-1}) and 30 pulses of 3.5 kV cm^{-1} (3.93 kJ kg^{-1}), which remained unaltered. On the other hand, 5 and 12 pulses of 0.8 kV cm^{-1} (0.14 and 0.38 kJ kg^{-1}, respectively)

showed a decrease. After 48 h, a significant ($p < 0.05$) decrease in phenolic content occurred in carrots subjected to a field strength equal or higher than 2 kV cm^{-1}, whereas after the application of 0.8 kV cm^{-1} phenolic content remained unaltered.

The maintenance of the phenolic content immediately after PEF suggests that these compounds were not released through the formed pores, although media conductivity (**Figure 1.1**) and weight loss (**Figure 1.2**) increased. Probably, PEF conditions applied were not enough to electroporate vacuoles, where phenolic compounds are mainly located. Galindo et al. (2009) reported that PEF-treated potato slices had a metabolic profile similar to that of untreated ones immediately after treatment. These results are consistent with the mechanism of action of plant stress defence. As an immediate response, during the first minutes, stress signaling molecules are produced [i.e. reactive oxygen species (ROS), ethylene, jasmonic acid, etc.] that would activate the expression of genes involved in the primary and secondary metabolism of the plant. Because of this activation, a long-term physiological process (hours or days) (Zhao et al., 2005) takes place to adapt to the new environment or to recover from damage. For instance, increasing the activity of certain enzymes, the biosynthesis of secondary metabolites (Jacobo-Velázquez et al., 2015), causing changes in respiration rate or in carbon sources. Nonetheless, in this study, a delayed defensive response must be considered. The increase in media conductivity after some treatments may involve an insufficient level of Ca^{2+} and K^{+} in the cytoplasm, which are important molecules to trigger the signaling network during stress response. These ions can be released during PEF treatments through formed pores as reported by Faridnia, Burritt, Bremer, & Oey (2015).

After 24 h of PEF treatments, variations in the phenolic content were observed among treatments. These differences are difficult to explain due to the complexity of metabolic networks in natural systems; several factors may be involved in these changes. Vallverdú-Queralt et al. (2013) based on a PLS-DA analysis in PEF-treated tomatoes, reported that changes in individual phenols were highly correlated to specific combinations between number of pulses and electric field strengths, causing differences in the total content. On the other hand, the type of formed pores and the way cells detect the damage intensity might cause these changes. By increasing field strength, pulse duration and number of pulses, the number and size of pores in the cell membrane increase (Bazhal et al., 2003; Janositz et al., 2011; Knorr & Angersbach, 1998). After 0.8 kV cm^{-1}, transient small pores are probably more common than irreversible ones. Hence, the observed decrease in phenolic content may be a plant defence way to fastly obtain energy through degradation of starch or antioxidants compounds and contribute to the hexose pool to recover from this weaker damage. More intense treatments (2 kV cm^{-1} and 3.5 kV cm^{-1}) cause a strong structural damage (i.e. less intercellular adhesion, permanent pores in membranes, intracellular content release). Furthermore, an increase in mass transfer may cause osmotic dehydration, an

additional stress that require a different metabolic strategy, which probably involves *de novo* biosynthesis of phenolic compounds to avoid oxidative damage. As a result, an increase in phenolic content was observed in these conditions.

A decrease of phenolic content in carrots was observed after 48 h of some PEF treatments. Soliva-Fortuny, Vendrell-Pacheco, Martín-Belloso, & Elez-Martínez (2017) reported similar results in apples submitted to PEF treatments and stored for 48 h at different temperatures. Several reasons would explain our results. Firstly, due to structural decompartmentalization, oxidative enzymes such as PPO or POD contact easily with their phenolic substrates. This idea is supported by obtained colour data (**Table 1.3**). L* value was decreased in the same treatments characterized by the lowest phenolic content at 48 h. In addition, a higher weight loss indicates a major intracellular liquid release and cellular destructuration that favoured this contact. Secondly, the decrease in phenolic compounds after 2 kV cm^{-1} treatments may be related to lignification process given the intracellular liquid release and hardness maintenance in cortical tissues observed at this time. Becerra-Moreno et al. (2015) reported that the combination of water loss and wounding stress might entail the accumulation of phenolic compounds in plants depending on their biosynthesis and utilization rate for lignin biosynthesis. Finally, electrical pulses could affect the three-dimensional structure of proteins and enzymes, constituted by weak non-covalent forces, hydrogen bonds and hydrophobic interactions (Ohshima, Tamura, & Sato, 2007). In the most intense treatments, 3.5 kV cm^{-1}, the highest intracellular liquid release, weight loss and decrease in hardness suggest that phenolic compounds may be released through formed pores and damaged protein channels responsible of the active transport system. These structural changes may be useful to enhance the release of phenolic compounds of cells and improve their bioaccessibility.

4. Conclusions

Results reported in this study suggest that the electric field strength was the most influential variable in media conductivity, quality attributes, cell viability and phenolic content variations. Additionally, a high correlation was found among media conductivity and the specific energy input. Moreover, PEF and post-treatment time affected the amount of phenolic compounds in carrots as well as their quality attributes. Carrots treated by 5 pulses of 3.5 kV cm^{-1} or 30 pulses of 0.8 kV cm^{-1} and stored during 24 h at 4 ºC led to the highest increases (39.5 % and 40 %) in phenolic content compared to those in the untreated carrots. At such conditions, surface colour and hardness were maintained, but weight loss and softening occurred because of electropermeabilization and cell disruption promoted by the most intense PEF condition. Irreversible damage occurred mainly in the epidermis, thus leading to a release of intracellular

content. Therefore, both PEF treatments could be proposed as a pre-processing treatment of raw material to produce carrot-based products with high antioxidant potential.

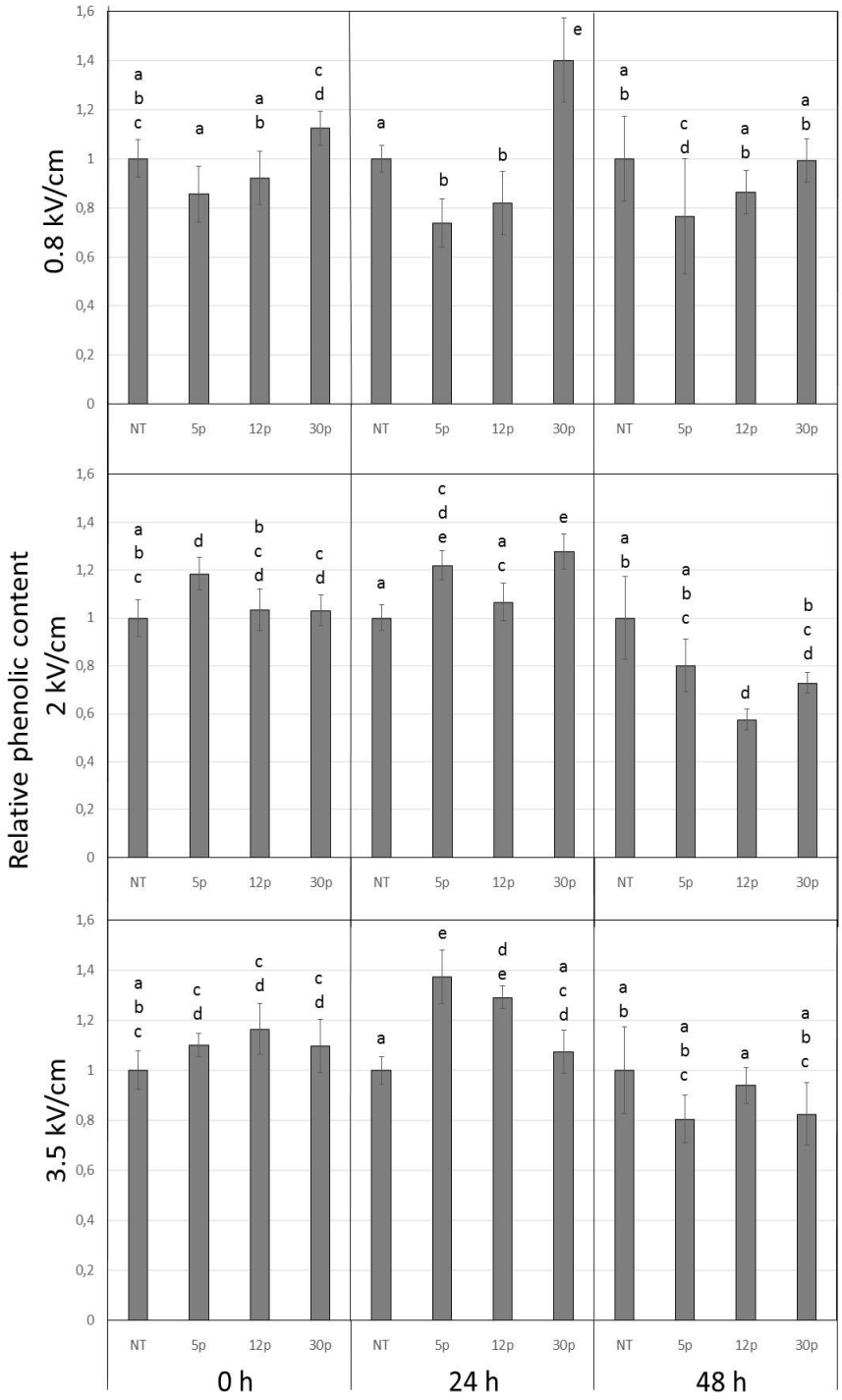


Figure 1.5. Relative phenolic content from non-treated (NT) and PEF-treated carrots throughout 48 h after treatments. Different letters indicate significant ($p < 0.05$) differences among groups at the same post-treatment time.

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SUPPLEMENTARY MATERIAL

Table S1.1. Correlation coefficients between specific energy input, phenolic content, cell viability, media conductivity and the quality attributes of carrots immediately after PEF treatments.

	Ws	***WL 0 h***	***MC***	***CV***	***L* 0 h***	***a*/b* 0 h***	***ΔE 0 h***	***VCF 0 h***	***CF 0 h***	***PC 0 h***
Ws										
WL 0 h	*0.820***									
MC	*0.774***	*0.931***								
CV	*-0.706***	*-0.525**	*-0.42**							
L* 0 h	*-0.544***	*-0.615**	*-0.501***	*0.331*						
a*/b* 0 h	*0.438**	*-0.575**	*-0.444**	*0.297*	*-0.44**					
ΔE 0 h	*0.525***	*0.426**	*0.432**	*-0.034*	*-0.511***	*0.153*				
VCF 0 h	*-0.576***	*-0.656***	*-0.509***	*0.502***	*0.552***	*0.189*	*-0.282*			
CF 0 h	*-0.542***	*-0.590***	*-0.667***	*0.489***	*0.579***	*0.367*	*-0.451**	*0.648***		
PC0 h	*0.276*	*0.219*	*0.162*	*-0.466**	*-0.26*	*-0.383**	*0.094*	*-0.226*	*-0.186*	

Significant correlation at $p < 0.05$ (*) and $p < 0.01$ (**). Ws: Specific energy input; WL: Weight loss; MC: Media conductivity; CV: Cell viability; L*: Lightness; ΔE: Total colour difference; VCF: Vascular cylinder hardness; CF: Cortical hardness; PC: Phenolic content.

Table S1.2. Correlation coefficients between specific energy input, phenolic content, cell viability, media conductivity and the quality attributes of carrots 24 h after PEF treatments.

	Ws	***WL 24 h***	***MC***	***CV***	***L* 24 h***	***a*/b* 24 h***	***ΔE 24 h***	***VCF 24 h***	***CF 24 h***	***PC 24 h***
Ws										
WL 24 h	*0.712***									
MC	*0.774***	*0.890***								
CV	*-0.706***	*-0.613***	*-0.42**							
L* 24 h	*-0.697***	*-0.634***	*-0.618***	*0.419**						
a*/b* 24 h	*0.043*	*-0.13*	*-0.008*	*0.153*	*-0.181*					
ΔE 24 h	*0.583***	*0.351*	*0.339*	*-0.227*	*-0.748***	*-0.016*				
VCF 24 h	*-0.527***	*-0.638***	*-0.655***	*0.433**	*0.551***	*0.032*	*-0.259*			
CF 24 h	*-0.453**	*-0.586***	*-0.541***	*0.250*	*0.378*	*0.162*	*-0.233*	*0.570***		
PC 24 h	*0.397**	*0.256*	*0.083*	*-0.503**	*-0.481**	*-0.303*	*0.410**	*-0.301*	*-0.222*	

Significant correlation at $p < 0.05$ (*) and $p < 0.01$ (**). Ws: Specific energy input; WL: Weight loss; MC: Media conductivity; CV: Cell viability; L*: Lightness; ΔE: Total colour difference; VCF: Vascular cylinder hardness; CF: Cortical hardness; PC: Phenolic content.

Table S1.3. Correlation coefficients between specific energy input, phenolic content, cell viability, media conductivity and the quality attributes of carrots 48 h after PEF treatments.

	Ws	***WL 48 h***	***MC***	***CV***	***L* 48 h***	***a*/b* 48 h***	***ΔE 48 h***	***VCF 48 h***	***CF 48 h***	***PC 48 h***
Ws										
WL 48 h	*0.383**									
MC	*0.774***	*0.672***								
CV	*-0.706***	*-0.275*	*-0.42**							
L* 48 h	*-0.400**	*-0.238*	*-0.394**	*0.595***						
a*/b* 48 h	*0.161*	*-0.356*	*-0.372*	*0.099*	*-0.023*					
ΔE 48 h	*0.148*	*0.197*	*0.292*	*-0.381**	*-0.76***	*0.331*				
VCF 48 h	*-0.415**	*-0.495***	*-0.534***	*0.454**	*-0.011*	*0.197*	*0.074*			
CF 48 h	*-0.381*	*-0.404**	*-0.476**	*0.372*	*-0.174*	*0.001*	*0.303*	*0.800***		
PC 48 h	*0.089*	*0.256*	*-0.12*	*-0.041*	*0.351*	*0.194*	*-0.175*	*-0.302*	*-0.329*	

Significant correlation at $p < 0.05$ (*) and $p < 0.01$ (**). Ws: Specific energy input; WL: Weight loss; MC: Media conductivity; CV: Cell viability; L*: Lightness; ΔE: Total colour difference; VCF: Vascular cylinder hardness; CF: Cortical hardness; PC: Phenolic content.

Chapter 2. Pulsed electric fields affect endogenous enzyme activities, respiration and biosynthesis of phenolic compounds in carrots

Gloria López-Gámez, Pedro Elez-Martínez, Olga Martín-Belloso, Robert Soliva-Fortuny

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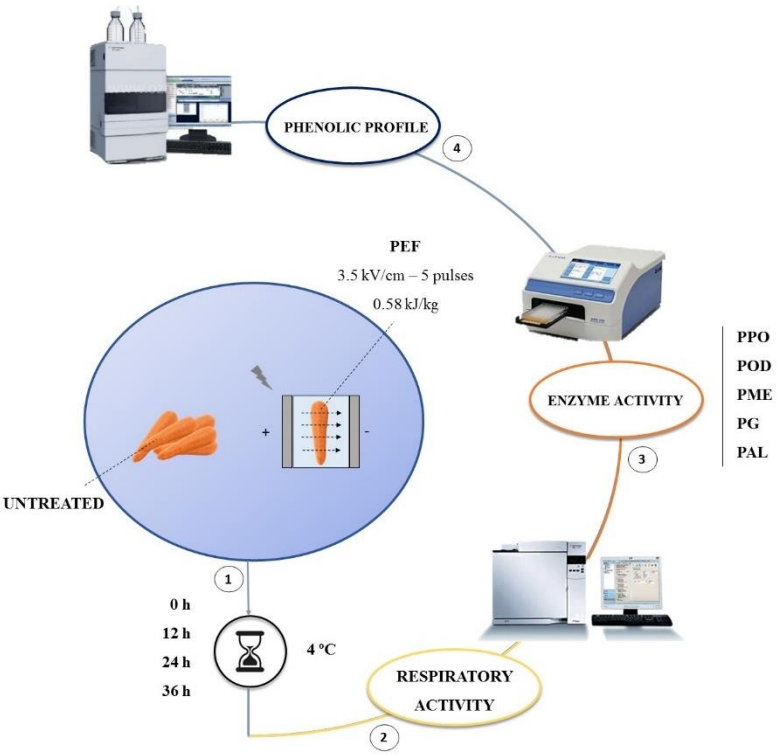
Abstract

Pulsed electric fields (PEF) can be applied to induce accumulation of bioactive compounds in plant tissues to obtain commodities with health-promoting properties. However, causes of this accumulation are not fully understood as it may result from either an improvement in extraction or an activation of stress-related biosynthetic pathways. The objective of this study was to investigate the effects of PEF on the physiological response and elucidating the causes underpinning changes in carrot phenolic contents. Respiration rate, oxidative and pectinolytic enzyme activities, synthesis, and content of phenolic compounds were evaluated in PEF-treated (580 J kg^{-1}) carrots after treatment and through storage (12, 24 and 36 h) at 4 ºC. The highest production of CO_2 and volatile organic compounds was observed 12 h after PEF treatment whereas the largest increases in total phenolic content (80.2 %), *p*-hydroxybenzoic (94.7 %), chlorogenic acid (74.9 %) and ferulic acid (52.2 %) occurred 24 h after treatment. Enhanced in phenylalanine ammonia lyase activity indicated that the increase in phenolic compounds may be mainly due to the triggering of biosynthesis pathways instead of structural modifications of the food matrix. Electropermeabilization also induced considerable changes in pectinolytic enzyme activities (increases in pectinmethylesterase and decreases in polygalacturonase) whereas no clear trends were observed for oxidative enzyme activities (peroxidase and polyphenol oxidase) during storage. These results suggest that volatile compounds generation, changes in respiration rate and the biosynthesis of phenolic compounds are induced by PEF application, as a plant defence response to stress.

Keywords: Pulsed electric fields; phenolic compounds; respiration; phenylalanine ammonia lyase; polyphenol oxidase; pectinmethylesterase

GRAPHICAL ABSTRACT



PEF: Pulsed Electric Fields
PPO: Polyphenol oxidase
POD: Peroxidase
PME: Pectinmethyl esterase
PG: Polygalacturonase
PAL: Phenylalanine ammonia lyase

1. Introduction

Consumers are increasingly demanding minimally processed products with health-promoting properties. Therefore, providing new strategies to enhance the antioxidant content of plant-based products is essential to meet consumer demands. Plant products have been proposed as biofactories to accumulate phenolic compounds after being submitted to abiotic stresses such as wounding, hyperoxia, and water stress (Jacobo-Velázquez et al., 2011; Becerra-Moreno et al., 2015). Damaged cells generate signalling molecules as an immediate response to stress (Jacobo-Velázquez et al., 2011), which elicit the production of secondary signalling molecules [e.g. ethylene, reactive oxygen species (ROS)] by undamaged cells (Jacobo-Velázquez et al., 2015). These transduction networks trigger the biosynthesis of secondary metabolites, such as polyphenols, for a few hours or days in response to an induced stress (Zhao et al., 2005). These changes are necessary for the acclimation to new environment and recovery of cells from damage (Jacobo-Velázquez and Cisneros-Zevallos, 2012).

In recent years, the application of non-thermal processing technologies such as pulsed electric fields (PEF) has been proposed as a promising tool to induce stress in horticultural crops, thus triggering the accumulation of phenolic compounds during postharvest life (Jacobo-Velázquez et al., 2017; López-Gámez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, 2020a). This treatment could be highly useful to obtain derived products from a commodity with enhanced antioxidant content. PEF involves the application of electrical pulses for a short time (microseconds to milliseconds) to foods located between two electrodes, which induces electropermeabilization. The application of low or moderate intensity treatments (0.1 – 5 kV cm^{-1}; 0.5 – 20 kJ kg^{-1}) has been reported to cause damage in cell membranes (reversible or irreversible formation of pores), which may activate the secondary metabolism of plant tissues as a way to overcome unfavourable conditions, that would end with the accumulation of phenolic compounds in fruit and vegetables (Soliva-Fortuny et al., 2009; Jacobo-Velázquez et al., 2017). However, PEF also affects cell permeability and may enable a greater recovery or extraction of bioactive compounds from plant material (Soliva-Fortuny et al., 2009). Wiktor et al., (2015) reported that applying PEF (1.85 kV cm^{-1}) to carrot slices increased carotenoid extraction up to 11 %. Aguiló-Aguayo et al., (2014) demonstrated that PEF application (1 – 4 kV cm^{-1}) enhanced the extractability of sugars from carrot. In addition, changes in cell permeability caused by PEF has been used to improve osmotic dehydration (Amami, Fersi, Vorobiev, & Kechaou, 2007), drying efficiency (Wiktor, Dadan, Nowacka, Rybak, & Witrowa-Rajchert, 2019) or reduce cutting force (Leong, Richter, Knorr, & Oey, 2014) of carrots.

Therefore, increased phenolic content in tissues may result either from the activation of their biosynthesis pathway as a stress response, or from an improvement in their extractability, or even from a combination of both factors. Information about the effects of PEF at a physiological level is limited, which is essential to develop strategies to obtain fruit and vegetables with a higher phenolic content. The modification of respiration rate and production of volatile organic compounds in plants (Rolle & Chism, 1987), the alteration of structural or oxidizing enzymes (Thipyapong et al., 1995; Kwak et al., 1996) and changes in the phenylpropanoid metabolism (Dixon & Paiva, 1995) are some effects related to the stress response.

The induced stress response after PEF in plant tissues has not been completely discerned. An increase in the phenylalanine ammonia lyase (PAL) activity, key enzyme in the phenylpropanoid metabolism, and in the phenolic content has been reported after applying some non-thermal technologies such as ultrasound (Cuéllar-Villarreal et al., 2016b; Yu, Engeseth, & Feng, 2016). However, information regarding the effects of PEF is yet scarce. Balaša (2014) reported increases of 54 % in PAL activity and 20 % in total phenolic content 9 h after applying PEF (4.1 J kg^{-1}) to apple cell cultures. In addition, Vallverdú-Queralt et al., (2013) reported that PEF-treated tomatoes (30 pulses of 1.2 kV cm^{-1}) exhibited an increase in total polyphenols and in some individual compound contents (39 % – 170 %) 24 h after treatment. Similar results were obtained by López-Gámez et al., (2020), who reported an increase in total phenolic content (40 %) at 24 h after applying PEF (5 pulses of 3.5 kV cm^{-1}) to whole carrots. On the other hand, González-Casado et al., (2018) reported changes in respiration rate and production of some volatile compounds (e.g. ethylene) in PEF-treated tomatoes.

In general, studies report the increase in bioactive compounds after PEF. However, these results are not supported with enzymatic or physiological data collected immediately and during post-treatment time, which would confirm stress reactions instead of better extractability. The main objective of this study was to gain an in-depth understanding of the effect of PEF on the stress response and provide evidence of the main cause of the increase in phenolic content.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol was acquired from Fisher Scientific Scharlau Chemie (Loughborough, UK), sodium chloride was purchased from POCH S.A. (Sowińskiego, Poland), EDTA, formic, acetic and ortho-phosphoric acids were acquired from Scharlau S.L. (Barcelona, Spain), ethyl acetate, diethyl ether, sodium hydroxide and 2-cyanoacetamide were purchased from

Acros Organics (New Jersey, USA), citrus pectin, bromothymol blue, bovine serum albumin, catechol, polygalacturonic acid, D-galacturonic acid, 2-mercaptoethanol and L-phenylalanine were acquired from Sigma Aldrich (St. Louis, MO, USA), *p*-phenylendiamine was purchased from Merck (Hohenbrunn, Germany), polyvinylpyrrolidone was acquired from Fischer Scientific (Geel, Belgium), hydrochloric acid was purchased from Panreac Química S.A. (Barcelona, Spain), hydrogen peroxide was acquired from Chemlab (Zedelgem, Belgium) and Coomassie brilliant blue G250 was purchased from Fluka Chemika (Buchs, Switzerland).

2.2. Carrot samples

Carrots (*Daucus carota* cv. Nantes) (caliber 25/35 mm and length 0.17 ± 0.02 m) were acquired in a local supermarket (Lleida, Spain). They were stored at 4 ºC until processing within a week. Before PEF treatments, carrots were washed with tap water and the excess was removed with a paper cloth.

2.3. Pulsed electric fields (PEF) treatments

Whole carrots were processed in a PEF batch system (Physics International, San Leandro, CA, USA), equipped with a TG-70 gas control unit and a PT55 pulse generator (Pacific Atlantic Electronics Inc., El Cerrito, CA, USA). The system supplied pulses of 4 μs with exponentially decaying waveform from a capacitor of 0.1 μF at a fixed frequency of 0.1 Hz. The treatment chamber, a parallelepiped methacrylate container, equipped with two stainless steel electrodes (0.2 × 0.05 m) with a gap between them of 0.05 m was filled with an aqueous solution (0.01 S m^{-1}) and carrots (~ 0.1 kg) were placed in parallel to electrodes in a ratio 1:3 (w: v) carrots: aqueous solution. Then, 5 pulses of 3.5 kV cm^{-1} (580 ± 80 J kg^{-1}) were applied in accordance to López-Gámez et al., (2020), who reported this treatment as optimum for increasing total phenolic content and Bazhal et al., (2003), who established that the optimum electric field strength for electropermeabilized carrot tissues was in the range of 2 and 4 kV cm^{-1}. Three replicates of six carrots per treatment (carrots were treated individually in chamber) were used. The specific energy input (Ws), expressed in J kg^{-1}, was calculated according to Eq. (1):

$$Ws = \frac{V^2\ C\ n}{2\ m} \quad (1)$$

where V [V], C [F], n, and m [kg] are the voltage, capacitance of the energy storage capacitor, number of pulses and mass of the sample in the treatment chamber, respectively. The temperature of the aqueous solution after PEF application did not exceed 20.0 °C.

Whole untreated and PEF-treated carrots immediately after treated and after 12, 24 and 36 h at 4 °C, were cut into slices and crushed before enzyme and phenolic extractions to overcome the possible heterogeneity within tissues.

2.4. Respiratory activity

A static system was used to determine the respiratory activity and volatile organic compound production of the carrots. Changes in headspace composition were measured over 36 h using a gas analyser (490 Micro GC, Agilent Technologies, Santa Clara, USA). Just after PEF treatment, carrots (ca. 0.12 ± 0.02 kg) were individually placed in hermetic containers of 2.25 L and gas sample (1.7 mL) was withdrawn from the headspace through an adhesive rubber septum with a syringe. Respiration as carbon dioxide production was expressed as mg kg^{-1} s^{-1} according to Tappi et al. (2014) and the production of ethylene, ethanol and acetaldehyde was expressed as ng kg^{-1} s^{-1} or pg kg^{-1} s^{-1}.

2.5. Pectinmethylesterase (PME) activity

The PME extraction and activity determination were performed as described by Hagerman and Austin (1986), adapted to 96-well microplates. All the solutions must be previously adjusted to pH 7.5. The reaction mixture consisted of 30 µL of PME extract, 70 µL of distilled water, 180 µL of citrus pectin 0.5 % (w/v) solution and 30 µL of bromothymol blue 0.01 % (w/v). PME activity was determined by monitoring the colour change during 3 min at 620 nm in a microplate spectrophotometer (Thermo Scientific Multiskan GO, Vantaa, Finland). Galacturonic acid was used to make a standard curve and calculate PME activity, which was expressed as nanomoles of galacturonic acid produced per minute and per milligram of protein (U mg^{-1} protein).

Protein content in crude enzyme extracts was measured according to Bradford (1976) using bovine serum albumin as a standard.

2.6. Polygalacturonase (PG) activity

PG extraction was based on the procedure described by Pressey (1988) and PG activity determination was carried out as described by Houben et al. (2014), by measuring the formation of reducing groups from a polygalacturonic acid substrate at 35 °C. The absorbance was determined spectrophotometrically in a quartz microplate (Thermo Scientific Multiskan GO, Vantaa, Finland) at 276 nm and 22 °C. A standard curve of monogalacturonic acid allowed to calculate the amount of formed reducing groups (Verlent, Van Loey, Smout, Duvetter, & Hendrickx, 2004). PG activity was expressed as nanomoles of reducing groups from polygalacturonic acid per minute and per milligram of protein (U mg^{-1} protein).

2.7. Polyphenol oxidase (PPO) activity

PPO extraction was based on the procedure described by Alegria et al. (2016) with slight modifications. In this case, PPO was extracted from carrot tissues (4 g) adding 15 mL of cold phosphate buffer (0.1 M; pH 6.5) and 0.4 g of polyvinylpyrrolidone. Then, samples were vortexed for 1 min and centrifuged at 20000 g for 15 min at 4 ºC. The resulting supernatant was filtered across Whatman No. 1 filter. During the whole procedure, samples were maintained in an ice-bath to prevent protein denaturation.

PPO activity was assayed spectrophotometrically measuring the catechol oxidation rate at 420 nm for 2 min (Thermo Scientific Multiskan GO, Vantaa, Finland). The reaction mixture was adapted to 96-well microplate, which contained 10 μL of enzymatic extract and 290 μL of catechol (0.05 M) prepared in extraction buffer just before the analysis. Results were expressed as nanomoles of enzymatic extract that causes an increase of one unit of absorbance at 420 nm on a protein basis (U mg^{-1} protein).

2.8. Peroxidase (POD) activity

POD extraction was carried out following the same procedure as previously described for PPO extraction. Determination was performed placing 10 μL of enzyme extract into 96-well microplate. Then, 260 μL of extraction buffer, 20 μL of *p*-phenylenediamine 1 % (w/v) and 10 μL of H_2O_2 1.5 % (v/v) were added. Spectrophotometric readings at 485 nm were registered every 10 s during 10 min of incubation in a spectrophotometer (Thermo Scientific Multiskan GO, Vantaa, Finland). POD activity was expressed as nanomoles of enzymatic extract causing an increase of one unit of absorbance at 485 nm on a protein basis (U mg^{-1} protein).

2.9. Phenylalanine ammonia-lyase (PAL) activity

PAL extraction was performed as described by Alegria et al. (2016) with slight modifications. After homogenizing carrot tissue (4 g) with polyvinylpolypyrrolidone and sodium borate buffer 100 mM (pH 8.7), samples were vortexed for 1 min and centrifuged at 20000 g for 15 min at 4 ºC. Then, supernatants were filtered across Whatman No. 1 filter. Extractions were also made using cold buffers and an ice-bath to prevent protein denaturation.

PAL activity determination was adapted to 96-well quartz microplate. L-phenylalanine substrate solution (100 µL), extraction buffer (100 µL) and PAL extract (50 µL) were mixed. Then, spectrophotometric readings at 290 nm were registered every 10 min during 1 h of incubation at 37 ºC (Thermo Scientific Multiskan GO, Vantaa, Finland) in a quartz microplate. PAL activity was expressed as nanomoles of *trans*-cinnamic acid per minute on a protein content basis (U mg^{-1} protein).

2.10. Extraction of phenolic compounds

Free and bound phenolic compounds were extracted following the methodology proposed by Mattila and Kumpulainen (2002) with slight modifications, in which acid and alkaline hydrolysis were performed. The modification consisted in adding a centrifugation step (8784 g for 5 min) before filtrating samples through a polytetrafluoroethylene (PTFE) membrane filter (0.45 µm, ø 13mm, Labbox Labware S.L., Barcelona) for the High-Performance Liquid Chromatography (HPLC) analysis. After quantification, the results from alkali and acid hydrolysates were calculated to represent total phenolic acids expressed on a dry weight basis (mg kg^{-1}).

2.11. Identification and quantification of phenolic compounds by High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD)

Phenolic compounds were separated following the procedure described by Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2011) using a reverse-phase C_{18} Spherisorb ODS2 (5 µm) stainless steel column (4.6 mm x 250 mm) at room temperature. The HPLC system comprised of a 600 Controller, a 486 Absorbance Detector programmed to scan from 200 to 350 nm, a thermostatic column compartment, and a 717 Plus Auto Sampler with cooling system (Waters, Milford, MA).

Flow rate of the mobile phase was 1 mL min^{-1} and the injection volume were 20 μL. Individual phenols were identified based on their UV-vis spectral data and retention times which were compared to their reference standards. Quantification of phenolic compounds was carried out by integration of the peak areas. Data were compared to calibration curves of each phenolic compound and results were expressed on a dry weight basis (mg kg^{-1}).

2.12. Statistical analysis

Statistical analyses were carried out using the SigmaPlot software (version 11.0, Systat Software Inc, Chicago, IL, USA). Three different replicates were assayed for every treatment condition. Each replicate consisted of 6 carrots. Enzymes and phenolic extractions were carried out twice from each replicate. Results are reported as the mean ± standard deviation. Normality and homoscedasticity criteria were evaluated by Shapiro-Wilk and Levene's tests, respectively. Repeated measures ANOVA and Tukey *post hoc* test were applied to establish differences between treatments and throughout time. The statistical significance level was $p < 0.05$.

3. Results and discussion

3.1. Respiration and production of volatile organic compounds

The effects of PEF on the respiratory activity of whole carrots are shown in **Table 2.1**. The application of PEF had a critical impact on the subsequent modification of the respiration rate and production of volatile compounds. The highest respiration rate was observed just after treatment, thereafter, it gradually decreased during storage in both untreated and PEF-treated carrots. However, PEF-treated carrots produced between 123 – 164 % more CO_2 than the untreated carrots from 12 h to 36 h of storage.

As carrot is a living tissue even after harvest, changes in respiration rate provide information about their overall metabolic activity. Some types of abiotic stresses may slow down or promote respiration and the accumulation of bioactive compounds in plant tissues (Cisneros-Zevallos, 2003; Saltveit, Choi, & Tomás-Barberén, 2005). Some authors have previously reported a decrease in O_2 consumption and CO_2 production in PEF-treated apple cylinders subjected to 0.25 and 0.4 kV cm^{-1} (Dellarosa et al., 2016a), attributing this effect to the loss of cell viability caused by electropermeabilization. However, the increase in CO_2 production by PEF-treated carrots observed in our study suggests that electroporation did not lead in a significant way to the loss of cell viability but to the triggering of a stress response in the injured cells.

In addition to changes in respiration rate, generation or overproduction of volatiles is strongly related to stress conditions (Sheshadri, Nishanth, & Simon, 2016). PEF application did not significantly affect volatile compounds production in carrots immediately after treatments. However, after 12 h of storage, PEF-treated carrots generated higher amounts of ethylene (50 ng kg^{-1} s^{-1}), ethanol (68 ng kg^{-1} s^{-1}) and acetaldehyde (7 pg kg^{-1} s^{-1}), whereas these metabolites were not found in untreated carrots. Increased volatile production after applying some non-thermal technologies has been reported, e.g. in ultrasound-treated carrots (Cuéllar-Villarreal et al., 2016). Thus, ethylene and acetaldehyde were found by González-Casado et al., (2018) 24 h after applying PEF to tomatoes and by Dellarosa et al., (2016) in fresh-cut apples treated with intensities ranging from 0.1 to 0.4 kV cm^{-1}.

Ethylene synthesis is enhanced by stress (Chervin, Triantaphylides, Libert, Siadous, & Boisseau, 1992) and plays an important role in respiration and the activation of secondary metabolism (Jacobo-Velázquez et al., 2015). Electropermeabilization caused by PEF may act as stress inductor, disrupting cells and inducing the release of ATP, a signalling molecule that would be diffused and detected by undamaged cells. Then, cytosolic Ca^{2+} would increase its concentration and ROS and ethylene would be produced by respiration (Jacobo-Velázquez et al., 2011, 2017). The presence of acetaldehyde and ethanol may indicate the triggering of anaerobic metabolism, which was possibly related to structural damage and intracellular content leakage caused by PEF. These signals would trigger the biosynthesis of secondary metabolites (e.g. phenolic compounds) to adapt its metabolism to environmental changes.

Table 2.1. Effects of PEF treatment on the respiratory activity and volatile organic compounds production of carrots.

Post-treatment time (h)	PEF-treatment energy (J kg^{-1})	Carbon dioxide production (µg kg^{-1} s^{-1})		Ethylene production (ng kg^{-1} s^{-1})		Acetaldehyde production (pg kg^{-1} s^{-1})		Ethanol production (ng kg^{-1} s^{-1})	
0	Untreated	10 ± 4		nd [1]		nd [1]		nd [1]	
	580	8 ± 0.9		nd [1]		nd [1]		nd [1]	
12	Untreated	1.3 ± 0.3		nd [1]		nd [1]		nd [1]	
	580	3.3 ± 0.7	*	50 ± 28	*	7 ± 6	*	68 ± 46	*
24	Untreated	0.7 ± 0.1		nd [1]		nd [1]		nd [1]	
	580	1.6 ± 0.4	*	34 ± 7	*	4 ± 2	*	43 ± 13	*
36	Untreated	0.4 ± 0.2		nd [1]		nd [1]		nd [1]	
	580	1.0 ± 0.3	*	24 ± 7	*	2 ± 1	*	46 ± 5	*

Asterisks (*) in the same column indicate statistical difference ($p < 0.05$) between the untreated and PEF-treated carrots at the same post-treatment time. Values are expressed as mean ± standard deviation (n=6). [1] nd: not detected.

3.2. POD and PPO enzyme activities

POD activity in both untreated and PEF-treated carrots changed over time without showing any clear trends (**Figure 2.1A**). POD activity of PEF-treated carrots was similar to that of untreated ones just after treatment. However, significant differences were noticed during storage. PEF application caused a 12 h delay in the peak of maximum POD activity. This effect was also observed by Yeoh and Ali (2017) in ultrasonicated fresh-cut pineapple. In this case, this delay may be due to the induction of reversible changes in enzyme conformation (Yu, Zeng, Zhang, Liao, & Shi, 2014). PPO activity of PEF-treated carrots trended upwards while they remained constant in untreated carrots (**Figure 2.1B**), but differences were not statistically significant. Higher activity of PPO in PEF-treated cell cultures of *Vitis vinifera* when increasing the electric field strength was attributed to stress induction by PEF (Balaša, 2014). In contrast, other authors have reported a decrease of 10 % in PEF-treated carrots (< 50000 J kg^{-1}) (Leong et al., 2014) or a reduction between 63 – 74 % in PEF-treated apricots (21200 – 162500 J kg^{-1}) (Huang et al., 2019). Results may seem conflicting as enzyme activities depend on diverse factors such as food source, sensitivity, molecular size, and structure (Giner et al., 2002), and their inactivation or enhancement depends also on PEF treatment conditions.

To maintain the concentration of ROS at a relatively low level, plants have developed mechanisms to scavenge these reactive substances with antioxidants such as phenolic compounds. However, certain enzymes (PPO and POD) are also helpful to achieve this purpose (Smith-Becker et al., 1998). Generally, enzymes are easily inactivated when higher electric field strength and treatment time are applied (Martín-Belloso & Elez-Martínez, 2005) due to lose of conformation, but after applying moderate electric fields (0.5 – 5 kV cm^{-1}, 1000 – 20000 J kg^{-1}) up- or downregulations may equally occur (Balaša, 2014) as a consequence of electropermeabilization. Substrates may be relocated, hence facilitating or hindering the contact with their respective enzymes, or they could be inhibited by substances with structural similarities.

To the best of our knowledge, there is scarce information about the effect of PEF on PPO and POD activity in solid food matrices. Due to the complexity of biological systems and involvement of many other enzymes, further investigation is necessary to clarify possible mechanisms of action caused by PEF treatments.

3.3. PME and PG enzyme activities

PME activity was affected by PEF application and post-treatment time (**Figure 2.1C**). PME activity increased in untreated carrots during the first 12 h of storage and then remained stable over time. However, PEF application caused an immediate increase of PME activity, being 164 % higher than that observed in untreated carrots at such time.

It is reported that PME activation strengthen vegetable tissues through cross-linking between pectin chains. This may occur by different ways that imply cell disruption e.g. mild thermal treatments. High temperature leads to cell wall disruption, thus leading to increase membrane permeability and migration of solutes from the cytoplasm to membrane, increasing potassium concentration and activating PME activity (Botero-Uribe, Fitzgerald, Gilbert, & Midgley, 2017). A similar mechanism may be proposed to explain the PME activation after PEF application, given that electroporation causes cell wall damage (Janositz and Knorr, 2010). In fact, some authors have previously reported an increase of PME activity after the application of PEF. Samaranayake and Sastry (2016) displayed that PME activity of tomato homogenates was enhanced after applying 0.008 kV cm^{-1} combined to thermal treatment (69.5 ºC), while at higher temperatures inactivation occurred, probably due to enzyme denaturation.

Besides, PG activity was also significantly affected by PEF application (**Figure 2.1D**) as PEF-treated carrots showed a decrease between 31 % – 32 % in PG activity compared with those untreated at the same storage time. High intensity pulsed electric fields technology has been widely applied to inactivate or reduce enzyme activity (e.g. PME and PG) in liquid products (Martín-Belloso, Marsellés-Fontanet, & Elez-Martínez, 2014). However, to the best of our knowledge, this is the first study in which PG activity is evaluated after PEF application to whole commodities. An increase in PG activity is associated with the solubilization of pectic substances and softening in many fruit and vegetables. However, PME and PG enzymes act differently depending on pH, ionic strength and methyl esterification grade of pectin (Micheli, 2001). PME may act randomly, demethylesterifying pectin chains, causing the release of protons and promoting PG activity, hence reducing cell wall rigidity. On the other hand, PME may act linearly allowing the interaction of free carboxyl groups with Ca^{2+}. In this situation, PG activity is reduced, and cell wall structure is maintained. This mechanism may be triggered by increases in intracellular pH, considered as signals of stress detection (Kader & Lindberg, 2010), which have been reported in some PEF-treated fruit (González-Casado et al., 2018). This suggests the second alternative as the most likely to occur.

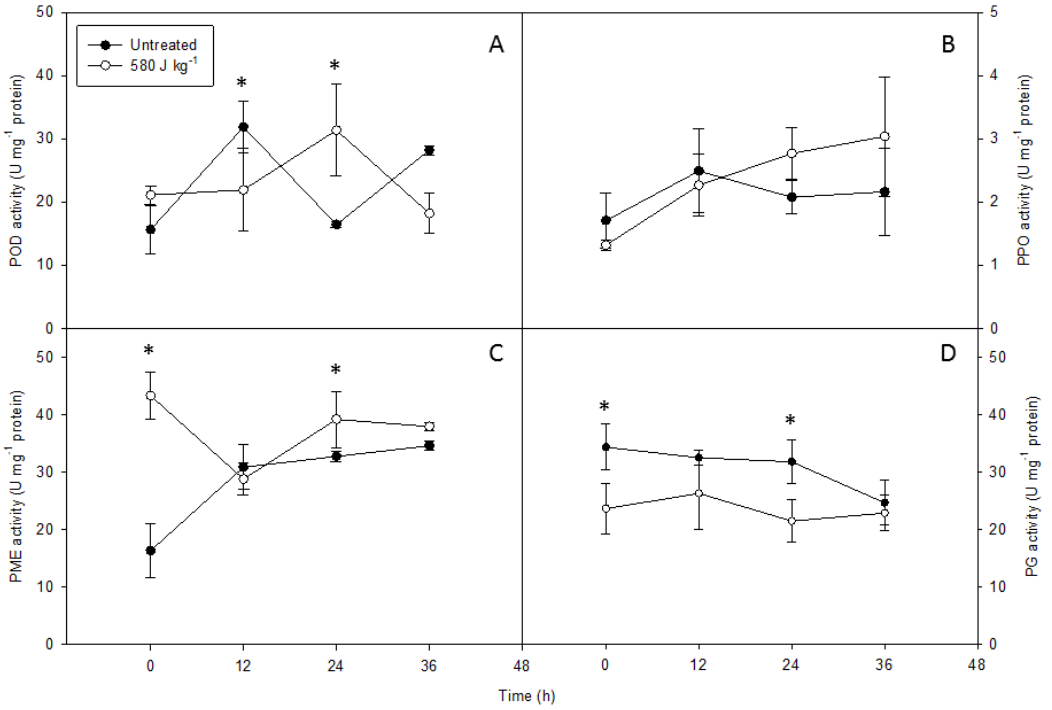


Figure 2.1. Effects of PEF and post-treatment time on the enzyme activity of (A) POD; (B) PPO; (C) PME and (D) PG in carrots stored at 4 ºC. Values represent the mean of 3 replicates with their standard error bars. Data points with an asterisk (*) indicate statistical difference ($p < 0.05$) between the untreated and PEF-treated carrots.

3.4. Phenolic compounds content and PAL activity

Phenolic content of carrots varied depending on storage time, type of compound and treatment. The total phenolic content in untreated carrots was maintained or decreased over time, whereas it peaked 24 h after PEF treatment (**Figure 2.2A**). Main phenolic compounds found in this study include hydroxybenzoic acids (*p*-hydroxybenzoic and protocatechuic acid) and hydroxycinnamic acids (chlorogenic acid, ferulic acid, and *p*-coumaric acid), which is consistent with literature (Jacobo-Velázquez et al., 2011; Becerra-Moreno et al., 2015) (**Table 2.2**). These compounds were only identified by HPLC-DAD after alkaline and acid hydrolysis, as most phenolic compounds in fruit and vegetables are bound to the cell wall polysaccharides. Esterification may be a mechanism to limit the pro-oxidant behaviour of phenylpropanoids, potentially serving as antioxidants (Grace & Logan, 2000).

Carrots just after PEF treatment had decreased ferulic (56.3 %), protocatechuic (78.1 %) and *p*-coumaric (42.3 %) acid contents compared with untreated carrots **(Table 2.2)**. These results may indicate their release through the formed pores, which is likely due to their high affinity to water (Sánchez-Rangel, Benavides, & Jacobo-Velázquez, 2014). Protocatechuic acid,

chlorogenic acid and total phenolic content **(Table 2.2** and **Figure 2.2A)** increased 12 h after PEF application, which may suggest an enhancement of the phenylpropanoid pathway through the activation of PAL. However, the maximum content was reached 24 h after treatment, when a considerable increase in total phenolic content (80.2 %) and in some individual compounds such as *p*-hydroxybenzoic (94.7 %), chlorogenic acid (74.9 %) and ferulic acid (52.2 %) were observed in comparison to those found in untreated carrots **(Table 2.2)**. Later on, 36 h after PEF treatment, *p*-hydroxybenzoic acid, chlorogenic acid, ferulic acid and total phenolic content decreased. These results are in accordance with those found by López-Gámez et al., (2020), who reported a decrease in total phenolic compounds 48 h after applying 610 J kg^{-1} to whole carrots.

Individual phenolic compounds that mainly accumulated 24 h after PEF were chlorogenic, ferulic and *p*-hydroxybenzoic acids (**Table 2.2**). Chlorogenic acid is one of the initial products formed during the transcriptional activation of the phenylpropanoid pathway by pathogen infection and abiotic stress events (Dixon & Paiva, 1995). It has been suggested that chlorogenic acid can be rapidly mobilized to form products such as lignin, antimicrobial phytoalexins, and cell wall cross-linking agents (Yao, Kening, De Luca & Brisson, 1995). Furthermore, phenolic compounds have different antioxidant potential depending on their conformation, number of hydroxyl groups and their distribution in the molecular structure (Heo et al., 2007). According to Grace and Logan (2000), chlorogenic acid has the most important role in scavenging $O_2^{-\cdot}$ and ABTS $^{\cdot+}$ radicals under adverse environmental conditions and ferulic acid also showed the highest ability of hydrogen-donating to scavenge the ABTS $^{\cdot+}$ radical (Rice-Evans et al., 1996). Therefore, it is likely that the damage induced by PEF in carrot tissue activated the biosynthesis of phenolic compounds in order to repair the tissue and scavenge ROS, as a defence mechanism similar to the one exerted by wounding (Cuéllar-Villarreal et al., 2016).

PAL activity of carrots was influenced by PEF treatments and post-treatment time. Results showed that PAL activity in untreated carrots remained stable through 36 h of storage, whereas in PEF-treated carrots it progressively increased, starting 12 h after treatment and reaching the highest activity, 153 % higher than that of untreated carrots 36 h after treatment (**Figure 2.2B**).

The enhancement in PAL activity at 24 h was in accordance with the accumulation of phenolic compounds at that time (**Figure 2.2**). However, the highest PAL activity at 36 h did not match with a further accumulation of phenolic compounds beyond 24 h. This would imply that these phenolics are synthesized at a lower rate compared with their rate of utilization. Therefore, it suggests that cells may have used phenolic substances to repair the damage caused by PEF

(Gürsul, Gueven, Grohmann, & Knorr, 2016) or have been degraded by other oxidative enzyme or released through irreversibly formed pores.

Little knowledge about the effect of PEF application on PAL activity in whole vegetables exists. Some authors have evaluated this activity in plant cell cultures, with similar results to those presented in the current study. Balaša (2014) reported that PAL activity of PEF-treated apple cell cultures (2 kV cm^{-1}; 4.1 J kg^{-1}) increased by 54 %, compared with untreated cell cultures, 9 h after treatment. In a similar way, Gürsul et al., (2016) reported that PAL activity and phenolic content of tomato cell cultures peaked 4 h after applying PEF (9 pulses of 1.2 kV cm^{-1}). They determined that PAL activity was higher with the increase in the intensity and extent of PEF application.

Changes in total and individual phenolic content after PEF and during storage may be a result of triggering the neutralization mechanism of ROS caused by abiotic stresses (Ribas-Agustí et al., 2019). Nevertheless, other authors attribute these changes to a better extractability caused by mechanical disruption of cell membranes (Fincan et al., 2004; Lebovka et al., 2005; Jaeger et al., 2012; Cuéllar-Villarreal et al., 2016; Nowacka and Wedzik, 2016). Further studies are needed to evaluate the effect of different PEF intensities on plant tissue metabolism. However, based on the ability of plants to develop and modulate the adaptive stress response (Dixon & Paiva, 1995), we could expect some tendencies. For instance, low intensities would likely cause less or slower phenolic increment in time. Conversely, larger intensities would induce irreparable damage in cells, which would prevent the development of defence response. Cell membrane rupture would lead to increase phenolic extractability rather than their biosynthesis.

Under the applied conditions in the present study, firmness was maintained for at least 24 h after treatment (López-Gámez et al., 2020). In addition, the phenolic content from untreated and just treated carrots was similar, which suggests that there is no better extraction. Besides, PAL activity was enhanced at 24 h, when the maximum phenolic content was observed. All these facts seem to point that the increase in phenolic content 12 and 24 h after PEF is mainly due to an enhancement of the plant defence response (López-Gámez et al., 2020).

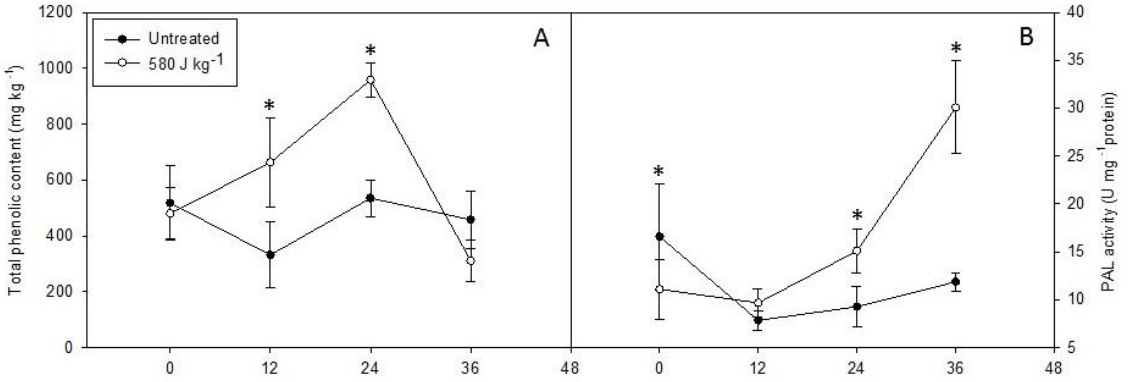


Figure 2.2. Effects of PEF and post-treatment time on (A) total phenolic content and (B) PAL activity of carrots stored at 4 ºC. Values represent the mean of 3 replicates with their standard error bars. Data points with an asterisk (*) indicate statistical difference ($p < 0.05$) between the untreated and PEF-treated carrots.

Table 2.2 Effects of pulsed electric fields (PEF) and post-treatment time on individual phenolic compounds of carrots stored at 4ºC.

		Phenolic compounds (mg kg^{-1})					
Post-treatment time (h)	**PEF treatment energy (J kg^{-1})**	**Gallic acid**	**Protocatechuic acid**	***p*-hydroxybenzoic acid**	**Chlorogenic acid**	**Ferulic acid**	***p*-coumaric acid**
0	**Untreated**	2.9 ± 0.6 a	10.8 ± 1.7 a	289 ± 44 a	229 ± 64 a	32.1 ± 2.1 a	26 ± 3 a
	580	2.3 ± 0.6 A	8.2 ± 1.7 A	247 ± 43 AC	194 ± 45 A	14 ± 3 A *	15 ± 0.0 A *
12	**Untreated**	2.5 ± 0.5 a	11.3 ± 1.5 a	249 ± 78 a	52 ± 7 b	15 ± 3 b	10 ± 6 b
	580	3.0 ± 0.5 A	18 ± 1.8 B *	340 ± 87 A	267 ± 70 A *	19 ± 3 A	17.8 ± 1.5 A
24	**Untreated**	2.4 ± 0.5 a	12.3 ± 1.8 a	298 ± 58 a	170 ± 50 a	43 ± 11 c	13 ± 9 ab
	580	2.2 ± 0.5 A	17.7 ± 1.7 B *	574 ± 48 B *	297 ± 42 A *	64.7 ± 1.1 B *	12.1 ± 6 A
36	**Untreated**	2.3 ± 0.2 a	14.9 ± 1.7 a	233 ± 38 a	170 ± 50 a	33 ± 3 c	5.9 ± 1.9 b
	580	3.5 ± 0.2 A	27 ± 7 B *	175 ± 53 C	71 ± 6 B *	35 ± 10 C	5.5 ± 1.5 A

Different letters in the same column indicate significant ($p < 0.05$) differences among different post-treatment time (Untreated: lowercase letters and PEF-treated: uppercase letters). Asterisks (*) in the same column indicate statistical difference ($p < 0.05$) between untreated and PEF-treated carrots at the same post-treatment time. Values represent the mean of 3 replicates with their standard error bars.

4. Conclusions

PEF and post-treatment time influenced phenolic compounds content, respiration rate, volatile organic compound production and enzyme activities of carrots, with remarkable changes 24 h after treatment. At such time, total phenolic content was clearly enhanced (80.2 %) and the amount of some individual compounds increased considerably [*p*-hydroxybenzoic (94.7 %), chlorogenic acid (74.9 %) and ferulic acid (52.2 %)]. Furthermore, the increase in the production of CO_2 and acetaldehyde, ethanol and ethylene, together with the enhancement of PAL activity suggest that the increase in phenolic content was most probably related to a stress-induced biosynthesis of hydroxycinnamic and hydroxybenzoic acids, instead of better extraction of these compounds. On the other hand, results regarding antioxidant (PPO and POD) and pectinolytic enzymes (PME and PG) throughout storage, may indicate that carrot metabolism had been triggered to adapt and recover from stress caused by PEF. Evidence is provided, for the first time, that the enhancement in phenolic content after PEF is mainly due to stress induction rather than to the increase in extractability caused by electroporation. Controlled stress offers a valuable tool to enhance the health benefits of fresh commodities. Additionally, post-treatment time need to be considered when the aim is obtaining plant products with enhanced bioactive content. Hence, according to our results, PEF application and 24 h of storage is proposed to obtain carrots, from which derived products with enhanced content in phenolic compounds can be obtained.

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Chapter 3. Pulsed electric fields to improve individual carotenoid contents in carrots

Gloria López-Gámez, Pedro Elez-Martínez, Olga Martín-Belloso, Robert Soliva-Fortuny

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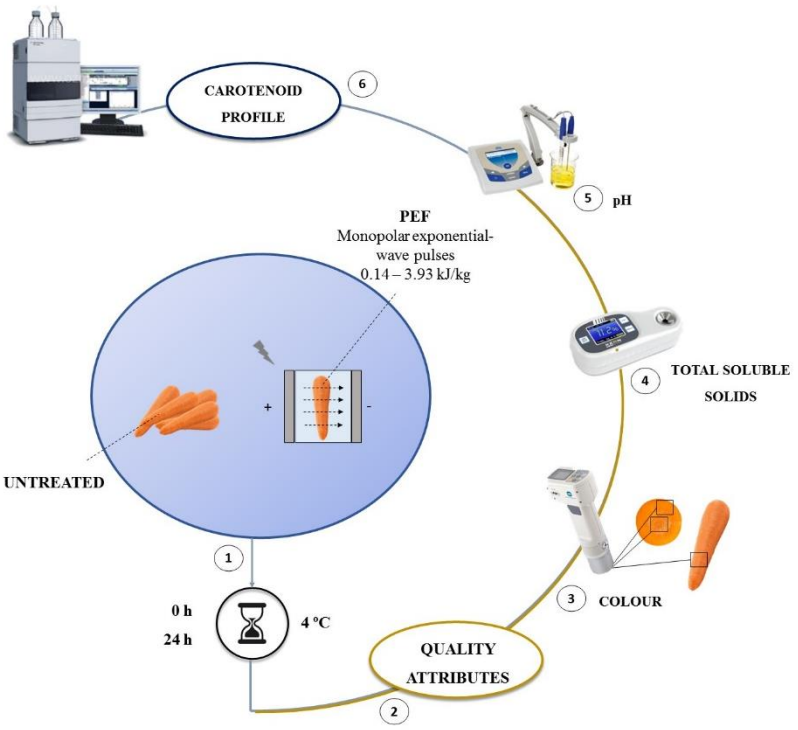
Abstract

Under certain conditions, pulsed electric fields (PEF) can promote the accumulation of phytochemicals through a stress-mediated response in plant tissues. Likewise, electropermeabilization of cell membranes can also improve the extraction of intracellular compounds. The objective of this study was to evaluate the main causes driving the increase in carotenoids content in PEF-treated carrots and to study the impact of those treatments on their relevant quality attributes. Carrots were treated with differing electric field strength (0.8, 2 and 3.5 kV cm^{-1}) and number of pulses (5, 12 and 30). Individual carotenoids content, colour, pH and total soluble solids (TSS) were determined after 0 h and 24 h after PEF treatment. Total and individual carotenoid contents (phytoene and β-carotene) increased just after applying $E \geq 2$ kV cm^{-1}, whereas lutein concentration decreased, and that of α-carotene remained similar to that found in untreated carrots. After applying 2 and 3.5 kV cm^{-1}, TSS and pH remained unaltered, but cortical browning index increased, which was correlated to carotenoid content. Increase in total carotenoid content is likely related to better extractability, but differences in the content of individual compounds suggest that PEF may also act by modulating their biosynthesis or causing their degradation.

Keywords: Carrot; carotenoids; pulsed electric fields; quality attributes

GRAPHICAL ABSTRACT



PEF: Pulsed Electric Fields

1. Introduction

Carrot is one of the most consumed root vegetables worldwide, containing ample carotenoids (e.g. α-carotene, β-carotene, lutein) (Arscott and Tanumihardjo, 2010). Provitamin A such as α-carotene and β-carotene play an important role in human health by acting as natural antioxidant, protecting tissues from free radicals and against some types of cancer or cardiovascular diseases (Maiani et al., 2009; Schweiggert & Carle, 2017). Besides, lutein intake is essential for the visual system and prevent macular degeneration (Schweiggert & Carle, 2017). Thus, their consumption is highly beneficial for health. However, they are hardly detached from carrot tissues, as cell wall and chromoplast membranes act as barriers that limit their release during digestion (Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012). Thermal treatments (e.g. blanching, cooking, pasteurization) may be beneficial to facilitate their liberation from the food matrix (Cilla et al., 2018; Palermo et al., 2014). However, heat also promotes carotenoids degradation and changes in quality attributes such as colour and flavour, especially in low water activity food systems (Maiani et al., 2009), which critically determines consumers' acceptance. Therefore, the search for processing technologies that help to preserve carrots fresh-like appearance while enhancing its bioactive content and accessibility is a current challenge.

Pulsed electric fields (PEF) is a non-thermal processing technology that involves the delivery of electric energy in the form of short pulses to food placed between two electrodes. Its application causes a transmembrane potential difference in biological tissues, which entails the increase in cell permeability through an electropermeabilization mechanism. High intensity treatments (15 – 40 kV cm^{-1}) have been widely applied to preserve liquid products as alternative to thermal processing. Besides, PEF has been applied on solid food products with the purpose of extracting intracellular metabolites (e.g. pigments or antioxidants) (Roohinejad et al., 2014; Wiktor et al., 2015), accelerating dehydration of either plant- or animal-based food commodities (Tylewicz et al., 2017), enhancing juice yield (Jaeger et al., 2012) or as a pre-treatment to soften tissues and facilitate further processing (e.g. cutting or peeling) (Leong and Oey, 2014). Despite being a non-thermal technology, structural changes caused by electropermeabilization are likely to impact carrot quality attributes. Therefore, treatments must be optimized in order to minimize these changes and avoid the degradation of bioactive compounds.

Apart from improving the extraction of bioactive compounds, PEF has been recently proposed as a tool to induce stress in plant products or cell cultures, thus stimulating the biosynthesis of bioactive compounds (Jacobo-Velázquez et al., 2017). Some studies have shown that PEF may cause the accumulation of flavonoids and flavan-3-oles in apples (Ribas-Agustí et al., 2019; Soliva-Fortuny et al., 2017), phenolic compounds in grapes (Balaša, 2014) and carrots

(López-Gámez et al., 2020) or carotenoids in tomato (González-Casado et al., 2018a; Vallverdú-Queralt et al., 2013). Although the mechanism is not yet fully understood, some hypotheses suggest that disruption of cell membranes, either reversible or irreversible, may elicit a defence response as other types of abiotic stressors (e.g. wounding) (Jacobo-Velázquez et al., 2017). This response would trigger an immediate burst of reactive oxygen species (ROS) and Ca^{2+}, among other stress-specific metabolites (Baxter et al., 2014; Carmody et al., 2016), which act as signals to activate defence mechanisms. Thereupon, signals would be transmitted from cell to cell and promote the accumulation of antioxidant compounds with the purpose of scavenging ROS (Fanciullino et al., 2014). This ultimately would enable obtaining commodities with higher nutritional value. Hence, PEF may be implemented with a dual purpose: 1) to obtain derived products from a commodity with enhanced antioxidant content; 2) to improve the extractability of bioactive compounds, thus enhancing their bioaccessibility.

The main aim of this study was to investigate the feasibility of PEF as a pre-treatment to improve carotenoids content and their extraction and to elucidate the main causes underpinning these changes. Additionally, colour, pH and TSS were evaluated to assess the feasibility of obtaining fresh-like derived products from PEF-treated carrots.

2. Material and methods

2.1. Chemicals and reagents

Phytoene and β-carotene standards were acquired from Carote-Nature (purity ≥ 95 %) (Ostermundigen, Switzerland), α-carotene was purchased from Supelco-Merck (purity ≥ 98 %) (Darmstadt, Germany) and lutein from Acros Organics (purity 90%) (New Jersey, USA).

2.2. Carrot samples

Carrots (*Daucus carota* cv. Nantes) were purchased in a local supermarket (Lleida, Spain). They were selected based on their uniform size and shape (caliber 25/35 mm and length 17 ± 2 cm), avoiding those with visual defects. Carrots were stored in darkness at 4 ºC, within a week until processing. Prior to treatments, carrots were washed with tap water to remove any soil residues and debris, and the excess of water was removed with a paper cloth.

2.3. Pulsed electric field (PEF) treatments

PEF treatments (**Table 3.1**) were conducted in a batch PEF system (Physics International, San Leandro, CA, USA) equipped with a TG-70 gas control unit and a PT55 pulse generator (Pacific Atlantic Electronics Inc., El Cerrito, CA, USA). The equipment delivers exponential-wave pulses of 4 μs from a capacitor of 0.1 μF at a fixed frequency of 0.1 Hz. Since 0, 5, 12 and 30 pulses were applied, treatment times were 0, 20, 48 and 120 μs, respectively. Whole carrots (0.11 ± 0.01 kg) were placed individually in the treatment chamber, which consists of a parallelepiped methacrylate container with two parallel stainless-steel electrodes (20 × 5 cm) separated by a gap of 5 cm. The chamber was filled with an aqueous solution containing NaCl (conductivity of 10 μS cm^{-1}), in which the ratio carrot : aqueous solution was 1:3 (w:v). The specific energy input (Ws), expressed in kJ kg^{-1}, was calculated according to Eq. (1) (Wiktor et al., 2015):

$$Ws = \frac{V^2 Cn}{2m} \quad (1)$$

where V, C, n, and m are the voltage, capacitance of the energy storage capacitor, number of pulses and mass of the sample in the treatment chamber, respectively. The temperature of the treatment water was measured before and immediately after PEF application and it did not significantly increase (18.0 ± 1.0 ºC).

Table 3.1. PEF processing treatment conditions.

Electric field strength (kV cm^{-1})	Number of pulses	Specific energy input (kJ kg^{-1})
0	0	Untreated
0.8	5	0.14
	12	0.38
	30	0.87
2	5	0.22
	12	0.50
	30	1.19
3.5	5	0.61
	12	1.92
	30	3.93

Untreated and PEF-treated carrots were characterized in terms of carotenoid contents and quality attributes just after processing (0 h) or after 24 h at 4 °C in darkness. These conditions were based on previous studies which determined that phenolic content peaked at such time (López-Gámez et al., 2020). For the analysis of carotenoids, carrots were frozen at -40 ºC at their corresponding post-treatment times (0 h or 24 h). Before freezing carrots, their top ends (2.5 ± 0.5 cm) were removed, and the rest was cut in slices (1 cm of thickness). Just before carotenoids extraction procedures they were crushed (Moulinex, 700 W) to overcome the possible heterogeneity within differently located tissues in PEF treatment chamber.

2.4. Quality attributes

Colour. The colour of peel, cortical and vascular tissues was measured by using a colorimeter (CR-400, Konica Minolta, Osaka, Japan). A white standard plate (Y=94.00, x=0.3158, y=0.3322) was used for calibration. Three measurements of each specific tissue were taken, and each measurement represents the average of three readings. The apparatus had an aperture size of 10 mm and it was set up for a D65 illuminant, 10° observer angle. CIEL*a*b* parameters were used to calculate chroma (C*) [Eq. (2)], hue angle (h) [Eq. (3)] and browning index (BI) [Eq. (4)] according to Huang et al., (2019).

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (2)$$

$$h = \arctan \frac{b^*}{a^*} \quad (3)$$

$$BI = \frac{100\,(X - 0.31)}{0.17} \quad (4)$$

where $X = \frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*}$

pH. Previous to pH measurements, whole carrots were cut in slices (1 cm of thickness) and ground in a blender to obtain a homogeneous sample, which was used for determining the pH values. A pH-meter (Crison 2001, Crison Instruments S.A., Alella, Barcelona, Spain) was used.

Total soluble solids (TSS). The same homogeneous sample previously described for measuring pH was used for measuring total soluble solids. TSS was determined by refractometry (RX-1000, Atago, Tokyo, Japan) at 25 ºC and results were expressed as % of total soluble solids.

2.5. Carotenoids extraction

Total carotenoids were extracted according to the method described by Sadler, Davis, & Dezman (1990), 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 $g \cdot L^{-1}$ BHT. Then, 15 mL of NaCl [10 % (w/v)] solution was added and the samples were stirred for 10 additional minutes. Samples were left to stand for ≥ 3 minutes, and the upper organic phase was analysed by high-performance liquid chromatography with Diode Array Detection (HPLC-DAD). All extractions were performed in duplicate, and samples were protected from light throughout extraction and analysis to avoid carotenoid degradation and isomerization.

2.6. Identification and quantification of individual carotenoids by HPLC-DAD

Carotenoids were quantified by HPLC-DAD, following a procedure validated by Cortés, Esteve, Frígola, & Torregrosa (2004). An aliquot of 20 µL of the extracted samples were injected into the 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 C_{18} 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. The flow rate was fixed at 1 mL min^{-1} and the total run time was 60 min. The column was set at 30 ºC, while sample amber vials on the auto sampler were preserved at 4 ºC. Carotenoids were identified by UV–vis spectral data and their retention times (Cortés et al., 2004; Mouly et al., 1999) and quantified by integration of the peak areas. Data were compared to calibration curves and results were expressed on a fresh weigh basis as mg of carotenoid compound in 100 g of carrot.

2.7. Statistical analysis

All experiments were conducted in triplicate, and results were reported as the mean ± standard deviation (SD). Statistical analyses were carried out using IBM SPSS Statistics 21 software (SPSS Inc., Chicago, IL) and SigmaPlot 11.0 (Systat Software Inc, Chicago, IL, USA). Results were subjected to an analysis of variance (ANOVA) followed by Tukey *post hoc* test to establish statistical differences among mean values. In order to establish differences between

carotenoid content at 0 h and 24 h, a two-way ANOVA was performed. Dependent variable was carotenoid content, whereas time and PEF treatments were those independent. The relationship between variables was determined using the Pearson (*r*) coefficient and calculation the partial square eta value (η_p^2) was reported to indicate the effect size of each processing parameter. Significance of differences were defined at $p < 0.05$.

3. Results and discussion

3.1. Carotenoid profile of carrots

The effect of PEF on total and individual carotenoid content is displayed in **Figure 3.1** and **Figure 3.2**, respectively. Total carotenoid contents of carrots were significantly affected by PEF treatment as a function of processing parameters. Statistical analysis indicated that electric field strength was the main parameter affecting total carotenoid concentrations ($\eta_p^2 = 0.727$; $p < 0.001$). Total content increased just after applying $E \geq 2$ kV cm^{-1} (**Figure 3.1**). Hence, the highest carotenoid content (83.8 %) compared to untreated carrots, was obtained just after applying 5 pulses of 3.5 kV cm^{-1} (0.61 kJ kg^{-1}). After 24 h of treatments, carotenoid content increased by 0.72- and 1.13-fold in carrots treated with 12 pulses of 3.5 kV cm^{-1} (1.92 kJ kg^{-1}) and 5 pulses of 2 kV cm^{-1} (0.22 kJ kg^{-1}), respectively. Nevertheless, the rest of PEF-treated carrots had similar carotenoid content as those untreated.

Carrot carotenoids are usually stored in crystalline form in chromoplasts. Hence, the main barriers limiting their release are cell wall and chromoplasts (Schweiggert et al., 2012). Pores formation and structural rearrangements caused by PEF application, may enhance the diffusion of solutes located inside the cells (Barba et al., 2015). PEF-treated carrots ($E \geq 2$ kV cm^{-1}) showed higher levels of total carotenoids than untreated carrots immediately after treatment, which suggests an enhancement of their extractability instead of their accumulation by *de novo* biosynthesis, which would require storage time. These results would be in accordance to those reported by Wiktor et al., (2015), in which carrots subjected to 3 and 5 kV cm^{-1} treatments had higher conductivity as those untreated, suggesting increased cell permeability. Likewise, other authors attributed the instant increase of total carotenoids to the mechanical disruption of carrot cell walls (Cuéllar-Villarreal et al., 2016; Nowacka & Wedzik, 2016).

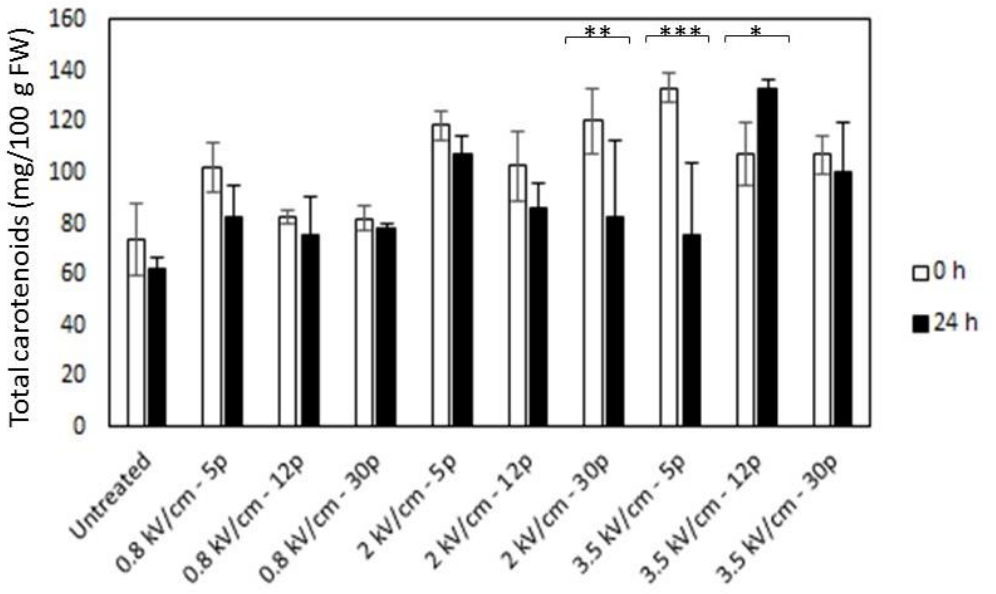


Figure 3.1. Total carotenoid contents in PEF-treated carrots just after and 24 h after treatment. All values are mean ± standard deviation of three replicates, each one composed by two carrots. Asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) stand for differences between just treated and stored carrots for each treatment condition.

Statistical analysis showed that changes in individual carotenoid content were mainly due to electric field strength applied [lutein ($\eta_p^2 = 0.914$; $p < 0.001$), phytoene ($\eta_p^2 = 0.718$; $p < 0.001$), β-carotene ($\eta_p^2 = 0.674$; $p < 0.001$) and α-carotene ($\eta_p^2 = 0.442$; $p < 0.01$)]. Phytoene and β-carotene increased just after treatments of $E \geq 2$ kV cm^{-1}. PEF-treated carrots had similar α-carotene content as those untreated, excepting carrots treated with 5 pulses of 3.5 kV cm^{-1} (0.61 kJ kg^{-1}), where a 63.3 % increase was observed (**Figure 3.2**). On the other hand, lutein content decreased between 15.1 % and 96.4 % when electric field strengths of 0.8 kV cm^{-1} and 2 kV cm^{-1} were applied. Furthermore, carotenoid content considerably changed 24 h after PEF treatments and differed depending on the individual compound. Some compounds increased whereas others decreased or remained similar to those found in untreated carrots. Carrots treated with 3.5 kV cm^{-1} or 2 kV cm^{-1} (0.22 and 1.19 kJ kg^{-1}) exhibited 1.05 – 1.57 times more phytoene than those untreated. Carrots treated with 12 pulses of 3.5 kV cm^{-1} (1.92 kJ kg^{-1}) showed twice as much β-carotene content than those untreated, although other treatments did not cause any significant change in their concentration (**Figure 3.2**). Besides, α-carotene contents increased 24 h after applying 5 pulses of 0.8 kV cm^{-1} (0.14 kJ kg^{-1}) or 2 kV cm^{-1} and 3.5 kV cm^{-1} (1.92 kJ kg^{-1} or 3.93 kJ kg^{-1}), whereas lutein decreased when applying electric field strengths of 0.8 kV cm^{-1} or 2 kV cm^{-1} (0.22 and 0.5 kJ kg^{-1}).

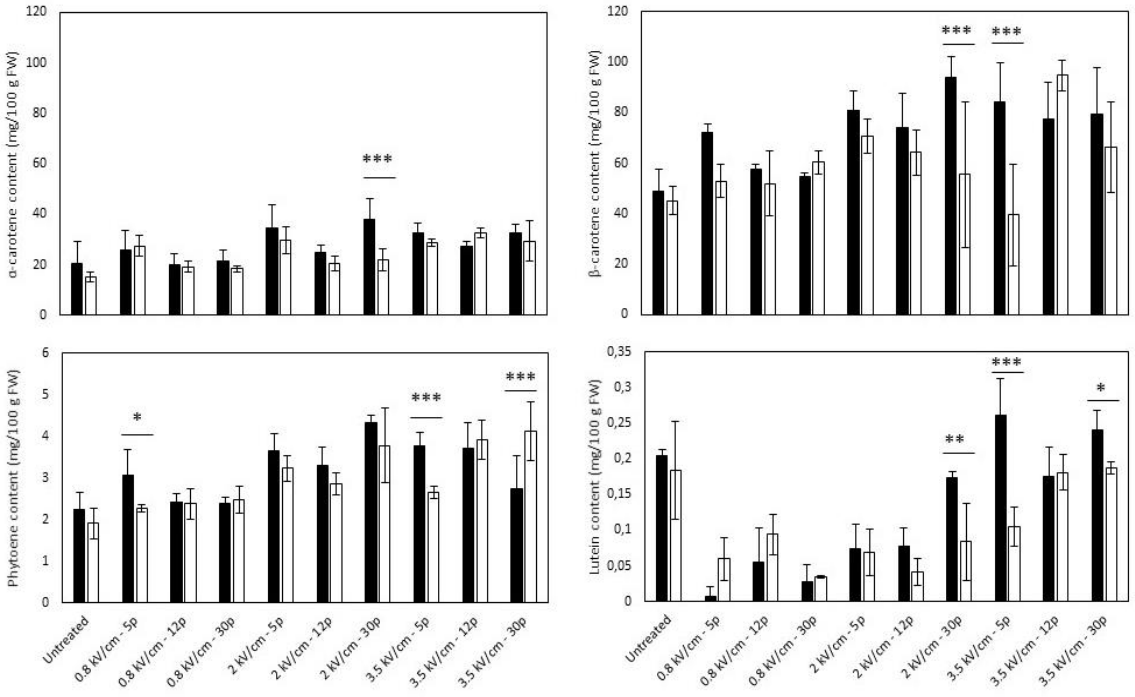


Figure 3.2. Individual carotenoid contents from PEF-treated carrots just after (black bars) and 24 h (white bars) after treatment. Asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) stand for differences between just treated (0 h) and stored carrots (24 h) for each treatment condition.

The increase in carotenoids content may result from either the activation of their biosynthesis pathway as a stress response, an improvement in their extraction, or a combination of both. Increases in the total content were likely related to better extractability due to electropermeabilization and disruptions in cell structure caused by PEF. However, differences observed in the content of individual carotenoids after PEF (**Figure 3.2**) may be attributed to adjustments in the carotenoid biosynthesis pathway, since pores formation may trigger the accumulation of ROS. These act as secondary signals belonging to the defence response of plants to stress (Baxter et al., 2014), which are necessary to synthesize secondary metabolites (e.g. carotenoids) (Gómez Galindo et al., 2009). Carotenoids scavenge singlet oxygen and lipid peroxy-radicals, as well as inhibit lipid peroxidation and superoxide generation under stress (Sarker and Oba, 2018a, 2018b). As stress signals are detected, carotenoid biosynthetic pathway is upregulated, thus triggering carotenoids accumulation (Nisar et al., 2015). The carotenoid biosynthetic pathway in plants has a bifurcation step after lycopene, the two branches are distinguished by different cyclic end-groups. Two beta rings lead to the β, β branch (β-carotene and its derivatives), whereas one beta and one epsilon ring define the β, ε branch (α-carotene and its derivatives). Significant correlations were found between specific energy applied and some carotenoids, such as phytoene ($r = 0.761$; $p < 0.001$), lutein ($r = 0.648$; $p < 0.001$) or α-carotene ($r = 0.402$; $p < 0.05$). According to that, some PEF treatments may modulate both the lycopene

β-cyclase (βLCY) and lycopene ε-cyclase (εLCY) enzyme activities, required to form α-carotene, lutein and β-carotene (Cunningham and Gantt, 1998) given that lutein and α-carotene both come from the same branch of the pathway.

Most studies about the effect of PEF on individual carotenoid contents have been carried out on tomatoes. Vallverdú-Queralt et al., (2013) and González-Casado, Martín-Belloso, Elez-Martínez, & Soliva-Fortuny, (2018) found an increment of α-carotene, β-carotene and lutein after 24 h of 1.2 kV cm^{-1} treatment. These authors attributed the accumulation of carotenoids to a stress response caused by PEF. However, no information about the carotenoid content just after treatment was provided, which is essential to elucidate the causes explaining this increase. The differences observed in the accumulation trend of each individual carotenoid of carrots subjected to intermediate electric field strengths (2 kV cm^{-1}) together with negligible softening (López-Gámez et al., 2020), indicate that changes in the extractability of carotenoids cannot be the only reason explaining carotenoid accumulation. In this line, Ramos-Parra et al., (2019) reported that after the application of another nonthermal technology, high hydrostatic pressure (HHP), papaya fruit accumulated carotenoids during storage (4 ºC for 2 days) and supported these results with gene expression of enzymes involved in the carotenoid biosynthetic pathway. In the present work, the greatest increases occurred after applying the most intense treatments and, generally, the content remained unaltered throughout time. Nonetheless, modification in individual carotenoid content seems to be related to changes at physiological level. These results suggest that the cause of increases is likely a combination between a better extraction and modulations of the carotenoids biosynthetic pathway. To confirm this hypothesis, further studies on gene expression of enzymes involved in their biosynthesis [e.g. phytoene synthase (PSY)] (Ramos-Parra et al., 2019) would be required.

3.2. Carrot quality-related parameters and their correlation with carotenoid content

Colour changes in three different tissues (peel, cortical and vascular tissues), TSS and pH of untreated and PEF-treated carrots are shown in **Table 3.2**. PEF processing did not trigger severe changes regarding pH and TSS. Only just after the application of 30 pulses of 2 kV cm^{-1} (1.19 kJ kg^{-1}) and 24 h after applying 12 pulses of 3.5 kV cm^{-1} (1.92 kJ kg^{-1}) a slight TSS decrease (11 %) was observed. Moreover, pH did not significantly vary between untreated (pH 6.2) and PEF-treated (pH 5.9 – 6.4) carrots. Conflicting information about these parameters in PEF-treated solid food is available. For instance, Amami, Vorobiev, & Kechaou, (2005) reported an increase in solid gain of PEF-treated apples when increasing electric field strengths (0.1 – 1.1 kV cm^{-1}) and specific energy inputs (0.26 – 36 kJ kg^{-1}). These results are in agreement with González-Casado et al., (2018), who disclosed increases in TSS (0.83 – 2.31 kJ kg^{-1}) and pH (0.09 – 2.31 kJ kg^{-1})

of PEF-treated tomatoes. TSS changes were attributed to cell membrane breakage, which may indirectly cause an osmotic imbalance and entail the increment of solids in tissues. Alternatively, they also proposed a faster ripening triggered by an increase in ethylene synthesis. Conversely, Ribas-Agustí et al., (2019) reported no alteration in TSS of PEF-treated apples, excepting those treated with the lowest energy (0.01 kJ kg^{-1}) and stored 24 h, which was attributed to the metabolic accumulation of organic acids and sugars. In these examples, changes in TSS might be related to the acceleration in their metabolism and climacteric ripening. Modifications in TSS of carrots, being non-climacteric vegetables, may be due to changes in membrane permeability, which may entail solid gain or water loss during PEF treatments or a better extraction of sugars and other water-soluble compounds. Regarding pH, Ribas-Agustí et al., (2019) reported an increase in apples subjected to treatments of 1.8 and 7.3 kJ kg^{-1}. On the other hand, Leong and Oey, (2014) did not observe any remarkable alteration in carrot puree treated by applying different specific energies (1.08 – 516.28 kJ kg^{-1}) neither Balaša, (2014) in PEF-treated (< 66.5 kJ kg^{-1}) *Vitis vinifera* cell cultures.

In general, colour differences among treated and untreated carrots were not significant, although some differences in BI of cortical and vascular tissues were noticed in carrots treated with different intensities. For instance, carrots treated by 0.8 kV cm^{-1} (0.38 and 0.87 kJ kg^{-1}) showed lower BI than those treated by 2 kV cm^{-1} or 3.5 kV cm^{-1}. Statistical analysis showed that electric field strength ($\eta_p^2 = 0.437$; $p = 0.006$), number of pulses ($\eta_p^2 = 0.335$; $p = 0.025$) and their interaction ($\eta_p^2 = 0.493$; $p = 0.012$) exerted a significant effect on the BI of vascular tissues, whereas cortical BI was mainly affected by electric field strength applied ($\eta_p^2 = 0.679$; $p < 0.001$). Some authors have reported browning increase after submitting products to PEF (González-Casado et al., 2018a; Ribas-Agustí et al., 2019) and they attributed these changes to cellular membrane disruption and greater contact between released phenolic substances and their oxidizing enzymes [e.g. polyphenol oxidase (PPO), peroxidase (POD)]. Since PPO is mostly present in carrot peel than in inner tissues (Alegria et al., 2016), changes in cortical BI may be likely related to carotenoid content, which is mainly stored in secondary phloem (Baranska et al., 2006). These results are consistent with statistical correlations found between cortical BI and phytoene ($r = 0.638$; $p < 0.05$) and β-carotene ($r = 0.683$; $p < 0.05$) contents. Besides, a correlation was also found between cortical h and lutein contents ($r = 0.701$; $p < 0.05$).

On the other hand, C* and h were not affected by PEF treatments in none of the studied tissues. However, a downward trend was observed in peel C* ($r = -0.467$; $p < 0.05$) and h ($r = -0.434$; $p < 0.05$) values when increasing the applied specific energy. Changes in C* may suggest more dullness in tissue because of electropermeabilization and leakage of intracellular content whereas modifications in h could be associated to an increment in redness, supported by the

increase in carotenoids (**Figure 3.1** and **Figure 3.2**). Some authors have also reported maintenance of C* and h parameters in PEF-treated blueberries (Jin et al., 2017), pumpkins (García-Parra et al., 2018), and juices obtained from just PEF-treated grapes (Leong et al., 2016). However, other studies found significant decreases in C* and increases in h of PEF-treated apples or alterations in a* and b* values of PEF-treated carrots depending on electric field strength applied (Wiktor et al., 2015). Discordant results are probably related to the intrinsic characteristics of each matrix and differences in processing parameters to which they are subjected. In this line, more intense colour changes may occur when PEF cause irreversible formation of pores, but they can be reversed if pores are resealed. Furthermore, it also depends on the content of coloured pigments, considering that they may be enhanced or degraded because of the treatment intensity. Finally, the extent of activation of enzymes related to browning (Ohshima et al., 2007) could also be decisive in colour modification.

Table 3.2. Quality attributes [colour, pH and total soluble solids (TSS)] of untreated and PEF-treated carrots just and 24 h after treatment.

Post-treatment time (h)	E [a] (kV cm^{-1})	n [b]	Peel			Cortical tissue			Vascular tissue			pH	TSS [f] (%)
			C* [c]	h [d]	BI [e]	C* [c]	h [d]	BI [e]	C* [c]	h [d]	BI [e]		
0	Untreated		45.5 ± 0.7 a	29.28 ± 1.0 a	149 ± 4 a	57.3 ± 1.2 a	30.4 ± 1.0 a	194.03 ± 1.7 ab	49 ± 3 a	28.11 ± 0.9 a	181 ± 17 ab	6.2 ± 0.0 a	8.6 ± 0.1 a
	0.8	5	41 ± 3 a	30.7 ± 1.2 a	132 ± 17 a	56.6 ± 2.6 a	28.0 ± 1.6 a	186 ± 12 ab	47 ± 4 a	26.7 ± 1.6 a	162 ± 10 ab	6.2 ± 0.1 ab	9.1 ± 1.1 a
		12	43.7 ± 1.2 a	29.6 ± 1.0 a	134 ± 16 a	51.3 ± 2.6 a	27.3 ± 2.5 a	169 ± 7 a	45 ± 3 a	26.2 ± 2.0 a	159 ± 5 b	6.3 ± 0.0 b	7.7 ± 1.1 ab
		30	40.5 ± 1.7 a	29.9 ± 1.2 a	131 ± 17 a	51.5 ± 2.3 a	26.4 ± 2.2 a	169 ± 13 a	49 ± 3 a	26.9 ± 1.6 a	180 ± 12 ab	6.3 ± 0.1 b	8.3 ± 0.6 ab
	2.0	5	43.8 ± 1.4 a	30.0 ± 2.1 a	141 ± 15 a	57 ± 6 a	27.3 ± 2.4 a	192 ± 6 ab	48.9 ± 2.9 a	27 ± 3 a	186 ± 7 a	6.3 ± 0.1 ab	8.4 ± 0.4 ab
		12	40 ± 3 a	30 ± 4 a	144 ± 24 a	57.1 ± 1.0 a	27 ± 2.7 a	207 ± 6 b	44.0 ± 1.8 a	25 ± 1.0 a	170 ± 4 ab	6.2 ± 0.0 ab	8.6 ± 0.2 ab
		30	40 ± 6 a	29.9 ± 0.5 a	130 ± 30 a	59.3 ± 2.9 a	28.8 ± 1.3 a	212 ± 6 b	47 ± 5 a	28.2 ± 1.6 a	179 ± 5 ab	6.2 ± 0.0 ab	7.6 ± 0.3 b
	3.5	5	43 ± 4 a	29.1 ± 1.2 a	144 ± 8 a	56 ± 5 a	28.1 ± 2.0 a	201 ± 16 b	42.7 ± 2.8 a	24.4 ± 1.5 a	176 ± 6 ab	6.2 ± 0.0 ab	8.0 ± 0.8 ab
		12	37 ± 4 a	27.3 ± 0.4 a	130 ± 13 a	55.2 ± 1.9 a	28.6 ± 1.1 a	199 ± 17 b	44 ± 4 a	26.1 ± 1.1 a	163 ± 10 ab	6.2 ± 0.2 ab	8.1 ± 0.4 ab
		30	38.0 ± 1.8 a	28.1 ± 1.3 a	133 ± 7 a	54.1 ± 2.0 a	28.9 ± 0.9 a	202 ± 11 b	43 ± 5 a	24.8 ± 2.8 a	159 ± 7 b	6.3 ± 0.1 b	8.6 ± 0.1 a

Table 3.2. Continuation.

Post-treatment time (h)	E [a] (kV cm^{-1})	n [b]	Peel			Cortical tissue			Vascular tissue			pH	TSS [f] (%)
			C* [c]	h [d]	BI [e]	C* [c]	h [d]	BI [e]	C* [c]	h [d]	BI [e]		
24	Untreated		42 ± 3 A	29.3 ± 1.3 A	131 ± 10 A	59.6 ± 2.8 A	28.5 ± 1.2 A	192 ± 15 AB	44.5 ± 2.9 AB	26.3 ± 1.2 A	154 ± 20 AB	6.2 ± 0.0 A	8.6 ± 0.4 A
	0.8	5	42.8 ± 2.5 A	31.0 ± 1.6 A	132 ± 11 A	58 ± 3 A	29.0 ± 0.3 A	175 ± 14 AB	42 ± 3 AB	28.1 ± 0.9 A	143 ± 20 A	6.3 ± 0.0 A	8.2 ± 0.3 AB
		12	43 ± 4 A	29.5 ± 0.0 A	140 ± 21 A	57.5 ± 2.0 A	27.6 ± 0.7 A	176 ± 6 B	42.4 ± 2.5 AB	26.6 ± 1.9 A	149 ± 15 AB	6.2 ± 0.1 A	8.7 ± 0.4 A
		30	40 ± 5 A	28.9 ± 1.1 A	132 ± 16 A	58.3 ± 2.1 A	28.2 ± 1.4 A	188 ± 8 B	45 ± 4 A	27.8 ± 1.6 A	162 ± 19 AB	6.3 ± 0.2 A	8.7 ± 0.5 A
	2.0	5	42.0 ± 2.1 A	30.1 ± 1.8 A	136 ± 7 A	55.0 ± 2.0 A	28.2 ± 2.5 A	193 ± 8 AB	41 ± 4 AB	26 ± 3 A	167 ± 8 AB	6.4 ± 0.2 A	8.6 ± 0.4 AB
		12	40 ± 3 A	29.1 ± 1.2 A	138 ± 10 A	54.6 ± 6 A	28.4 ± 1.1 A	190 ± 18 A	36.9 ± 1.7 B	24.4 ± 2.6 A	143 ± 7 A	6.3 ± 0.4 A	7.4 ± 0.8 AB
		30	33.2 ± 1.8 A	29.9 ± 0.7 A	116 ± 11 A	56.7 ± 4 A	28.9 ± 1.2 A	197 ± 7 AB	40.2 ± 1.1 AB	27.5 ± 2.6 A	156 ± 4 AB	6.3 ± 0.3 A	8.6 ± 0.5 AB
	3.5	5	40 ± 3 A	29.6 ± 1.2 A	145 ± 11 A	55.6 ± 1.9 A	27.9 ± 2.1 A	203 ± 14 AB	42 ± 3 AB	25 ± 4 A	180 ± 5 B	6.1 ± 0.2 A	7.9 ± 0.7 AB
		12	39 ± 3 A	29.0 ± 1.1 A	145 ± 14 A	56 ± 3 A	29.7 ± 2.5 A	205 ± 10 A	46 ± 2.5 A	26.6 ± 2.3 A	180 ± 10 B	6.1 ± 0.5 A	7.6 ± 0.3 B
		30	38 ± 4 A	29.7 ± 2.3 A	136 ± 13 A	54.6 ± 1.7 A	27.6 ± 2.0 A	202 ± 10 A	45.3 ± 2.1 A	25.8 ± 1.6 A	187 ± 12 B	5.9 ± 0.4 A	7.7 ± 1.1 AB

All values are mean ± standard deviation of three replicates, each one composed by two carrots. Different lowercase and uppercase letters within the same column indicate significant differences ($p < 0.05$) among treatments immediately after and 24 h after treatment, respectively. [a] Electric field strength; [b] number of pulses; [c] chroma; [d] hue angle; [e] browning index; [f] total soluble solids.

4. Conclusions

PEF treatments positively influenced carrots quality, since carotenoid contents were enhanced without major changes in colour, pH or TSS for at least 24 h. Total carotenoids increased by 39.3 – 81.3 % just after treatment ($E \geq 2$ kV cm^{-1}), which may suggest their better extractability. However, changes in carotenoid individual contents seem to point out that PEF may also modulate their biosynthesis pathway, which is supported by the high correlation found between specific energy input, lutein, and phytoene. In order to know to what extent each factor participates in carotenoid fluctuation, further studies related to enzymes activity involved in their synthesis are necessary. Obtained results demonstrate that PEF can be effectively exploited to enhance carotenoids extractability and/or content without altering carrot quality attributes. Furthermore, PEF stands as a potential pre-treatment to obtain derived products (juices or purees) with enhanced characteristics, since carotenoids would probably be easily released and become more available for absorption during digestion.

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Chapter 4. Carotenoid and phenolic compounds bioaccessibility as affected by pulsed electric fields and their effects in carrot structure

Gloria López-Gámez, Pedro Elez-Martínez, Amparo Quiles-Chuliá, Olga Martín-Belloso, Isabel Hernando-Hernando, Robert Soliva-Fortuny

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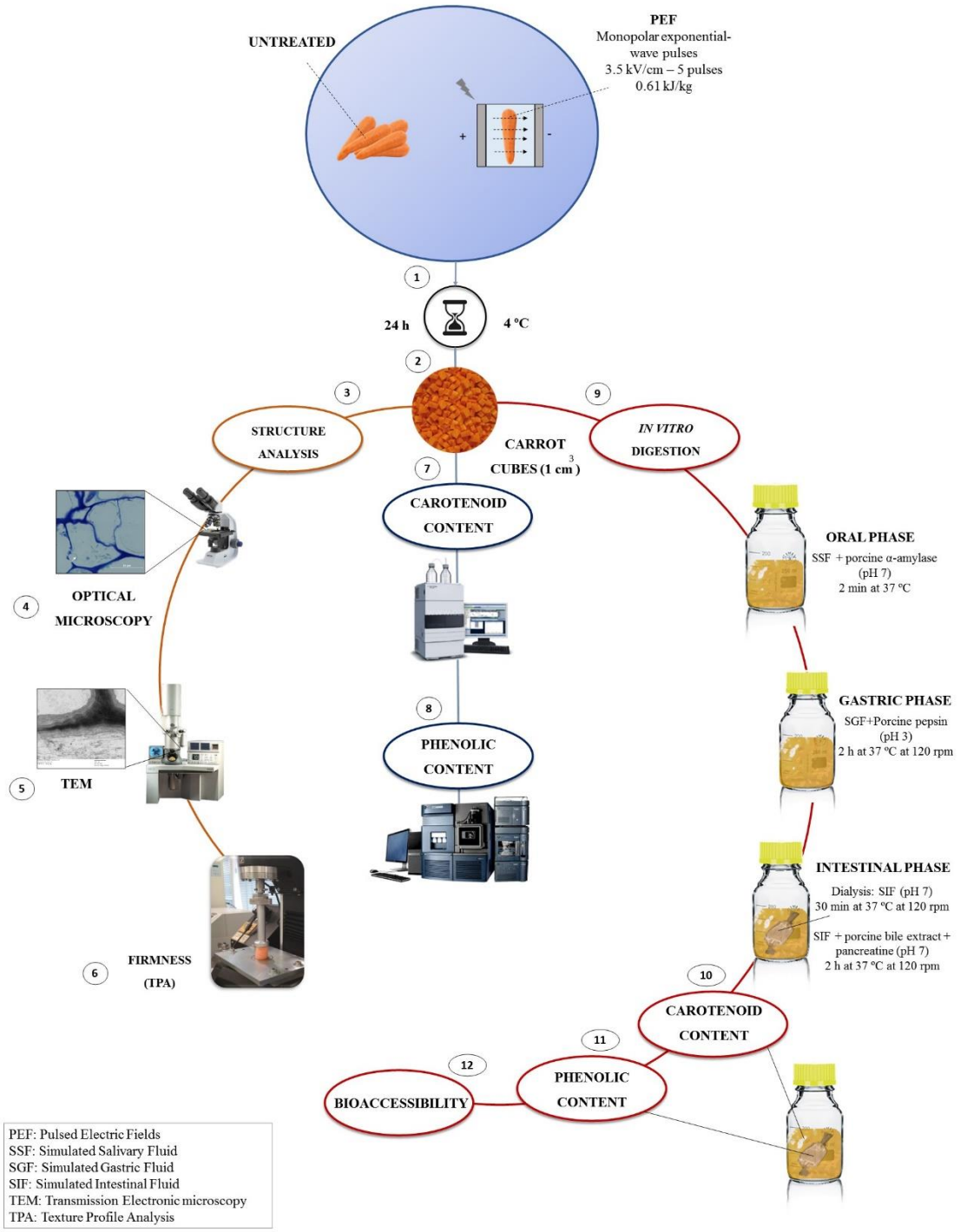
Abstract

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 ºC and microstructure was evaluated before submitting them to an *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 (163.21 %), 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, others may be degraded or entrapped by other compounds during digestion. Therefore, PEF is an effective technology for obtaining carrots with enhanced carotenoids and phenolic bioaccessibility.

Keywords: Bioaccessibility; phenolic compounds; carotenoids; microstructure; carrot; texture; pulsed electric fields

GRAPHICAL ABSTRACT



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) (Arscott & Tanumihardjo, 2010). High dietary intakes of carotenoids and phenolic compounds have been associated with protective effects against some types of cancer and cardiovascular diseases (Rao & Rao, 2007) due to their antioxidant, anti-inflammatory and antitumoral activities (Scalbert & Williamson, 2000). 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 (Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Blanco-Navarro, et al., 2007). 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 (Bohn, 2008, 2014). Carotenoids are lipophilic compounds that need to be released from the food matrix, solubilized and incorporated into micelles for absorption (Sy, Gleize, Dangles, Landrier, Veyrat & Borel, 2012). In carrots, these compounds are stored in crystalline form in chromoplasts. Hence, together with cell walls, chromoplasts are the main barriers limiting carotenoids bioaccessibility (Schweiggert et al., 2012). 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, Phan, Bucknall, & Arcot (2019) demonstrated that the bioaccessibility of hydrophilic compounds in a food matrix can be increased when it is digested with other carotenoid-rich vegetables due to their antioxidant activities, which may serve as protection against degradation.

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 (Hornero-Méndez & Mínguez-Mosquera, 2007; Knockaert, Lemmens, et al., 2012; Ribas-Agustí et al., 2019b; Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015). 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 (Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010), though degradation has also been reported (Hornero-Méndez & Mínguez-Mosquera, 2007; Knockaert, Pulissery, Lemmens, et al., 2012). 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 (Lemmens, Colle, Buggenhout, Loey, & Hendrickx, 2014; Moelants et al., 2012; Palmero, Lemmens, et al., 2014; Palmero et al., 2016).

Effects of processing on phenols bioaccessibility vary depending on food matrix, processing parameters or the chemical characteristics of individual compounds (Ribas-Agustí et al., 2018). It has been reported that chemical or physical changes in the food matrix may induce their better release (Carrillo, Buvé, Panozzo, Grauwet, & Hendrickx, 2017).

Pulsed electric fields (PEF) is a non-thermal technology based on the application of short duration pulses of external electric fields that induce electropermeabilization of cell membranes. Modification of cell membrane permeability has been applied to more efficiently extraction of lipophilic (Luengo, Álvarez, & Raso, 2014; Roohinejad et al., 2014; Wiktor et al., 2015) or hydrophilic compounds (Donsì, Ferrari, & Pataro, 2010; Gachovska et al., 2010; Puértolas, Cregenzán, Luengo, Álvarez, & Raso, 2013). Therefore, PEF application can also be a potential tool to facilitate bioactive compounds release during digestion and promote their bioaccessibility (Bot et al., 2018; Jayathunge et al., 2017; Ribas-Agustí et al., 2019b).

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 caliber 25/35 mm and length of 17 ± 2 cm were purchased in a local supermarket in Lleida (Spain). They were stored at 4 ºC until treatment within a week. Carrots were washed with tap water and water excess was removed 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), which delivers pulses of 4 μs exponentially from a capacitor of 0.1 μF 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 μS 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 ºC for 24 h. The specific energy input was calculated based on Wiktor et al., (2015) and medium temperature did not exceed 20.0 ºC after PEF application.

The treatment selection was based on the results of previous studies, aimed at maximizing the accumulation of phenolic compounds in carrot (López-Gámez et al., 2020a) without altering its quality attributes.

2.4. Texture analysis

In order to perform compression tests a TA-XT2 texture analyser (Stable Micro Systems, England), equipped with a loading cell (25 kg-f), a cylindrical flat-head aluminum probe (25 mm diameter, Stable Micro Systems) 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 center. 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) (Leong, Du, & Oey, 2018).

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 min 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 ºC. Samples were cut into 70 – 80 nm and 1 µm sections using a 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 adopted according to the standardized COST Infogest protocol (Minekus et al., 2014), 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 cm^3 cubes) for 1 min (Taurus aromàtic 150 W) and transferred to a glass bottle. Then, 10 mL of simulated salivary fluid (pH 7 and 37 ºC) with α-amylase (Minekus et al., 2014) were added to stir the mix for 1 min at 37 ºC. The gastric phase started adding 20 mL of simulated gastric fluid (pH 3 and 37 ºC) and pepsin (Minekus et al., 2014). 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 ºC) (Minekus et al., 2014). 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) (Minekus et al., 2014; Ribas-Agustí et al., 2019b). After a period of 30 min to reach pH 7, a solution containing simulated intestinal fluid (pH 7 and 37 ºC), 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 at 5000 g for 15 min at 4 °C (Brodkorb et al., 2019; Eriksen et al., 2017; Svelander et al., 2011) 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; Anoia S.A., Barcelona, Spain) and the resulting solution corresponded to the micellar fraction. Resulting digested fractions were freeze-dried and stored at -40 ºC 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 (Ovan, Badalona, Spain) at 37 °C and 120 rpm. Electrolyte concentrations and enzyme activities were prepared following the indications provided by Minekus et al. (2014). 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, Davis, & Dezman (1990), 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 with Diode Array Detection (HPLC-DAD) 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.

2.7.2. 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 at 4000 g for 5 min (Svelander, 2011). 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 by HPLC-DAD

Carotenoids were quantified by HPLC-DAD, following a procedure validated by Cortés, Esteve, Frígola, & Torregrosa (2004). An aliquot of 20 µL of the extracted samples was injected into the 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 C_{18} 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. The flow rate was fixed at 1 mL min^{-1} and the total run time was 60 min. The column was set at 30 ºC, while sample amber vials on the auto sampler were preserved at 4 ºC. The carotenoids were identified by UV–vis spectral data and their retention times (Cortés et al., 2004; Mouly et al., 1999). Quantification of carotenoids was carried out by integration of the peak areas and comparison to calibration curves.

2.9. Extraction of phenolic compounds

Phenolic compounds were extracted from freeze-dried non-digested 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 min, 4 ºC) , and the clear supernatant was microfiltered using polyvinylidene difluoride (PVDF) filters (0.2 µm) (Scharlab, Barcelona, Spain) prior to injection to the chromatographic system.

2.10. Identification and quantification of individual phenolic compounds by Ultra-Performance™ Liquid Chromatography (UPLC-MS/MS)

Phenolic compounds and their generated metabolites were determined in methanolic extracts obtained from freeze-dried non-digested 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 C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C_{18} (2.1 × 5 mm, 1.7 μm), also from Waters. During the analysis, the column was kept at 30 °C, and the flow rate was 0.3 mL min^{-1}. Mobile phases were acetic acid (0.2 %) and acetonitrile, and elution gradients are shown in **Table 4.1**. 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.

Table 4.1. Chromatographic conditions for identifying phenolic compounds in UPLC.

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

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 dialyzed fraction of digested samples.

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

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

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

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). Three different replicates were submitted to each assayed treatment condition and each analysis was conducted twice. Results were reported as the mean ± standard deviation. Pairwise comparisons of means (Student's t test) were 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 4.1 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 4.1A** and **B**). Using the TEM technique, the observed cell walls were bonded to each other by a firm middle lamella (black arrows in **Figures 4.1C** 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 4.1A** 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 4.1A** and **B**). On the one hand, carotenoids were found, near the cell wall, inside the chromoplasts, adopting a globular structure (white arrows in **Figure 4.1B**). On the other hand, they appeared forming crystal clusters both inside the cell and on the cell wall (black arrow in **Figure 4.1A**). These clusters have also been detected in mango and carrot tissue (Vásquez-Caicedo, Heller, Neidhart, & Carle, 2006) and in persimmon (Vázquez-Gutiérrez, Quiles, Hernando, & Pérez-Munuera, 2011; Vázquez-Gutiérrez et al., 2016).

PEF treatment caused structural modifications in both, outer and inner parenchyma of carrot (**Figure 4.2**). PEF caused the breakdown and degradation of cell walls and membranes (**Figure 4.2A** and **B**). In some areas, the cell walls appeared thin and weakly stained (**Figures 4.2C** 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" favored the walls of adjoining cells to separate (black arrows in **Figure 4.2B**). Moreover, in both, outer and inner parenchyma, the middle lamella appeared dissolved and degraded in some areas (black arrows in **Figures 4.2C** 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 4.2A** and **B**). All these structural modifications could improve solutes transport through parenchyma, which favored bioactive compounds bioaccessibility (**Tables 4.2** and **4.3**).

Carotenoid components were mainly located in the cytoplasm, further away from the cell walls than in the untreated carrot (white arrows in **Figures 4.2A** and **B**). Cell wall and membranes degradation produced by the PEF treatment favored 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.

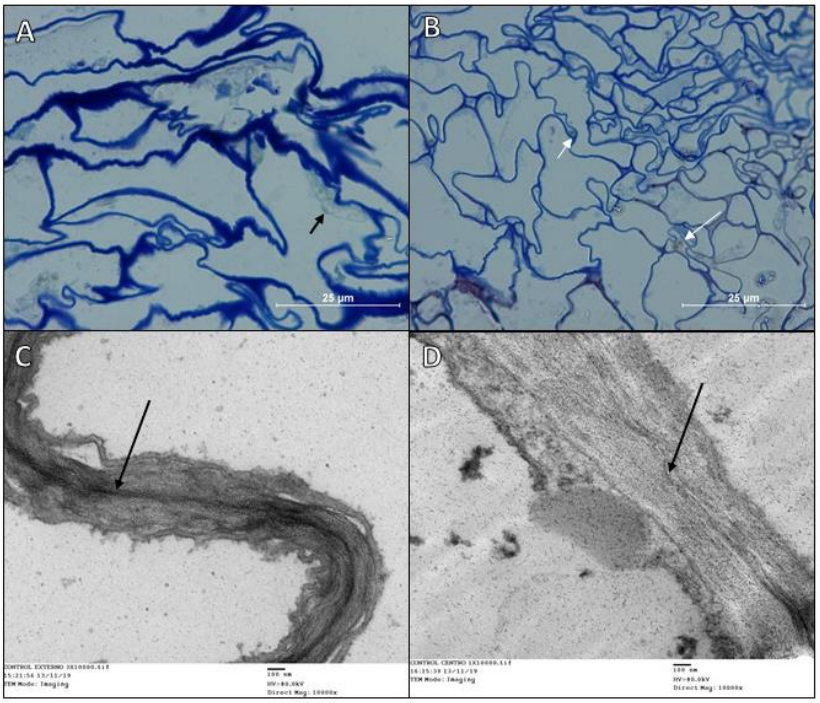


Figure 4.1. Light microscopy and TEM images of outer (A, C) and inner parenchyma of untreated carrots (B, D).

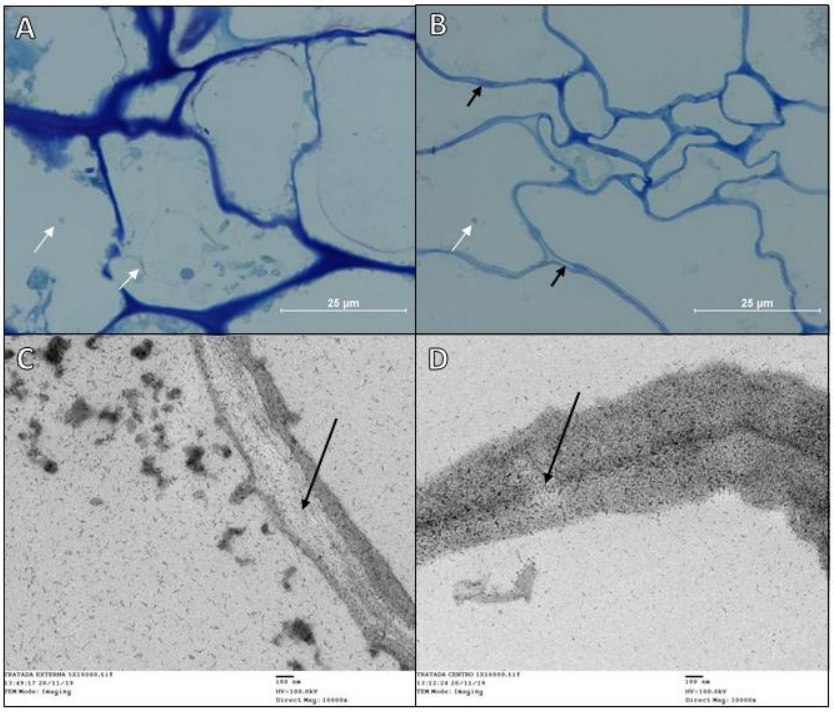


Figure 4.2. Light microscopy and TEM images of outer (A, C) and inner parenchyma of PEF-treated (5 pulses of 3.5 kV cm^{-1}) carrots (B, D).

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 4.3**). 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 $kV \cdot cm^{-1}$ can induce severe tissue damage through membrane breakdown (Asavasanti, Ersus, Ristenpart, Stroeve, & Barrett, 2010). Leong, Du, & Oey (2018) and Wiktor et al. (2018) 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 (González-Casado et al., 2018b; Jin et al., 2017; Ribas-Agustí et al., 2019a). This fact is supported by obtained microstructure images (**Figures 4.1 and 4.2**).

The increase in adhesiveness of PEF-treated carrots seems to be caused by permeabilization and cytoplasmic content leakage. Aguiló-Aguayo et al. (2014a) studied water mobility in PEF-treated carrots and reported that cellular content moved from vacuoles to cytoplasm and outside the cell. In addition, Aguiló-Aguayo et al. (2014b) demonstrated that sugar content was easily extracted from cells of PEF-treated carrot purees. The release of this intracellular juice rich in sugars may cause the higher adhesion to the texture analyser probe.

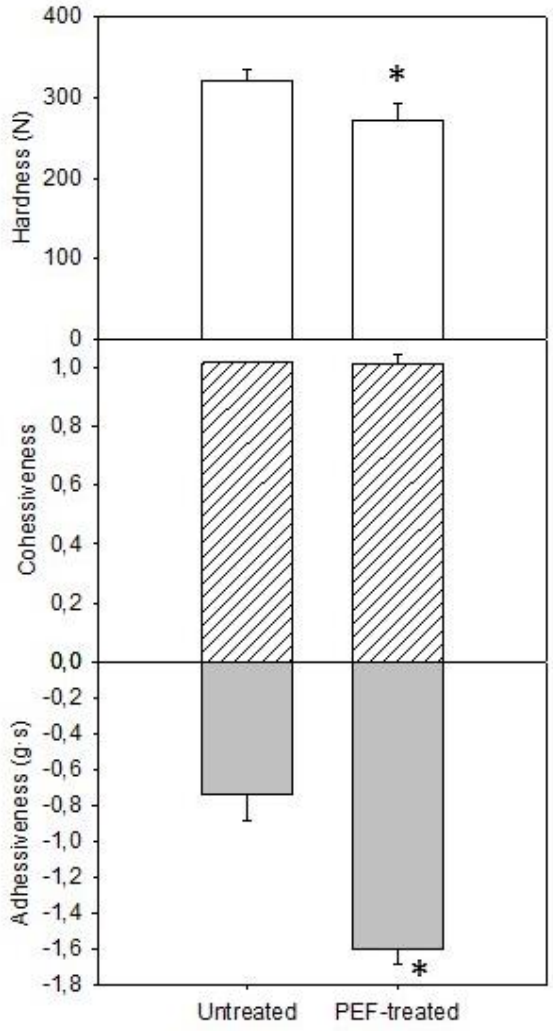


Figure 4.3. Hardness, cohesiveness and adhesiveness (mean ± SD) of PEF-treated (5 pulses of 3.5 kV cm^{-1}) and untreated carrots. Asterisks (*) indicate significant differences ($p < 0.05$) between treated and untreated carrots.

3.3. Effect of PEF on carrot carotenoids content

Carotenoid content of PEF-treated and untreated carrots is shown in **Table 4.2**. Main carotenoids present in carrots are α-carotene and β-carotene, with a lesser presence of lutein and phytoene (**Table 4.2**), which is in agreement with what was found in other previous studies (Mapelli-Brahm et al., 2017; Reboul et al., 2006; Schweiggert et al., 2012). Carotenoid content of PEF-treated carrots before digestion was similar to that of those untreated. Some authors have reported that electropermeabilization could enhance the liberation of carotenoids (Barba et al., 2015) depending on the applied conditions. For instance, Wiktor et al., (2015) 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 4.2**). 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 (5.75 mg/100 g FW) was higher than that obtained in those untreated (3.35 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 non-digested 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 (7 mg/100 g FW) was significantly higher than that observed in untreated carrots (4.3 mg/100 g FW). Even if carotenoids were better released from food matrix due to structural modifications caused by PEF (**Figures 4.1** and **4.2**), 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.

Carotenoid bioaccessibility from untreated carrots varied between individual compounds: lutein (23.6 %) > phytoene (17 %) > α-carotene (6 %) and β-carotene (7.5 %). 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 4.2**). 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 (Borel, 2003; Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Pérez-Sacristán, et al., 2007; Sy, Gleize, Dangles, Landrier, Veyrat & Borel, 2012; Tyssandier, Lyan, & Borel, 2001) and its deposition form in plastoglobuli (Lado, Zacarías, Gurrea, Page, Stead, Rodrigo, 2015; Nogueira, Mora, En, Bramley, & Fraser, 2013). 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 (Mapelli-Brahm et al., 2017) and in a low-fat tomato puree obtained from PEF-treated tomatoes (González-Casado et al., 2018a).

To the best of our knowledge, this is the first study evaluating the bioaccessibility of individual 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 (Jeffery et al., 2012). The increase in carotenoids bioaccessibility from PEF-treated carrots may suggest that electropermeabilization 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 4.1** and **4.2**) (Barba et al., 2015). Besides, during digestion, pectin may also interact with bile salts or digestive enzymes involved in the formation of micelles, which comprises carotenoids bioaccessibility (Cervantes-Paz, Ornelas Paz, Pérez-Martínez, Reyes-Hernández, Zamudio-flores, & Rios-velasco, Ibarra-Junquera, Ruiz-Cruz, 2016; Jeffery, Holzenburg, & King, 2012). In this line, PEF could also activate some pectic enzymes [e.g. pectinmethylesterase (PME) and polygalacturonase (PG)] related to tissue softening, which would be helpful for a better transference of carotenoids to micelles.

Table 4.2. Carotenoid content and bioaccessibility (mean ± SD) in the non-digested, released and micellar fractions from PEF-treated (5 pulses of 3.5 kV cm^{-1}) 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	1.8 ± 0.3	0.4 ± 0.0	0.3 ± 0.0	17 ± 0.7
	PEF-treated	1.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	19 ± 3

α-carotene	Untreated	15.8 ± 1.6	1.4 ± 0.2		0.7 ± 0.5		6 ± 3	
	PEF-treated	12 ± 3	2.7 ± 1.0		1.2 ± 0.1	***	9.9 ± 0.9	**
β-carotene	Untreated	38 ± 5	4.3 ± 1.3		2.3 ± 0.9		7.5 ± 2.4	
	PEF-treated	35 ± 5	7 ± 2	*	4.2 ± 0.3		11.9 ± 0.9	*
Lutein	Untreated	$6.3 \cdot 10^{-2} \pm 0.9 \cdot 10^{-2}$	$1.2 \cdot 10^{-2} \pm 0.8 \cdot 10^{-2}$		$1.5 \cdot 10^{-2} \pm 0.0$		23.6 ± 0.5	
	PEF-treated	$8.2 \cdot 10^{-2} \pm 3 \cdot 10^{-2}$	$2 \cdot 10^{-2} \pm 0.5 \cdot 10^{-2}$		$1.8 \cdot 10^{-2} \pm 0.7 \cdot 10^{-2}$		22 ± 5	
Total carotenoids	Untreated	51 ± 8	6.8 ± 1.1		3.4 ± 0.3		6.6 ± 0.5	
	PEF-treated	49 ± 8	10.1 ± 1.7	*	5.8 ± 0.5	**	11.9 ± 1.1	***

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

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 4.3**). 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 (163.21 %), caffeoylferuloylquinic acid (422.57 %), caffeic acid arabinoside glucoside (94.46 %), ferulic acid (116 %), 3-feruloylquinic acid (27.81 %) and feruloylquinic acid derivative (30.55 %).

Application of PEF causes electropermeabilization of cell membranes, leading to microstructural modifications in carrot matrix **(Figures 4.1** and **4.2**), which seem to be directly related to changes in phenolic content. On the one hand, these rearrangements in tissues may entail greater contact between 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 (Kilmartin, Zou, & Waterhouse, 2001). 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. On the other hand, the increase could be attributed to a better extraction due to membrane breakdown

(Wang, He, & Chen, 2014). 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 (López-Gámez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, 2020b).

3.6. 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 4.3**). Regarding the sum of coumaric and caffeic acids, their content was, respectively, 50.5 % and 68.6 % lower than that found in the dialyzed fraction of untreated carrots. Nevertheless, the dialyzed 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 dialyzed whereas they were not detected in dialyzed fraction from untreated carrots.

A higher content in dialyzed fraction does not necessarily entail a greater bioaccessibility, since it must also be calculated according to the amount found in the non-digested fraction. After submitting carrots to PEF, the bioaccessibility of some phenolic compounds dramatically decreased. 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 (Ribas-Agustí et al., 2019b). Given that the most abundant compounds found in non-digested 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 non-digested fractions had also a higher dialyzed 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 (Palafox-Carlos et al., 2011), avoiding their crossing through the dialysis membrane.

Table 4.3. Phenolic compounds bioaccessibility and content (mean ± SD) in the non-digested and dialyzed fractions from PEF-treated (5 pulses of 3.5 kV cm^{-1}) and untreated carrots.

Phenolic compound	Treatment	Non-digested fraction (mg kg^{-1} DW)		Dialyzed fraction (mg kg^{-1} 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	8.8 ± 1.52	***	4.84 ± 0.24	**	55.08 ± 9.34	
Coumaric acids and derivatives	Untreated	16.75 ± 2.8		14.26 ± 1.36		85.16 ± 18.6	
	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 ± 0.0		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	**	17.4 ± 5.7	***
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	
Caffeoylferuloylquinic 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		0.05 ± 0.01		69.14 ± 14.51	
	PEF-treated	0.14 ± 0.01	***	0.05 ± 0.01		36.4 ± 11.1	***
Caffeic acid Glu Acetyl glucoside	Untreated	3.17 ± 0.41		1.12 ± 0.05		35.5 ± 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		100 ± 14.2	
	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 ± 0.1		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	0.3 ± 0.04		0.08 ± 0.01		25.57 ± 5.75	
	PEF-treated	0.1 ± 0.01	***	0.05 ± 0.0	***	52.21 ± 7.84	***
Ferulic acid derivative	Untreated	1.04 ± 0.08		0.35 ± 0.02		33.28 ± 3.31	
	PEF-treated	1.0 ± 0.13		0.33 ± 0.03		32.9 ± 5.65	
Ferulic acid coumaroyl glucoside	Untreated	1.33 ± 0.27		0.6 ± 0.03		45.1 ± 9.02	
	PEF-treated	0.99 ± 0.18	*	0.49 ± 0.04	***	49.28 ± 10.55	
	Untreated	7.41 ± 0.57		1.59 ± 0.1		21.5 ± 1.82	

Ferulic acid caffeoyl glucoside	PEF-treated	4.73 ± 0.61	***	1.3 ± 0.13	***	27.52 ± 4.84	*
Feruloylquinic acid derivative	Untreated	3.95 ± 0.41		1.61 ± 0.18		40.79 ± 7.32	
	PEF-treated	4.3 ± 0.6		1.28 ± 0.16	**	29.8 ± 7.16	*
Feruloylquinic acid derivative (2)	Untreated	0.85 ± 0.09		0.23 ± 0.01		27.23 ± 4.19	
	PEF-treated	1.1 ± 0.12	**	0.27 ± 0.01	***	24.63 ± 3.67	
Ferulic acid and its derivatives	Untreated	26.29 ± 1.72		10.8 ± 0.3		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 with respect to untreated carrot (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).[1] nd: Not-detected.

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 %). Regarding total bioaccessibility, it was enhanced in both types of compounds, reaching 11.9 % in carotenoids and 20.8 % in phenols. 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.

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.

Acknowledgments

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Chapter 5. Feasibility of applying pulsed electric fields to whole carrots for improving bioaccessibility of carotenoid and phenolic compounds in their derived products

Gloria López-Gámez, Pedro Elez-Martínez, Olga Martín-Belloso, Robert Soliva-Fortuny

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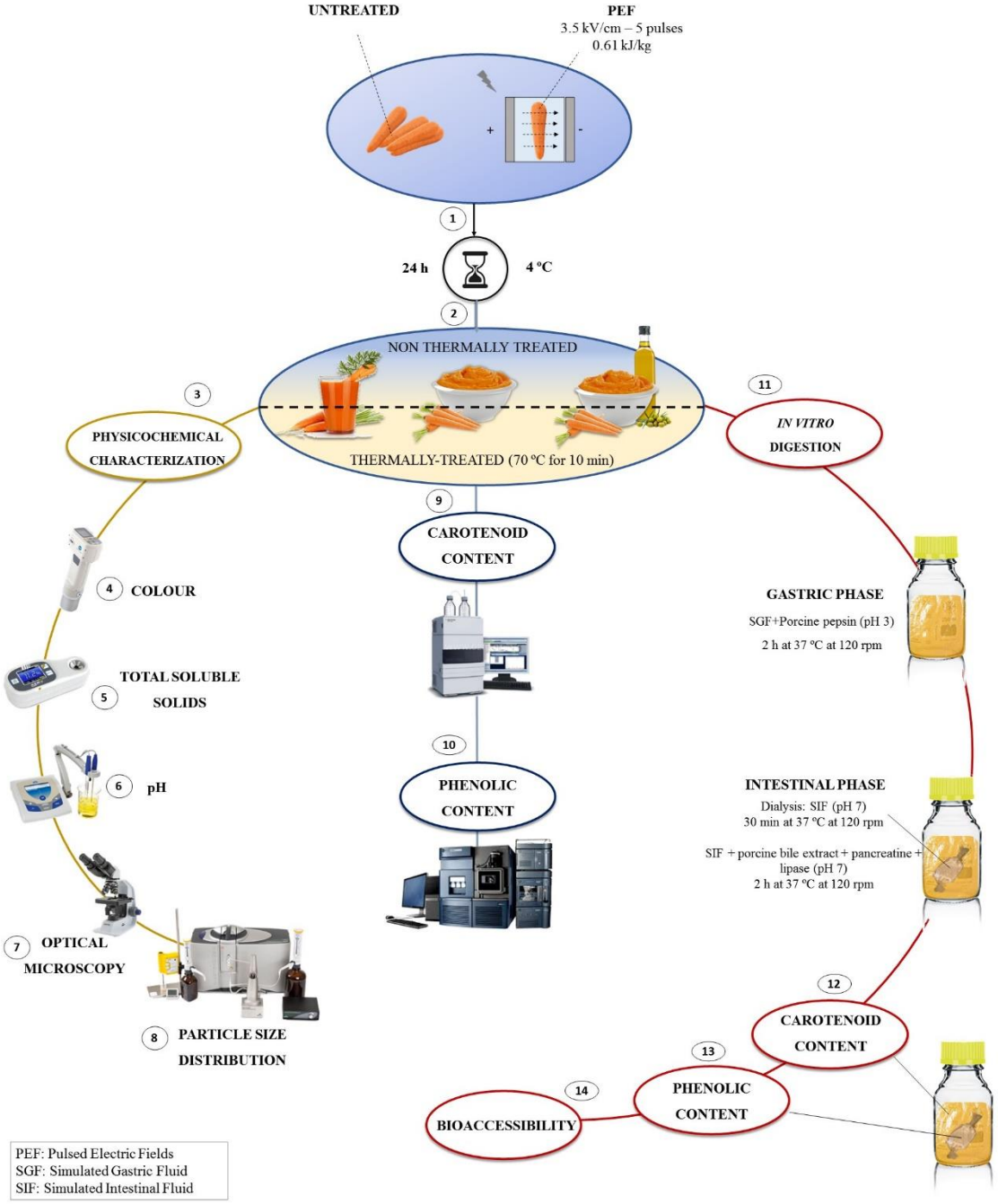
Abstract

The application of pulsed electric fields (PEF) to carrots is proposed to obtain derived products with increased phenolic and carotenoid bioaccessibility. With this purpose, whole carrots were submitted to 5 pulses of 3.5 kV cm^{-1} and stored for 24 h at 4 ºC. Different derived products, namely juices, purees and oil-added purees, were obtained. The products were subjected to a thermal treatment (70 ºC for 10 min) for obtaining a shelf-stable product. Carotenoid and phenolic contents as affected by PEF were determined before and after an *in vitro* digestion. Additionally, quality attributes, microstructure and particle size of each product were evaluated. Interactions between PEF application and critical aspects concerning processing of the derived products were also studied. Quality attributes were maintained in all derived products, although slight changes in colour were reported after thermal treatments, probably due to cell disruption. Carrot juice had the highest carotenoid (43.4 mg/100 g fresh weight) and phenolic content (322 mg kg^{-1} dry weight). However, the latter was reduced by half after applying PEF to whole carrots. Regarding phenolic compounds, ferulic acid derivatives were generally less affected than caffeic and coumaric acids. The highest phenolic bioaccessibility was reported in purees obtained from PEF-treated carrots (100 %), whereas further thermally-treated oil-added purees exhibited the greatest carotenoid bioaccessibility (7.8 %). The increase in carotenoid bioaccessibility could be related to their better release from matrix and solubilization into oil and micelles. Otherwise, results suggest that food matrix aspects apart from particle size, such as pectin state and quantity, are involved in phenolic bioaccessibility.

Keywords: Carotenoids; phenolic compounds; puree; juice; carrot; bioaccessibility; pulsed electric fields

GRAPHICAL ABSTRACT



1.Introduction

Today's life pace leads consumers to increasingly demand healthier minimally processed products that are easy to prepare and consume. Carrot is one of the most consumed vegetables worldwide, thus being a significant source of antioxidants, including carotenoids and phenolic compounds. Clinical studies have demonstrated that α-carotene and β-carotene, the most abundant carotenoids in carrots, prevent from suffering atherosclerosis, cancer, or macular degeneration (Rao & Rao, 2007). Likewise, chlorogenic acid, the main phenolic compound found in carrot, possesses anti-diabetic and cardioprotective properties (Hogervorst Cvejić, Atanacković Krstonošić, Bursać, & Miljić, 2017). Therefore, due to their health-promoting properties, carrots are a potential commodity for developing functional derived products and meet consumer demands.

Both carotenoids and phenolic compounds are usually enclosed by cell walls and organelle structures that hinder their release during digestion. Bioaccessibility is referred to the percentage of a compound released from food matrix and absorbed during digestion (Granado-Lorencio et al., 2007), which is more important than the actual content in a food matrix. Chemical structure, concentration, matrix structure and processing are the most important factors that determine bioactive compounds bioaccessibility (Van Buggenhout et al., 2010). Therefore, mechanical and thermal processes could disrupt the natural matrix, thus modifying their bioaccessibility (Parada & Aguilera, 2007; Van Buggenhout et al., 2010). Decrease in particle size and depolymerization of pectin have been shown to improve β-carotene bioaccessibility in carrot purees (Hedrén, Diaz, & Svanberg, 2002; Knockaert, Lemmens, Van Buggenhout, Hendrickx, & Van Loey, 2012; Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010). Furthermore, oil addition favour carotenoid micellarization during digestion (Hornero-Méndez & Mínguez-Mosquera, 2007) and a thermal treatment can enhance their release from cells and further solubilization into micelles (Knockaert, Lemmens, et al., 2012). Nonetheless, some studies have reported that cell wall fragments formed after thermal treatments may entrap carotenoids and compromise their bioaccessibility (Palmero, Lemmens, Hendrickx, & Loey, 2014). The bioaccessibility of phenolic compounds has been reported to increase in thermally-treated grape and orange juices, whereas it was shown to decrease in pomelo (80 ºC for 30 min) and fruit juice-based beverages (90 ºC for 1 min) (He et al., 2016; Quan et al., 2020; Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015). The effect of the presence of oils and fats on phenolic bioaccessibility is limited, although literature works report positive effect on phenolic bioaccessibility when whole milk was added to juices (He et al., 2016; Quan et al., 2020; Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015).

Pulsed electric fields (PEF) is a non-thermal processing technology that delivers short pulses (ms or μs) of electric energy to a food product that is located between two electrodes. Electropermeabilization causes reversible or irreversible structural changes in matrix depending on the applied intensity. Low and moderate intensities (0.1 – 5 kV cm^{-1}, 0.5 – 20 kJ kg^{-1}) have been reported to trigger a stress defence response in plant tissues, leading to the accumulation of bioactive compounds (e.g. carotenoids and phenolic compounds) in fruit and vegetables (Jacobo-Velázquez et al., 2017, Ribas-Agustí, Martín-Belloso, Soliva-Fortuny, & Elez-Martínez, 2019a, López-Gámez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, 2020b). On the other hand, severe changes in structure may facilitate the extraction of bioactive compounds and consequently, their bioaccessibility (Bot et al., 2018; Jayathunge et al., 2017). Results regarding the application of PEF to whole apples (Ribas-Agustí et al., 2019b) and carrots to enhance the bioaccessibility of carotenoid and phenolic compounds are promising, thus suggesting that derived products, such as juices or purees, with a higher and more accessible antioxidant content could be obtained from these commodities (González-Casado, Martín-Belloso, Elez-Martínez, & Soliva-Fortuny, 2018; Jayathunge et al., 2017).

Therefore, the main aim of this study was to investigate the feasibility of applying PEF to whole carrots as a treatment to enhance bioaccessibility of carotenoids and phenolic compounds in different shelf-stabled derived products (juices, purees and oil-added purees). Additionally, the effects of the interactions between PEF application and different processing strategies were investigated.

2. Material 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). Ammonium carbonate, acetonitrile, hexane and ethanol (HPLC grade), magnesium chloride hexahydrate, acetic acid and ammonium acetate were acquired from Scharlab (Sentmenat, Spain). Butyl hydroxytoluene (BHT) was purchased from Scharlau Chemie S.A. (Barcelona, Spain). Calcium chloride dihydrate was obtained from Merck (Darmstadt, Germany). Sodium hydrogen carbonate and potassium dihydrogen phosphate were acquired from VWR (Llinars del Vallès, Spain). Potassium chloride was obtained from Panreac (Castellar del Vallès, Spain). Digestive enzymes (porcine pepsin, porcine bile extract, porcine pancreatin and porcine lipase) were acquired from Sigma-Aldrich (Darmstadt, Germany).

Caffeic acid, ferulic acid, *p*-coumaric acid commercial patterns were obtained from Sigma-Aldrich (St. Louis, MO, USA). B-carotene standard was acquired from Carote-Nature (Ostermundigen, Switzerland) and α-carotene was acquired from Supelco-Merck (Darmstadt, Germany).

2.2. Carrot samples

Carrots (*Daucus carota* cv. Nantes) (17 ± 2 cm and 106 ± 7 g) were purchased in a local supermarket (Lleida, Spain) and stored at 4 ºC within a week until processing. Carrots were washed with tap water and the excess was removed with a paper cloth before PEF application.

2.3. Pulsed electric fields (PEF) treatments

PEF treatments were conducted in a batch PEF system (Physics International, San Leandro, CA, USA) equipped with a TG-70 gas control unit and a PT55 pulse generator (Pacific Atlantic Electronics Inc., El Cerrito, CA, USA). The system delivers exponential pulses of 4 μs from a capacitor of 0.1 μF at a frequency of 0.1 Hz. 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. Whole carrots (~ 0.1 kg) were immersed in an aqueous solution (conductivity of 10 μS cm^{-1}) and placed in parallel to the electrodes. Then, carrots were subjected to 5 pulses of 3.5 kV cm^{-1} (0.61 kJ kg^{-1}) and were stored at 4 ºC for 24 h. Treatment conditions were selected based on previous results in which phenolic and carotenoid bioaccessibilities were enhanced in whole carrots (not yet published).

The specific energy input was calculated based on Wiktor et al., (2015) and medium temperature was measured after treatment application to assure that it remained constant after PEF application.

2.4. Preparation of carrot derived products

Untreated and PEF-treated carrots were washed with tap water and the excess was removed with a paper cloth before discarding their top and bottom ends. Two types of puree were obtained: one batch from untreated (U) and other from PEF-treated carrots (PEF/-). Purees were prepared by mixing approximately 500 g of 1-cm thick carrot slices with water (1:1) (w/w) in a food processor (Taurus Mycook) operated in crushing function at full power in two 10-second intervals. To prepare oil-added carrot purees, extra virgin olive oil (Borges Branded Foods,

S.L.U., Tàrrega, Lleida) was added (5 % w/w) and the homogenates were stirred for 15 min at 8000 rpm with an Ultra-Turrax IKA equipped with a 3-blade stirring rod.

Two types of carrot juices were obtained from approximately 500 g of 1-cm thick carrot slices using a cold blender (Imetec Succovivo SJ1000 accoupled with a filter of 0.4 mm). One batch was obtained from untreated carrots and another from PEF-treated carrots.

The resulting purees and juices were divided into two similar fractions. One was subjected to a thermal treatment (U/T or PEF/T) and the other served as a control (U or PEF/-) for the thermally-treated products.

Thermal treatment was applied in order to inactivate pectinmethylesterase and peroxidase activities (Balogh, Smout, Nguyen, Van Loey, & Hendrickx, 2004; Houben, Jamsazzadeh Kermani, Van Buggenhout, Van Loey, & Hendrickx, 2014; Soysal & Söylemez, 2005) thus obtaining stable products. Carrot purees or juices (200 g) were packed in re-sealable polyethylene bags (20 × 15 cm) and heated in a water bath for 10 min at 70 °C. Product temperature was monitored during treatment to assure that purees/juices did not exceed 70 ºC. Thereafter, purees were cooled under a constant flow of cold water for 3 min. Aliquots (20 mL) of non-digested fractions were stored at -40 ºC until extraction and analysis of carotenoids was performed. Then, samples were freeze-dried in order to extract phenolic content. Additional aliquots (20 mL) were subjected to an *in vitro* digestion to determine their carotenoid and phenolic contents in digesta.

2.5. Evaluation of quality attributes

Colour. Colour was evaluated by measuring the CIEL*a*b* parameters with a colorimeter (Minolta CR-400, Konica Minolta Sensing, INC., Osaka, Japan). Total colour difference (ΔE) was also calculated by Eq. (1).

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{0.5} \quad (1)$$

where L^*_0, a^*_0 and b^*_0 refer to untreated carrot products and L*, a* and b* correspond to data collected after treatments.

pH. pH was assessed in products by using a pH-meter (Crison Instruments S.A., Alella, Barcelona, Spain).

Total soluble solids (TSS). TSS was measured using a refractometer (Atago Company LTD., Tokyo, Japan) and expressed as % of total soluble solids.

2.6. Particle size distribution

A Mastersizer 3000™ (Malvern Instruments Ltd., Worcestershire, UK) was used to measure particle size distribution of juices and purees. Results were expressed in terms of volume and surface diameter, D [4, 3] and D [3, 2], respectively. The refractive index of water was 1.33 and particle calculation was set for irregular particles.

2.7. Microstructure

Microstructure was investigated using a light microscope (BX41, Olympus, Göttingen, Germany) equipped with UIS2 optical system. 10 μL drops were mounted on glass slides without staining and were microscopically observed. A general inspection of the samples was made, and representative photos with 10x lens were taken. All images were processed using the instrument software (Olympus CellSense, Barcelona, Spain).

2.8. *In vitro* digestion

The *in vitro* digestion procedure was performed according to the standardized COST Infogest protocol (Minekus et al., 2014), in which electrolyte and enzymatic solutions to simulate the phases of human digestion is described. Digestions were performed in darkness, in absence of oxygen (bottles were flushed with nitrogen gas) in an orbital incubator (Ovan, Badalona, Spain) at 37 °C and 120 rpm. Electrolyte concentrations and enzyme activities were prepared following the indications provided by Minekus et al. (2014) and blank samples consisting in water instead of carrot products, were made in identical conditions.

Oral phase was omitted due to the very short residence times of purees and juices in the oral cavity (Minekus et al., 2014). Then, the gastric phase started by adding 20 mL of simulated gastric fluid (pH 3 and 37 ºC) and pepsin to 20 g of juice/puree. This mixture was incubated at 37 °C for 2 h in agitation. The duodenal phase was initiated by inserting a cellulose-membrane dialysis bag (molecular weight cut-off 12,000 Da, Sigma-Aldrich), which contained simulated intestinal fluid (pH 7 and 37 ºC). This dialysis bag simulates the intestinal epithelium and it harbor phenolic compounds released from matrix (bioaccessible fraction) (Minekus et al., 2014; Ribas-Agustí et al., 2019b). After 30 min of incubation, pH was adjusted to 7 and a solution containing simulated intestinal fluid (pH 7 and 37 ºC), bile extract, pancreatin (and lipase in case of purees

containing oil) was added and the mixture was further incubated for 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 at 5000 g for 15 min at 4 °C (Brodkorb et al., 2019; Eriksen, Luu, Dragsted, & Arrigoni, 2017; Svelander, Lopez-Sanchez, Pudney, Schumm, & Alminger, 2011) and supernatant was also collected. Resulting digested fractions were freeze-dried and stored at -40 ºC until analysis.

Phenolic and carotenoid content of olive oil was determined. It was composed by 0.86 mg/100 g FW of α-carotene, 2.59 mg/100 g FW of β-carotene, 0.18 mg kg^{-1} DW of coumaric acid, 0.02 mg kg^{-1} DW of caffeic acid, 0.02 mg kg^{-1} DW of ferulic and isoferulic acids.

2.9. Carotenoids determination

2.9.1. Carotenoids extraction

Carotenoids were extracted following the method described by Sadler, Davis, & Dezman, (1990), with slight modifications. Extraction solution (50 mL) composed by hexane : acetone : ethanol (50 : 25 : 25) and 1 $g \cdot L^{-1}$ BHT was added to carrot puree (2 g) or juice (1 g) and were stirred for 20 minutes. Then, 15 mL of NaCl [10 % (w/v)] solution was added and the samples were stirred for 10 additional minutes. Samples were left to stand for ≥ 3 minutes, and the upper organic phase was microfiltrated across a nylon filter (0.45 μm, ø 13 mm, Labbox Labware S.L., Barcelona) and analysed by High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD).

Recovery of the carotenes from the micellar digested fraction was performed by adding 5 mL of the extraction solution 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 at 4000 g for 5 min (Svelander, 2011). An aliquot of the upper organic phase was microfiltrated across a nylon filter (0.45 μm, ø 13 mm, Labbox Labware S.L., Barcelona) and analysed by HPLC using the same method as for non-digested fractions.

All extractions were performed in duplicate, and samples were protected from light throughout extraction and analysis to avoid carotenoid degradation and isomerization.

2.9.2. Identification and quantification of carotenoids by HPLC-DAD

Carotenoids were quantified by HPLC-DAD, following a procedure validated by Cortés, Esteve, Frígola, & Torregrosa, (2004). HPLC system 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). An aliquot of 20 µL of the extracted samples were injected and carotenoids were separated using a reverse-phase C_{18} Spherisorb ODS2 (5 µm) stainless steel column (4.6 mm × 250 mm). The mobile phase consisted of: (A) methanol/ammonium acetate 0.1 M, (B) milli-Q water, (C) methyl tert-butyl ether and (D) methanol. The flow rate was fixed at 1 mL min^{-1} and the total run time was 60 min. The column was set at 30 ºC, while sample amber vials on the auto sampler were preserved at 4 ºC. Identification was carried out by UV–vis spectral data and their retention times (Cortés et al., 2004; Mouly, Gaydou, & Corsetti, 1999). Carotenoids were quantified by using calibration curves and integrating peak areas. Results were expressed on a fresh weight basis.

2.10. Phenolic compounds determination

2.10.1. Phenolic compounds extraction

Phenolic compounds were extracted from freeze-dried non-digested or digested carrot puree/juice (0.2 g). For non-digested juices and digested fractions of purees and juices 1 mL of methanol (80:20 v/v) was added, whereas 1.5 mL were needed for non-digested purees. Samples were vortexed for 1 minute and then centrifuged (16209 g, 15 min, 4 ºC). The clear supernatant was microfiltered using polyvinylidene difluoride (PVDF) filters (0.2 µm) (Scharlab, Barcelona, Spain) prior to injection to the chromatographic system.

2.10.2. Identification and quantification of phenolic compounds by Ultra-Performance™ Liquid Chromatography (UPLC-MS/MS)

Phenolic compounds and their generated metabolites were determined in methanolic extracts obtained from freeze-dried non-digested or digested fractions. 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 C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C_{18} (2.1 × 5 mm, 1.7 µm). During the analysis, the column was kept at 30 °C, and the flow rate was 0.3 mL min^{-1}. Mobile phases were acetic acid (0.2 %) and acetonitrile. 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). The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1 software.

2.11. Bioaccessibility calculation

The bioaccessibility of each individual compound was determined using Eq. (2) and results were expressed as percentage.

$$\text{Bioaccessibility (\%)} = \frac{CC_{digested}}{CC_{non-digested}} \times 100 \quad (2)$$

where $CC_{digested}$ corresponds to the overall concentration of each compound in the absorbable fraction and $CC_{non\text{-}digested}$ is the concentration in non-digested samples.

Carotenoid bioaccessibility was calculated referred to the concentration found in the digested micellar fraction, whereas phenolic compounds bioaccessibility was calculated in reference to their concentration in the dialyzed digested fraction.

2.12. Statistical analysis

Statistical analyses were carried out using the SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA). Three different replicates were submitted to each assayed treatment condition and each analysis was conducted thrice, excepting extraction and determination of phenolics and carotenoids, which were conducted twice. Results were reported as the mean ± standard deviation and were subjected to an analysis of variance (ANOVA) followed by Tukey *post hoc* test to establish statistical differences among treatments. A two-way ANOVA was carried out for establishing differences between derived products. In case that results showed no homogeneity in their variance, they were subjected to ANOVA on ranks by Kruskal Wallis test. The statistical significance level was set up at $p < 0.05$.

3. Results and discussion

3.1. Quality attributes

Quality attributes from differently treated carrot juices and purees are shown in **Table 5.1**. TSS and pH were not generally influenced neither by PEF treatment nor by further processing conditions, although colour was differently affected. PEF application to carrot matrix did not cause significant changes in L* nor a* values of any derived product, although b* was lower in juices. Hence, ΔE value of PEF/- purees was lower than 2, whereas that of PEF/- juices reached 3.8. In the latter case, colour changes would be visually apparent, as ΔE > 2 have been reported to be noticeable by consumers (Barba, Esteve, & Frígola, 2012). Similar results were reported by Aguiló-Aguayo et al., (2014) and Xiang et al., (2013) in PEF-treated carrot purees and juices, respectively.

Oil addition and thermal treatment also affected colour of different products. On the one hand, oil addition increased values of CIEL*a*b* coordinates compared to purees without added oil. These differences would be detectable by consumers, given that ΔE values were between 3.3 and 4.7. On the other hand, temperature application did not affect L* nor b* in any derived product. However, thermally-treated juices (U/T and PEF/T) showed higher a* values than those untreated and ΔE > 2. Moreover, U/T and PEF/T oil-added purees had lower a* than untreated ones. Colour differences could be associated to the disruption of cells and breakage of the chromoplast carotenoid–protein complexes, leading to release of carotenoids (Aguiló-Aguayo et al., 2014; Yan et al., 2017). Likewise, non-enzymatic Maillard reactions could also be responsible of colour changes after thermal treatments, since carrot juice had greater sugar contents and high temperatures could favour this reaction (Kebede et al., 2014). In addition, Mutsokoti, Panozzo, Loey, & Hendrickx, (2016) demonstrated that carotenoid transfer to oil was enhanced by thermal treatments although carotenoid degradation was also more pronounced in presence of oil (Knockaert, et al., 2012), which could explain decreases in a* values.

3.2. Particle size distribution

Mean particle size of differently treated products is displayed in **Table 5.1**. Purees were constituted by 3 – 5 % of particles smaller than 100 μm, which may suggest that they were mainly formed by cell fragments or single cells, since carrot cells size has been reported to average ca. 125 μm (Lemmens et al., 2010). On the other hand, juices had between 13 – 17 % of particles below 100 μm, which is probably due to cell clusters were removed during juicing and they were mainly composed of small fragments of disrupted tissue and cells.

PEF treatment before further processing did not cause significant changes in mean particle size of any product. Untreated juices and those PEF/- were characterized by D [3, 2] of 66 ± 17 μm and 74.3 ± 0.9 μm, respectively. Otherwise, untreated and PEF/- purees showed D [3, 2] = 207 ± 48 μm and 183 ± 34 μm, respectively. Some authors have reported that PEF treatment may facilitate juice-pressing due to structural rearrangements. Thus, derived products with smaller particles than those untreated could be obtained (Rybak et al., 2020). However, results obtained in this work suggest that PEF treatment was not intense enough to cause lower resistance to mechanical load and promote changes in particle size. Similarities in particle size distribution between PEF/- and untreated products could indicate that they will have similar stability during storage.

Thermal treatments (U/T and PEF/T) caused changes in the area-based mean diameter of juices and purees without oil, whereas those with oil added were not affected. These purees showed higher D [3, 2] values (472 ± 20 and 431 ± 53 μm) compared to that of untreated purees (207 ± 48 μm). This increase in the size of small particles after thermal treatments has been reported in thermally-treated tomato suspensions (Li et al., 2016), which was attributed to the swelling of cells or formation of aggregates from cellular components (Palmero et al., 2014). However, it could also mean that the largest cell clusters were disintegrated in smaller cell aggregates, which increased D [3, 2]. On the other hand, juices (U/T and PEF/T) had between 29 – 34 % of particles below 100 μm and D [3, 2] in the range of 32 – 46 μm. These results indicate that thermal treatments increased the number of small particles, reducing the mean particle size (D [3, 2]). This was also observed in pasteurized orange juices (Stinco et al., 2020; Velázquez-Estrada, Hernández-Herrero, Guamis-López, & Roig-Saguès, 2019). It has been reported that thermal treatments between 65 to 95 °C cause disruptions between chromoplasts membrane structure and/or carotenoid protein interactions, which could cause a decrease in the particle size of juices (Greve, McArdle, Gohlke, & Labavitch, 1994; Palmero et al., 2014).

On the other hand, particle sizes (D [4, 3] and D [3, 2]) of added-oil purees were significantly lower than that of purees without oil. Oil-added purees had between 29 – 37 % of particles below 100 μm. These values indicate that purees would be mainly formed by cell fragments or single cells together with oil droplets (20 μm) (Lemmens et al., 2010). Any treatment affected oil-added purees particle size. Probably, these results were highly influenced by presence of oil droplets, which likely has a major influence on particle size distribution and masked changes in the particle size of purees.

3.3. Microstructure

Microstructure of juices, purees and oil-added purees is shown in **Figure 5.1**. Both purees showed clusters of whole cells with carotenoids inside. On the other hand, whole cells were rarely detected in juices (**Figure 5.1**) given that cells and chromoplasts are probably comminuted during processing. Juices are mainly composed by cloud particles of different densities formed by chromoplasts fragments and carotenoids, which was verified through isopycnic gradient centrifugation by Marx, Stuparic, Schieber, & Carle, (2003).

The application of PEF to whole carrots did not cause significant changes in the microstructure of juices obtained from them. However, PEF/- purees showed some starch grains inside cells that were absent in untreated purees. Gómez Galindo et al., (2009) demonstrated that PEF can trigger changes in hexose pool due to stress induction. Specifically, it could have affected AGPase activity, which is involved in starch biosynthesis, or in starch-degrading enzymes (Blenkinsop, Yada, & Marangoni, 2010). To the best of our knowledge, microstructural studies of derived products obtained from PEF-treated matrices are limited. However, some authors have observed irregular cell walls, such as folds and loss of smoothness in PEF-treated whole tomatoes and carrots (Jayathunge et al., 2017; Wiktor et al., 2018).

Thermal treatment of the derived products led to thin cell walls in purees, probably as a consequence of depolymerization and pectin degradation (Ribas-Agustí, Buggenhout, Palmero, Hendrickx, & Loey, 2014). Additionally, temperature likely favours interactions between dissolved particles (Augusto, Ibarz, & Cristianini, 2012), which would explain the increases in particle size, D [3, 2] (**Table 5.1**). On the other hand, thermally-treated juices had greater content of small particles than those untreated or PEF/-. Temperatures between 65 – 95 ºC are able to break carotenoid-chromoplasts complexes (Greve, McArdle, Gohlke, & Labavitch, 1994), which could explain the decrease in particle size (**Table 5.1**) and increased carotenoid release (**Figure 5.1**).

Table 5.1. Quality attributes and particle size of untreated carrot purees and juices (U), those obtained from PEF-treated carrots (PEF/-) and those thermally-treated (U/T and PEF/T).

Product	Treatment	L*	a*	b*	ΔE	pH	TSS (%)	D [4, 3] (μm)	D [3, 2] (μm)
Puree	U	41.3 ± 0.3 aA	11.0 ± 0.3 aA	24.4 ± 0.2 aA	-	6.4 ± 0.1 abcA	3.6 ± 0.3 aA	596 ± 12 aA	207 ± 48 aA
	PEF/-	40.5 ± 0.1 aA	10.3 ± 0.1 aA	23.1 ± 0.2 aA	1.6 ± 0.2 aA	6.5 ± 0.1 bA	3.4 ± 0.2 aA	589 ± 16 aA	183 ± 34 aA
	U/T	42.0 ± 0.2 aA	10.4 ± 0.5 aA	24.3 ± 0.5 aA	1.3 ± 0.1 aA	6.1 ± 0.1 dA	3.7 ± 0.0 aA	608 ± 11 aA	472 ± 20 bA
	PEF/T	41.0 ± 0.4 aA	9.9 ± 0.5 aA	23.1 ± 0.8 aA	1.8 ± 0.7 aA	6.3 ± 0.0 cA	3.5 ± 0.3 aA	601 ± 12 aA	431 ± 53 bA
Oil-added puree	U	56.0 ± 1.7 a′B	15.2 ± 0.7 a′B	44.8 ± 1.9 a′B	-	6.3 ± 0.0 a′A	4.0 ± 0.6 a′A	449 ± 41 a′B	15 ± 3 a′B
	PEF/-	55.7 ± 0.3 a′B	14.5 ± 0.5 a′b′B	45.6 ± 3.9 a′B	3.3 ± 2.4 a′A	6.4 ± 0.1 a′A	3.8 ± 0.4 a′A	422 ± 15 a′B	13.8 ± 2.0 a′B
	U/T	55.3 ± 0.7 a′B	13.8 ± 0.4 b′B	45.6 ± 3.9 a′B	3.6 ± 1.9 a′B	6.0 ± 0.0 b′A	4.2 ± 0.4 a′A	460 ± 30 a′B	20 ± 4 a′B
	PEF/T	55.2 ± 0.7 a′B	12.4 ± 0.9 d′B	47 ± 3 a′B	4.7 ± 2.1 a′B	6.1 ± 0.0 c′B	3.9 ± 0.1 a′A	408 ± 39 a′B	16 ± 4 a′B
Juice	U	43.0 ± 0.5 a″	15.8 ± 0.7 a″b″	29.2 ± 0.7 a″	-	6.2 ± 0.1 a″	8.2 ± 0.5 a″	487 ± 43 a″	66 ± 17 a″ b″
	PEF/-	41.3 ± 0.3 a″	13.4 ± 0.7 a″b″	26.9 ± 0.7 b″	3.8 ± 1.9 a″	6.2 ± 0.1 a″	8.2 ± 06 a″	499 ± 4 a″	74.3 ± 0.9 a″
	U/T	40.7 ± 2.3 a″	18.6 ± 1.6 c″	28.1 ± 1.3 a″b″	4.8 ± 1.7 a″	6.1 ± 0.0 a″	7.7 ± 0.4 a″	359 ± 48 b″	32 ± 2 b″
	PEF/T	41.7 ± 0.3 a″	17.9 ± 0.6 c″	27.7 ± 0.7 a″b″	2.86 ± 0.2 a″	6.3 ± 0.2 a″	7.5 ± 0.2 a″	408 ± 61 b″	46 ± 3 b″

Values are means ± standard deviation. Different letters in the same column within the same product indicate significant ($p < 0.05$) differences among treatments (puree: lowercase letters; puree with oil: lowercase letters′, juice: lowercase″). Different uppercase letters in the same row indicate significant differences between both purees.

Oil-added purees showed lipid droplets surrounding carrot clusters (**Figure 5.1**). Oil was especially internalized in thermally-treated and PEF/- purees, given that disruption of cell membranes may facilitate the efflux and influx of intra- and extracellular content. These results have been previously reported in excipient emulsions of olive oil and tomato by Li et al., (2016), who attributed this lipid entry to capillary forces generated by pores in tissues (Zhang et al., 2015).

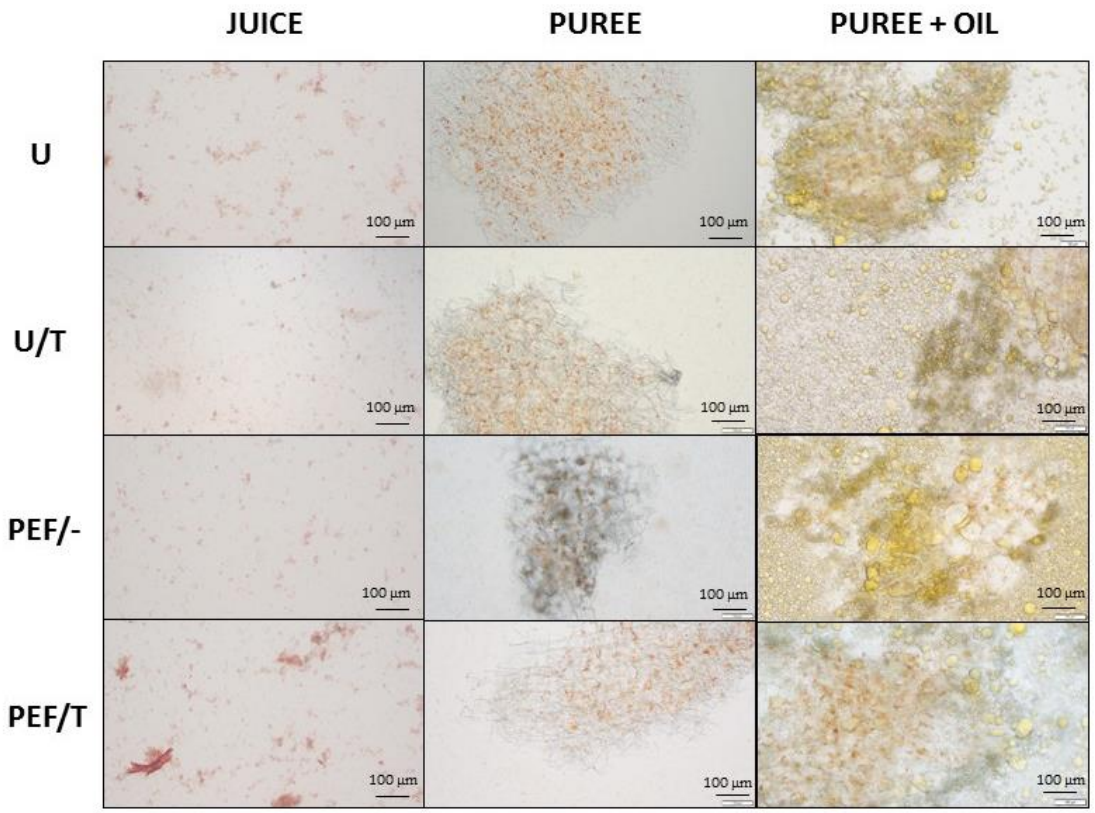


Figure 5.1. Representative light micrographs of juices and purees obtained from untreated carrots (U), obtained from PEF-treated carrots (PEF/-) and thermally-treated (U/T and PEF/T).

3.4. Carotenoid content

Carotenoid content from differently treated products is shown in **Figure 5.2**. PEF application to whole carrots did not affect content nor carotenoid profile, although further processing caused a significant effect in carotenoid concentration. Hence, different carotenoid content was obtained depending on the type of derived product, but neither thermal treatment nor oil addition caused significant changes. Carrot juices had higher carotenoid contents (43.4 ± 7 – 48 ± 8 mg/100 g FW) than both types of puree (20.7 ± 2.5 – 23.6 ± 1.8 mg/100 g FW). However, these differences were mainly related to dilution during puree preparation. Obtained results are in accordance to Hedrén, Diaz, & Svanberg, (2002), given that mechanical processing was more determinant to the release of carotenoids than thermal treatment or oil addition.

Main carotenoids present in carrot-derived products were α-carotene and β-carotene. These results are in agreement to those previously reported (Knockaert et al., 2011; Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012), although the presence of lutein in carrots was also found in some studies. Panozzo et al., (2013) hypothesised that crystalloid chromoplasts were easily disrupted by mechanical processing than globular chromoplasts, in which lutein is usually dissolved. In addition, lutein is more susceptible to degradation due to the presence of oxygen in their chemical structure (Morales-De La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011). Hence, it could be degraded during mechanical processing or be entrapped in chromoplasts.

Carotenoid contents of derived products obtained from PEF-treated whole commodities have been scarcely studied. However, increases in carotenoid content of purees and juices obtained from PEF-treated tomatoes have been reported (González-Casado et al., 2018; Jayathunge et al., 2017). Such increment was attributed to two main causes: 1) their accumulation in the whole product resulting from the induction of a stress defence response and 2) their better extractability due to electropermeabilization. On the other hand, Rybak et al., (2020) observed a decreased content in juices obtained from PEF-treated peppers (3 kJ kg^{-1}), which was attributed to promoted oxidation or isomerization reactions. Therefore, our results suggest that selected PEF conditions did not induce carotenoid biosynthesis during 24 h of storage nor improve their extractability in derived products.

Thermal treatment did not enhance carotenoid extractability nor induce their degradation in any derived product. Similar results were obtained in thermally-treated oil-added carrot purees (Knockaert, Lemmens, et al., 2012). However, decreases in carrot juices (Kim, Park, Cho, & Park, 2001) and increases in carrot purees (Patras, Brunton, Da Pieve, Butler, & Downey, 2009) have been reported. Differences between our results and those previously mentioned are likely due to variations in processing parameters (time or temperature) or derived products preparation procedures. These processing conditions probably also caused differences in particle size, structural properties, or enzyme activities, which are closely related to carotenoid extractability or degradation.

3.5. Carotenoid bioaccessibility

Carotenoids bioaccessibility was affected by PEF treatment and further processing. Mechanical processing was the main factor that influenced bioaccessibility, followed by oil addition and thermal treatment (**Figure 5.2**). The highest bioaccessibility was obtained in oil-added purees (5.3 %), whereas purees (2.6 %) and juices (0.4 %) had lower bioaccessibility. The

application of PEF to carrots did not affect total bioaccessibility in any derived product. Generally, thermal treatments were those that led to further enhancement of total bioaccessibility: oil-added purees (10.7 %) > purees (3.8 %) > juices (0.9 %). Regarding the bioaccessibility of individual carotenoids, α-carotene and β-carotene similarly increased in any of the studied products as a consequence of the application of thermal treatments, whereas PEF only caused a decrease in β-carotene bioaccessibility from juices (**Figure 5.2**).

Regarding results obtained in derived products obtained from PEF-treated carrots, they contrast with those obtained by González-Casado et al., (2018). Authors reported increases in total carotenoids and β-carotene bioaccessibility of purees obtained from PEF-treated tomatoes, which were attributed to electropermeabilization and better carotenoids release. The initial content of such tomato purees was considerably higher than in those untreated, which could make a difference with our study since content in non-digested products was similar regardless the applied treatment. In addition, observed decrease in β-carotene bioaccessibility from juices was also reported by Bot et al., (2018) in PEF-treated tomato chromoplast fractions. This was attributed to induced modifications in carotenoid-protein complexes, which limit their bioaccessibility. Finally, some authors have also suggested that released carotenoids can be broken-down during digestion to non-detected metabolites (e.g. oxidation products) (Blanquet-Diot, MahaSoufi, Rambeau, Rock, & Alric, 2009).

Carotenoids are generally located inside chromoplasts or bound to membrane (Faulks & Southon, 2005). Hence, chromoplasts are probably comminuted during juicing or blending, which make carotenoids more available to be absorbed when particle size decreases as a consequence of processing (**Table 5.1**). Apart from particle size decrease, carotenoids bioaccessibility also depends on their chemical structure, interactions with other macromolecules, micellarization, content and characteristics of pectin (Gence, Servent, Poucheret, Hiol, & Dhuique-Mayer, 2018). The increase in bioaccessibility after thermal treatments has been related to carotenoids isomerization (Marx et al., 2003), given that *cis* isomers are better assimilated than *trans* (Meléndez-Martínez, Paulino, Stinco, Mapelli-Brahm, & Wang, 2014). On the other hand, thermal processing can degrade pectin and may improve carotenoids bioaccessibility (Lemmens, Van Buggenhout, Oey, Van Loey, & Hendrickx, 2009; Ribas-Agustí et al., 2014), given that a high pectin content would entrap carotenoids or act as a barrier for lipase (Palmero et al., 2016) hindering their micellarization.

Solubilization of carotenoids into micelles is another critical step in carotenoids absorption. The low amount of lipids in purees and juices could hinder their micellarization, given that carotenoids are not water-soluble. Hence, the presence of lipids facilitates carotenoids

transference to micelles (Huo, Ferruzzi, Schwartz, & Failla, 2007). Obtained results indicate that the application of a thermal treatment to oil-added purees caused the highest bioaccessibility. Previous studies have showed that thermal-treated carrot cell clusters (95 – 110 °C) improved carotenoids transfer to oil (Palmero, Panozzo, Simatupang, Hendrickx, & Loey, 2014b).

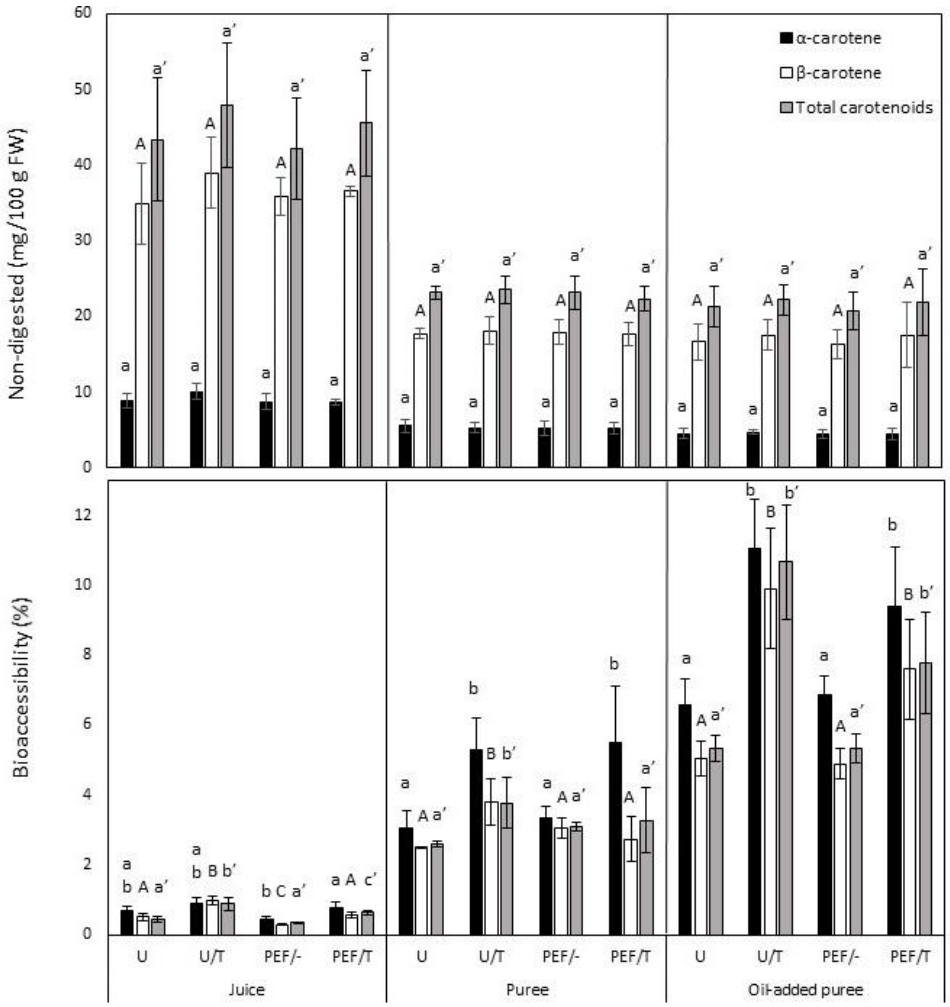


Figure 5.2. Carotenoid content and bioaccessibility of untreated purees and juices (U), those obtained from PEF-treated carrots (PEF/-) and those thermally-treated (U/T and PEF/T). Values are means ± standard deviation. Different letters within the same product and compound indicate significant ($p < 0.05$) differences among treatments (α-carotene: lowercase letters; β-carotene: uppercase letters; total carotenoids: lowercase′). Olive oil was composed by 0.86 mg/100 g FW of α-carotene and 2.59 mg/100 g FW of β-carotene.

3.6. Phenolic content

Phenolic content was affected by both PEF treatment and further processing (**Table 5.2**). Carrot juices showed the highest phenolic content (322 ± 56 mg kg^{-1} DW), whereas oil-added

purees (81 ± 36 mg kg^{-1} DW) and purees (62 ± 23 mg kg^{-1} DW) exhibited similar contents. PEF treatment applied to whole carrots generally did not affect phenolic content of both types of puree, although it decreased the content in juices (38.5 %). Despite this reduction, phenolic content in juices remained higher than in purees. On the other hand, the total phenolic content in purees without added oil was doubled when a thermal treatment was applied, whereas juices and oil-added purees were not significantly affected.

The main phenolic compounds found in derived products were hydroxycinnamic acids, namely 5-caffeoylquinic acid, coumaroylquinic acid or 5-feruloylquinic acid (**Table 5.2**). These results are in accordance to previous studies performed in whole carrots (Becerra-Moreno et al., 2015; López-Gámez et al., 2020b) or juices (Quitão-Teixeira, Odriozola-Serrano, Soliva-Fortuny, Mota-Ramos, & Martín-Belloso, 2009; Szczepańska, Barba, Skąpska, & Marszałek, 2020). Extractability of phenolic compounds may be enhanced in juices due to their lower particle size (**Table 5.1**). Likewise, differences in composition between purees and juices could also be related to mechanical processing. The procedure applied to obtain juices may have enhanced the extraction of compounds tightly linked to cell walls whereas cells from puree were not totally disrupted. In addition, it has been reported that low dietary fibre content in juices is also beneficial for releasing phenolic compounds (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011).

PEF treatment caused different effects depending on the type of derived product and phenolic chemical structure. Hence, the application of PEF to whole carrots did not affect most of compounds from both purees obtained from such carrots, although juices had lower content of coumaric, ferulic and caffeic acid derivatives [e.g. 5-caffeoylquinic (68.5 %)] (**Table 5.2**). Selected PEF treatment was based on previous results obtained by López-Gámez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, (2020a) in which total phenolic content of whole carrots was enhanced. Nevertheless, such increase was not observed in derived products obtained from PEF-treated whole carrots. This may suggest that mechanical processing could affect phenolic stability or favour their interactions with cell wall debris, which would hinder their extractability.

Results indicate that individual compounds were also differently affected by thermal treatment, depending on their structure and derived product. Hence, caffeic acid derivatives were strongly enhanced in purees [e.g. 5-caffeoylquinic acid (231 %)], although caffeic acid decreased in those with oil added (**Table 5.2**). Juices showed increases in some caffeic acid derivatives [e.g. 3- caffeoylquinic acid (400 %)], but lower content was observed in some ferulic acid derivatives (e.g. 5-feruloylquinic acid). Increases in phenolic content have been previously reported in thermally-treated fruit juices (e.g. caffeoyl glucoside or caffeic acid) (He et al., 2016; Quan et al., 2020; Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015).

This was attributed to their better release due to cell wall disruptions, which is in agreement with the lower particle sizes observed in juices (**Table 5.1**). The higher content in caffeic acid derivatives could result from the partial inactivation of enzymes responsible of phenolic degradation (e.g. PPO) (Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015).

3.7. Phenolic bioaccessibility

Phenolic compounds bioaccessibility was affected by PEF treatment application to whole carrots and further processing. Bioaccessibility differed depending on the evaluated derived product. Carrot purees had the highest bioaccessibility (52 %) followed by oil-added purees (31%) and juices (16.1 %) (**Table 5.3**). PEF application to carrots caused a large enhancement of bioaccessibility, hence reaching 100 % in purees, whereas that of juices and oil-added purees was not affected. On the other hand, thermal treatment did not significantly influence total bioaccessibility in any derived product (**Table 5.3**).

Individual compounds were differently affected depending on their chemical structure and processing. PEF treatments increased bioaccessibility of most ferulic (e.g. ferulic acid glucoside) and caffeic acid derivatives (e.g. 5-caffeoylquinic acid) in purees, although some decreases were also observed (e.g. coumaric acid). Likewise, oil-added purees were similarly affected as those without lipids, but the bioaccessibility of certain compounds decreased [e.g. 5-caffeoylquinic acid or feruloylquinic acid derivative (2)]. On the other hand, phenolics bioaccessibility in juices obtained from PEF-treated carrots was not affected. Limited information about phenolic bioaccessibility in juices and purees obtained from PEF-treated matrices is available in literature. Generally, phenolic compounds should be easily released from juices, due to their low content in dietary fibre (Palafox-Carlos et al., 2011). However, obtained results are controversial since bioaccessibility in juice was the lowest (**Table 5.3**). Juices had lower particle size (D [4, 3] = 487 μm) than purees (D [4, 3] = 596 μm), which favoured phenolic release from matrix. Phenolic compounds are likely more exposed to degradation or entrapment by other macromolecules during digestion in juices than in purees, hence limiting their bioaccessibility (Jakobek, 2015). PEF application to carrots strongly enhanced bioaccessibility in purees without oil, whereas treatment was not effective for juices or oil-added purees. Despite no correlation was found between particle size and bioaccessibility values, cell permeability changes could have caused a better release and dialysis of phenols in purees. Besides, variations caused by PEF in the initial phenolic content of carrots would directly affect bioaccessibility in derived products.

Table 5.2. Phenolic content in untreated purees and juices (U), those obtained from PEF-treated carrots (PEF/-) and those thermally-treated (U/T and PEF/T).

Phenolic compounds	Puree				Oil-added puree				Juice			
	U	PEF/-	U/T	PEF/T	U	PEF/-	U/T	PEF/T	U	PEF/-	U/T	PEF/T
Coumaric acid	5.1 ± 0.2 a	7.2 ± 1.8 b	2.9 ± 0.5 c	1.03 ± 0.2 d	4.3 ± 0.6 A	2.8 ± 0.3 BC	1.7 ± 0.0 C	2.2 ± 0.7 C	0.7 ± 0.1 a′	0.8 ± 0.0 a′	0.7 ± 0.1 a′	0.8 ± 0.1 a′
Coumaroylquinic acid	17 ± 8 a	8 ± 3 ab	9.0 ± 2.5 ab	5.9 ± 1.2 b	3.2 ± 0.6 A	3.6 ± 0.7 A	6.2 ± 2.2 A	10 ± 4 A	15 ± 7 a′	6.0 ± 0.4 b′	19 ± 3 a′	6.4 ± 0.5 b′
Coumaric acid and its derivatives	**22 ± 8 a**	**15 ± 5 ab**	**12 ± 3 ab**	**6.9 ± 1.5 b**	**7.6 ± 0.8 A**	**6.4 ± 1.0 A**	**7.9 ± 2.2 A**	**12 ± 4 A**	**15 ± 7 a′**	**6.8 ± 0.4 b′**	**19 ± 4 a′**	**7.2 ± 0.5 b′**
Caffeic acid	1.5 ± 0.9 a	0.8 ± 0.1 a	0.9 ± 0.3 a	0.4 ± 0.1 b	1.7 ± 0.6 A	0.6 ± 0.1 B	0.5 ± 0.2 B	0.4 ± 0.1 B	1.3 ± 0.2 a′	0.9 ± 0.1 a′	2.5 ± 0.3 b′	1.1 ± 0.1 a′
Caffeic acid arab/xiloside	nd [1] a	nd [1] a	nd a	nd a	nd [1] A	nd [1] A	nd [1] A	nd [1] A	0.1 ± 0.0 a′	0.1 ± 0.0 a′	0.3 ± 0.1 b′	0.1 ± 0.0 a′
Caffeoylshikimic acid	nd [1] a	nd [1] a	nd [1] a	nd [1] a	nd [1] A	nd [1] A	nd [1] A	nd [1] A	0.1 ± 0.0 a′	0.02 ± 0.0 b′	0.1 ± 0.0 a′	0.02 ± 0.0 b′
3-caffeoylquinic acid	nd [1] a	nd [1] a	0.2 ± 0.1 b	0.2 ± 0.0 b	nd [1] A	nd [1] A	nd [1] A	0.1 ± 0.0 A	0.2 ± 0.0 a′	0.1 ± 0.0 a′	1.0 ± 0.1 b′	0.6 ± 0.1 c′
5-caffeoylquinic acid	32 ± 17 a	15.1 ± 1.0 a	106 ± 23 b	85 ± 13 b	67 ± 35 A	65 ± 20 A	79 ± 6 A	59 ± 19 A	264 ± 47 a′	83 ± 7 b′	264 ± 32 a′	92 ± 7 c′
4-caffeoylquinic acid	nd [1] a	nd [1] a	1.7 ± 0.7 b	1.6 ± 0.1 b	0.2 ± 0.1 A	0.3 ± 0.1 A	0.8 ± 0.1 B	0.7 ± 0.1 B	0.8 ± 0.1 a′	0.3 ± 0.1 a′	7.7 ± 1.2 b′	3.4 ± 0.3 c′
Dicaffeoylferuoylquinic acid	nd [1] a	nd [1] a	0.3 ± 0.1 b	0.2 ± 0.0 b	nd [1] A	nd [1] A	nd [1] A	nd [1] A	0.3 ± 0.1 a′	0.1 ± 0.0 b′	0.6 ± 0.1 c′	0.1 ± 0.0 b′
Caffeoylferuoylquinic acid	0.4 ± 0.1 a	0.4 ± 0.1 a	0.3 ± 0.1 a	0.4 ± 0.1 a	0.2 ± 0.1 A	0.1 ± 0.0 A	0.2 ± 0.1 A	0.2 ± 0.1 A	0.3 ± 0.1 a′	0.6 ± 0.1 b′	0.4 ± 0.1 a′	0.6 ± 0.1 b′
Caffeic acid arabinoside glucoside	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	nd [1] a′	0.2 ± 0.0 b′	nd [1] a′	0.2 ± 0.0 b′

Caffeic acid Glu Acetyl glucoside	0.8 ± 0.4 a	0.7 ± 0.2 a	0.8 ± 0.5 a	0.8 ± 0.1 a	0.5 ± 0.0 A	1.0 ± 0.4 A	0.8 ± 0.1 A	1.0 ± 0.1 A	3.8 ± 0.2 a′	4.3 ± 0.2 a′b′	3.3 ± 0.6 a′	4.7 ± 0.4 b′
Caffeic acid and its derivatives	**35 ± 17 a**	**17.2 ± 1.3 a**	**111 ± 23 b**	**89 ± 13 b**	**69 ± 35 A**	**67 ± 20 A**	**81 ± 5 A**	**62 ± 19 A**	**271 ± 48 a′**	**90 ± 7 b′**	**280 ± 34 a′**	**103 ± 7 c′**
Ferulic acid	0.3 ± 0.1 a	0.4 ± 0.1 a	0.4 ± 0.1 a	0.4 ± 0.0 a	0.3 ± 0.0 A	0.2 ± 0.0 A	0.5 ± 0.2 A	0.5 ± 0.1 A	1.2 ± 0.1 a′	1.7 ± 0.1 a′	2.9 ± 0.6 b′	2.6 ± 0.2 b′
Isoferulic acid	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	0.2 ± 0.0 a′	0.1 ± 0.0 b′	0.2 ± 0.1 a′b′	0.1 ± 0.0 b′
3-feruloylquinic acid	nd [1] a	nd [1] a	0.2 ± 0.0 b	0.1 ± 0.0 b	0.1 ± 0.1 A	0.1 ± 0.0 A	0.2 ± 0.0 A	0.1 ± 0.0 A	0.9 ± 0.1 a′	0.5 ± 0.1 b′	0.9 ± 0.1 a′	0.7 ± 0.1 b′
5-feruloylquinic acid	1.3 ± 0.4 a	1.3 ± 0.1 a	2.5 ± 0.5 a	2.4 ± 0.2 a	1.1 ± 0.2 A	1.2 ± 0.1 A	1.3 ± 0.3 A	1.5 ± 0.5 A	13.2 ± 1.9 a′	8.9 ± 0.4 b′	9.7 ± 1.2 b′	9.2 ± 0.8 b′
4-feruloylquinic acid	0.1 ± 0.0 a	0.1 ± 0.0 a	0.3 ± 0.1 a	0.3 ± 0.0 a	0.1 ± 0.0 A	0.1 ± 0.0 A	0.2 ± 0.0 A	0.2 ± 0.0 A	1.7 ± 0.3 a′	1.2 ± 0.0 b′	1.6 ± 0.3 a′	1.6 ± 0.1 a′
Ferulic acid glucoside	nd [1] a	nd [1] a	0.1 ± 0.0 a	0.1 ± 0.0 a	nd [1] A	nd [1] A	nd [1] A	0.5 ± 0.3 B	0.3 ± 0.0 a′	0.3 ± 0.0 a′	0.3 ± 0.1 a′	0.2 ± 0.0 a′
Ferulic acid coumaroyl glucoside	2.0 ± 0.5 a	1.5 ± 0.2 a	1.7 ± 0.4 a	1.4 ± 0.2 a	1.5 ± 0.2 A	1.4 ± 0.8 A	2.2 ± 0.5 A	1.3 ± 0.0 A	3.7 ± 0.4 a′	3.4 ± 0.2 a′	2.5 ± 0.4 b′	1.7 ± 0.1 c′
Ferulic acid caffeoyl glucoside	0.1 ± 0.0 a	0.1 ± 0.0 a	0.2 ± 0.1 a	0.1 ± 0.1 a	0.1 ± 0.0 A	nd [1] A	0.1 ± 0.0 A	0.1 ± 0.0 A	8.6 ± 0.6 a′	4.3 ± 0.3 b′	7.3 ± 1.0 a′c′	7.0 ± 0.5 c′
Feruloylquinic acid derivative	0.9 ± 0.2 a	0.8 ± 0.1 a	0.7 ± 0.3 a	0.8 ± 0.1 a	0.4 ± 0.1 A	0.4 ± 0.0 A	0.4 ± 0.0 A	0.4 ± 0.1 A	2.9 ± 0.3 a′	4.2 ± 0.1 b′	2.5 ± 0.4 a′	2.4 ± 0.2 a′
Feruloylquinic acid derivative (2)	0.2 ± 0.1 a	0.1 ± 0.0 a	0.2 ± 0.1 a	0.1 ± 0.0 a	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	0.1 ± 0.0 A	1.2 ± 0.1 a′	1.3 ± 0.1 a′	0.9 ± 0.1 b′	1.3 ± 0.1 a′
Ferulic acid and its derivatives	**5.1 ± 0.1 a**	**4.4 ± 0.3 a**	**6.3 ± 1.3 a**	**5.9 ± 0.3 a**	**3.9 ± 0.4 A**	**3.7 ± 0.7 A**	**5.1 ± 0.8 A**	**4.8 ± 0.8 A**	**36 ± 4 a′**	**26.8 ± 1.0 b′**	**30 ± 4 b′**	**27.8 ± 1.9 b′**
Total phenolic compounds	**62 ± 23 a**	**37 ± 5 a**	**129 ± 25 b**	**101 ± 14 b**	**81 ± 36 A**	**77 ± 20 A**	**94 ± 6 A**	**79 ± 24 A**	**322 ± 56 a′**	**124 ± 8 b′**	**329 ± 40 a′**	**138 ± 9 b′**

Values are means ± standard deviation. Different letters within the same product and compound indicate significant ($p < 0.05$) differences among treatments (puree: lowercase letters; puree with oil: uppercase letters; juice: lowercase′). Olive oil contained 0.18 mg kg^{-1} DW of coumaric acid, 0.02 mg kg^{-1} DW of caffeic acid, 0.02 mg kg^{-1} DW of ferulic and isoferulic acids. Blank sample did not contain phenolic compounds. nd [1]: not detected.

Thermal treatment effect also depended on food matrix and chemical structure of each compound (He et al., 2016). Generally, they increased bioaccessibility of caffeic and ferulic acid derivatives in all derived products, although some decreases were also obtained in purees [e.g. ferulic acid (23.2 %)] or juices [e.g. ferulic acid (38 %)] (**Table 5.3**). Besides, coumaric acid bioaccessibility decreased in purees by 41.8 % and in juices by 50 %, whereas in oil-added purees bioaccessibility of coumaroylquinic acid was reduced by 50 %. Decreases in particle size or changes in pectin properties could lead to improve bioaccessibility.

To the best of our knowledge, scarce studies about the impact of adding oil in phenolic bioaccessibility from plant-based products has been reported. Our results show that bioaccessibility was differently affected when oil was added. Some authors have reported that the fat content can protect phenols during digestion, improving their bioaccessibility (Ortega, Reguant, Romero, Macía, & Motilva, 2009; Quan et al., 2020) as in the case of feruloylquinic acid derivative (2), although it depends on the chemical structure of such compound. Probably, PEF or thermal treatments caused membrane changes that facilitate phenolic release and interactions with oil droplets. Likewise, released phenols would be more exposed to be entrapped by polysaccharides. Larger molecules with much hydroxyl groups would be more propense to interact and form complexes with other macromolecules, which makes them less bioaccessible than those with low molecular weight (Jakobek, 2015).

Table 5.3. Phenolic bioaccessibility of untreated purees and juices (U), those obtained from PEF-treated carrots (PEF/-) and those thermally-treated (U/T and PEF/T).

Phenolic compounds	Puree				Oil-added puree				Juice			
	U	PEF/-	U/T	PEF/T	U	PEF/-	U/T	PEF/T	U	PEF/-	U/T	PEF/T
Coumaric acid	91 ± 11 a	56 ± 12 b	53 ± 10 b	100 ± 0 a	63 ± 4 A	99.4 ± 1.1 B	79 ± 7 A	77 ± 28 A	100 ± 0 a′	100 ± 0 a′	47 ± 7 b′	45.1 ± 1.1 b′
Coumaroylquinic acid	80 ± 20 a	100 ± 0 b	100 ± 0 b	100 ± 0 b	84 ± 14 A	97.4 ± 2.2 B	42.7 ± 0.4 C	26.3 ± 2.2 D	42 ± 4 a′	35.9 ± 2.1 a′	25 ± 4 b′	50 ± 4 c′
Coumaric acid and its derivatives	**84 ± 18 a**	**96.2 ± 6.5 a**	**100 a**	**100 a**	**74 ± 8 A**	**100 ± 0 B**	**48.8 ± 0.1 C**	**31.2 ± 0.2 D**	**56 ± 6 a′**	**60 ± 3 a′**	**25 ± 4 b′**	**50 ± 4 a′**
Caffeic acid	0 a	0 a	52 ± 32 b	24 ± 4 b	0 A	0 A	43 ± 13 B	24 ± 5 C	0 a′	0 a′	47 ± 7 b′	34 ± 5 b′
Caffeic acid arab/xiloside	0* a	0* a	0 a	0 a	0* A	0* A	0* A	84.4 ± 18.6 B	0 a′	0 a′	23 ± 7 b′	55 ± 9 c′
Caffeoylshikimic acid	0 a	0 a	0 a	0 a	0 A	0 A	0 A	0 A	0 a′	0 a′	0 a′	0 a′
3-caffeoylquinic acid	0* a	0* a	100 ± 0 b	100 ± 0 b	0* A	0* A	0* A	0* A	0 a′	0 a′	0 a′	0 a′
5-caffeoylquinic acid	15 ± 9 a	56 ± 10 b	22 ± 4 a	12 ± 4 a	16 ± 11 A	8.4 ± 1.6 B	29 ± 3 A	28 ± 11 A	0 a′	0 a′	26.1 ± 1.7 b′	23 ± 5 b′
4-caffeoylquinic acid	100 ± 0 a	100 ± 0 a	100 ± 0 a	66 ± 6 b	100 ± 0 A	73 ± 35 B	100 ± 0 A	100 ± 0 A	10.6 ± 1.8 a′	19 ± 5 a′	93 ± 6 b′	84 ± 14 b′
Dicaffeoylferuoylquinic acid	0 a	0 a	0 a	0 a	0 A	0 A	0 A	0 A	0 a′	0 a′	18 ± 5 b′	0 a′
Caffeoylferuoylquinic acid	39 ± 5 a	69 ± 16 b	46 ± 14 a	66 ± 18 ab	48 ± 17 A	48 ± 12 A	36 ± 12 A	25.9 ± 1.7 A	67 ± 5 a′	47 ± 1.7 a′b′	43 ± 13 a′b′	28.7 ± 1.8 b′

Caffeic acid arabinoside glucoside	73 ± 27 a	55.2 ± 2.4 a	62 ± 6 a	48.0 ± 2.4 a	84 ± 27 A	42 ± 28 B	100 ± 0 A	71 ± 22 A	74 ± 29 a′	57 ± 3 a′	86 ± 8 a′	35.8 ± 1.9 a′
Caffeic acid Glu acetyl glucoside	89 ± 19 a	100 ± 0 a	99.1 ± 1.5 a	100 ± 0 a	100 ± 0 A	100 ± 0 A	100 ± 0 A	100 ± 0 A	55 ± 3 a′	45 ± 7 a′	35 ± 9 a′	31.9 ± 0.9 a′
Caffeic acid and its derivatives	**19 ± 9 a**	**66 ± 9 b**	**26 ± 5 a**	**16 ± 4 a**	**20 ± 13 AB**	**11.7 ± 2.3 B**	**33 ± 3 A**	**33 ± 12 A**	**1.3 ± 0.2 a′**	**3.4 ± 0.4 a′**	**29.1 ± 1.9 b′**	**27 ± 5 b′**
Ferulic acid	99 ± 0.7 a	100 ± 0 a	76 ± 24 b	99.2 ± 1.3 a	100 ± 0 A	100 ± 0 A	100 ± 0 A	95.1 ± 8.5 A	100 ± 0 a′	100 ± 0 a′	68 ± 15 b′	45 ± 6 c′
Isoferulic acid	100 ± 0 a	100 ± 0 a	98 ± 4 a	100 ± 0 a	100 ± 0 A	100 ± 0 A	100 ± 0 A	100 ± 0 A	70 ± 16 a′	100 ± 0 b′	91.3 ± 15 b′	99.2 ± 1.3 b′
3-feruloylquinic acid	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	93.8 ± 11 AB	81 ± 19 B	100 ± 0 A	100 ± 0 A	79 ± 11 a′	100 ± 0 a′	98 ± 4 a′	98.0 ± 2.0 a′
5-feruloylquinic acid	81 ± 17 a	99.6 ± 0.7 b	95 ± 5 ab	96 ± 4 ab	100 ± 0 A	91 ± 9 A	100 ± 0 A	100 ± 0 A	40 ± 6 a′	43 ± 3 a′	56 ± 9 b′	35 ± 5 a′
4-feruloylquinic acid	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 A	100 ± 0 A	100 ± 0 A	99.5 ± 0.8 A	86 ± 12 a′b′	83 ± 7 a′b′	94 ± 5 b′	66 ± 6 c′
Ferulic acid glucoside	56 ± 4 a	99.1 ± 1.5 b	100 ± 0 b	91 ± 15 b	0 A	0 A	60.5 ± 35 B	8 ± 3 C	34.7 ± 0.9 a′	38 ± 6 a′	36 ± 8 a′	37 ± 7 a′
Ferulic acid coumaroyl glucoside	71 ± 19 a	100 ± 0 a	76 ± 20 a	94 ± 12 a	81 ± 24 A	57 ± 37 A	78 ± 22 A	82 ± 24 A	44 ± 8 a′	41.2 ± 1.7 a′	99 ± 3 b′	57.9 ± 1.9 a′
Ferulic acid caffeoyl glucoside	38 ± 15 a	65 ± 3 b	100 ± 0 c	92 ± 14 c	71 ± 31 A	92 ± 14 A	61 ± 14 A	78 ± 19 A	36 ± 3 a′	43.3 ± 0.9 a′	37 ± 7 a′	24 ± 7 a′
Feruloylquinic acid derivative	46 ± 11 a	89 ± 9 b	89 ± 19 b	93 ± 12 b	73 ± 27 A	78 ± 10 A	68 ± 14 A	78 ± 9 A	46 ± 7 a′	33 ± 3 a′	41 ± 14 a′	33.5 ± 0.3 a′
Feruloylquinic acid derivative (2)	18 ± 7 a	57 ± 12 b	53 ± 24 b	89 ± 19 c	77 ± 21 A	42 ± 17 B	39 ± 17 B	38 ± 10 B	38.3 ± 2.4 a′	33.6 ± 1.2 a′	31 ± 5 a′	19 ± 4 b′

Ferulic acid and its derivatives	**77 ± 4 a**	**100 ± 0 b**	**97 ± 5 b**	**100 ± 0 b**	**98 ± 3 A**	**89 ± 12 A**	**100 ± 0 A**	**100 ± 0 A**	**45 ± 4 a′**	**50.0 ± 1.8 a′b′**	**60 ± 10 b′**	**37 ± 5 a′**
Total phenolic compounds	**52 ± 14 a**	**100 ± 0 b**	**49 ± 8 a**	**48 ± 6 a**	**31 ± 15 A**	**24 ± 5 A**	**40 ± 4 A**	**40 ± 17 A**	**16.1 ± 2.5 a′**	**27.9 ± 2.0 a′**	**34 ± 3 a′**	**33 ± 2 a′**

Values are means ± standard deviation. Different letters within the same product and compound indicate significant ($p < 0.05$) differences among treatments (puree: lowercase letters; puree with oil: uppercase letters; juice: lowercase′). The asterisk (*) indicates that compound was detected in dialysed fraction, but it was not present in those non-digested.

4. Conclusions

The content and bioaccessibility of carotenoids and phenolic compounds were affected by both PEF application to whole carrots and further processing conditions. Carrot juices had the highest phenolic and carotenoid content. However, just phenolic amount of purees was further increased after thermal treatment and decreased in juices obtained from PEF-treated carrots. On the other hand, treating whole carrots with PEF before obtaining a puree from them was a potential method for enhancing their phenolic bioaccessibility (100 %). Otherwise, thermal treatment and oil addition were more effective to improve carotenoid bioaccessibility (10.7 %) in purees. Regarding physicochemical properties, TSS and pH were similar among differently processed products, although slight changes in colour occur after oil addition or after thermal treatments in juices. Further studies focused on matrix structure and composition (e.g. pectin characteristics) are necessary to understand the mechanisms governing changes in the bioaccessibility of health-related compounds in the different studied products.

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Chapter 6. Pulsed electric field treatment strategies to increase bioaccessibility of phenolic and carotenoid compounds in oil-added carrot purees

Gloria López-Gámez, Pedro Elez-Martínez, Olga Martín-Belloso, Robert Soliva-Fortuny

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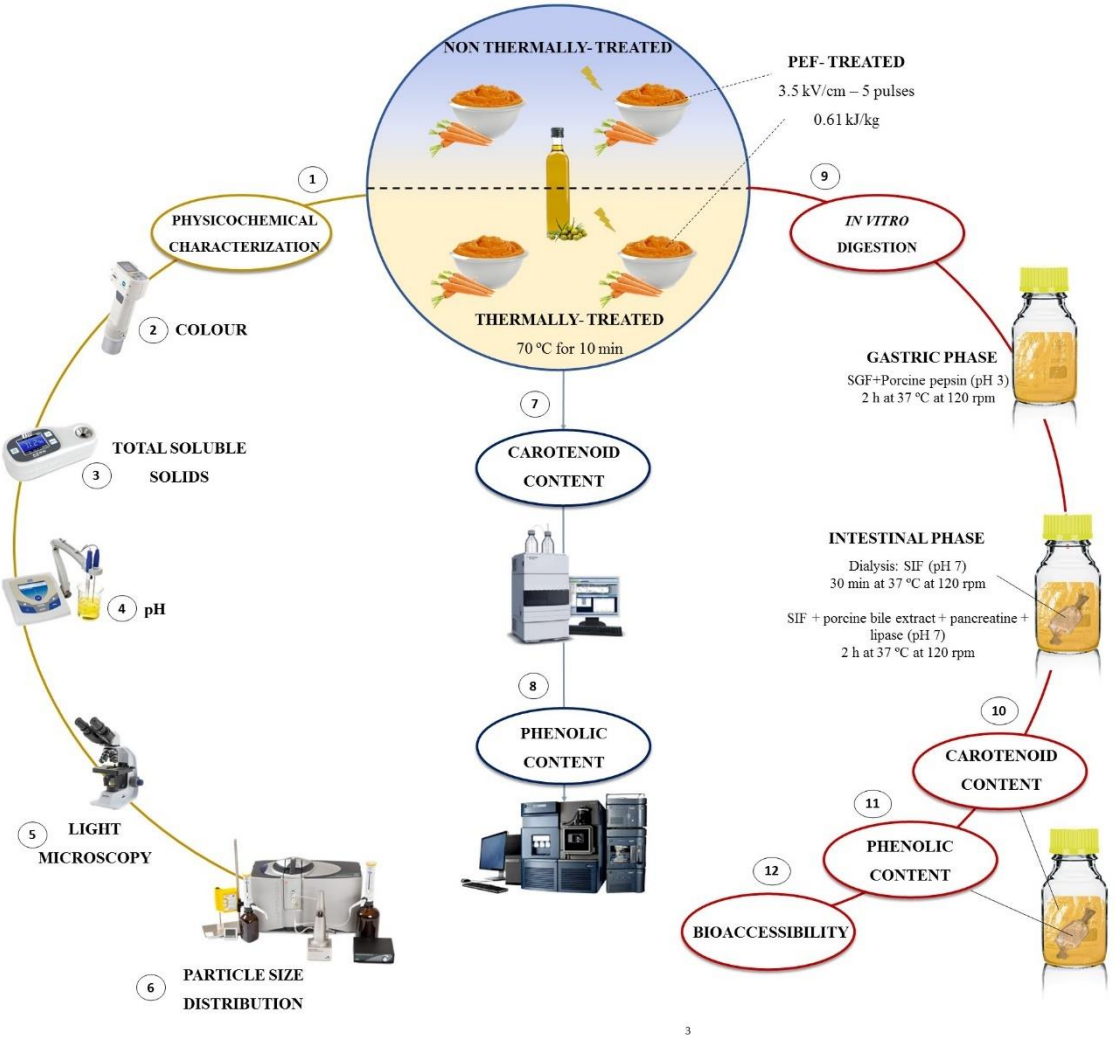
Abstract

The influence of mild intensity pulsed electric field (PEF) and their combination with a thermal treatment on the bioaccessibility of phenolic and carotenoid compounds in carrot purees with added oil was investigated. Puree was produced by blending carrot slices with water [1:1 (w/w)] and mixing them with olive oil (5 %). Fractions of such puree were differently treated: subjected to PEF (5 pulses of 3.5 kV cm^{-1}) (PEF); thermally treated (70 ºC for 10 min) (T) and first treated by PEF and then by thermal treatment (PEF/T). Colour, pH, total soluble solids and viscosity were analysed, as well as their microstructure and particle size distribution. Likewise, carotenoid and phenolic content were determined before and after an *in vitro* digestion to calculate their bioaccessibility. Quality attributes were not generally affected by the treatments, excepting viscosity and colour in those thermally treated, probably caused by changes in pectin state and higher oxidative enzymes activity. Carotenoid content was maintained regardless the treatment applied, whereas total phenolic content dramatically decreased in PEF- and PEF/T-treated purees. In general, caffeic acid derivatives were more sensitive than ferulic and coumaric acid derivatives. In addition, all treatments enhanced carotenoids and phenolic bioaccessibilities, which were doubled in PEF-treated purees. Observed cell disruption and decreased particle size (D [4, 3] = 305.21 ± 4.67 μm) at such conditions may suggest that microstructural changes could be responsible of bioaccessibility increases. Therefore, these results suggest that PEF could be a feasible treatment to obtain carrot derivatives with enhanced phenolic and carotenoid bioaccessibility without altering their quality attributes.

Keywords: Pulsed electric fields; bioaccessibility; phenolic compounds; carotenoids; oil

GRAPHICAL ABSTRACT



PEF: Pulsed Electric Fields
SGF: Simulated Gastric Fluid
SIF: Simulated Intestinal Fluid

1.Introduction

Consumption of fruit and vegetables has been extensively shown to prevent the development of degenerative or cardiovascular diseases because of their high content in antioxidant phytochemicals, e.g. phenolic compounds or carotenoids (Rao & Rao, 2007). These substances are not synthesized by humans; hence their dietary consumption is essential to human health. Bioactive compounds must be released and absorbed when digested in order to exert their health-promoting properties (Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Blanco-Navarro, et al., 2007). For this reason, the bioaccessible fraction is even more relevant rather than their native concentration in a food product (Knockaert, Lemmens, et al., 2012). Thus, offering attractive products with increased bioaccessible content of bioactive compounds could be a good strategy to prevent the previously described diseases.

Carrot is an economically important crop, highly consumed as a minimally processed product or further processed (e.g. juice, puree). Besides, it is a good source of carotenoids (e.g. β-carotene) and phenolic compounds (e.g. chlorogenic acid) (Ahmad, Iqbal, & Batool, 2019), despite their accessible contents are rather low. For being absorbed, both compounds need to be released from the matrix in which they are embedded. Carotenoids must be thereafter dissolved in the oil phase and incorporated into micelles, whereas phenolic compounds do not need to be micellarized. Carotenoids absorption is conditioned by cell wall and chromoplasts structures (Schweiggert et al., 2012). Likewise, phenolic compounds are generally stored in vacuoles or bound to dietary fibre (Shahidi & Yeo, 2016). Hence, tissue disruption and particle size reduction are usually favourable strategies to enhance their bioaccessibility (Moelants et al., 2012; Ribas-Agustí et al., 2018).

Processing can be therefore exploited to improve carotenoids and phenolic compounds bioaccessibility (Ribas-Agustí et al., 2018; Van Buggenhout et al., 2010). Results about the effects of thermal and mechanical processing in bioaccessibility are conflicting since they depend on amount and characteristics of individual carotenoids, matrix structure or treatment intensity (Van Buggenhout et al., 2010). Generally, in carrot derived products, lutein has higher bioaccessibility than α-carotene and β-carotene (Courraud, Berger, Cristol, & Avallone, 2013; Schweiggert et al., 2012). Regarding processing, an increase in β-carotene has been reported in thermal treated carrot purees due to softening of cell walls (Hedrén et al., 2002; Knockaert, Lemmens, et al., 2012). Conversely, decreases have also been observed by Palmero et al., (2014a), which are usually attributed to their degradation or isomerization (Knockaert et al., 2012a). Another critical step in carotenoids bioaccessibility is their transfer into the oil phase and micelles. The positive effect of dietary lipids addition on their bioaccessibility has been described in literature (González Casado,

2018; Hornero-Méndez & Mínguez-Mosquera, 2007; Knockaert, Pulissery, Colle, et al., 2012). However, type of lipids, hydrophobicity of carotenoids or further processing (e.g. thermal treatments, pulsed electric fields, high pressure processing) are important factors involved in transfer efficiency (Colle, Buggenhout, Lemmens, Loey, & Hendrickx, 2012; Palmero et al., 2014a). Hence, it has been reported that lutein bioaccessibility increased after blending carrot and sunflower oil, whereas that of β-carotene remained unaltered (Schweiggert et al., 2012). In addition, a high proportion of polyunsaturated acids and long chain fatty acids favour their incorporation to micellar fraction (e.g. olive oil) (Colle, Buggenhout, et al., 2012; Roohinejad et al., 2014). Likewise, high temperatures may induce a better transference to oil or conversely, carotenoids oxidation (Colle et al., 2013; Mutsokoti et al., 2016).

The effect of processing on phenolic bioaccessibility depends on compound characteristics, especially their size and molecular structure, protective effect of matrix and new interactions generated during digestion or processing. For instance, chlorogenic acid bioaccessibility decreased in thermal treated apple juice, whereas it was unaltered in kiwi juice (He et al., 2016; Quan et al., 2020b). Likewise, caffeoyl glucoside in thermally-treated orange juice was better absorbed than in kiwi juice (He et al., 2016; Quan et al., 2020b). Phenolic bioaccessibility can be enhanced when polysaccharides or proteins that compose the matrix structure are modified, entailing higher extractability. Increases up to 38 % in caffeic and *p*-coumaric acids of PEF-treated fruit juices were attributed to such cause (Rodríguez-Roque , Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015). Nevertheless, some compounds are sensitive to high temperatures and can also be degraded or entrapped by other macromolecules, such as chlorogenic and *p*-hydroxybenzoic acids in thermally-treated fruit juices (Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015; Ribas-Agustí et al., 2018). Phenolics could also interact with other molecules during digestion such as lipids, which may protect them, leading to increase bioaccessibility (Ortega et al., 2009; Quan et al., 2020a).

Applying cost-effective, faster and environmentally friendlier technologies as an alternative to heat treatments to achieve cell disruption and increase the bioaccessibility of health-related compounds is a thrilling challenge for food scientists. The application of pulsed electric fields (PEF) could be a valuable strategy to facilitate carotenoid and phenolic release based on its mechanism of action. PEF is a non-thermal technology that induces electropermeabilization in food products throughout the application of microsecond or nanosecond electrical pulses. This causes the reversible or irreversible formation of pores in cell membranes, among other structural and metabolic changes in tissues (Soliva-Fortuny et al., 2009). Recently, some studies have demonstrated that moderate-intensity PEF could be applied to increase the bioaccessibility of

carotenoids and phenolic compounds when applying to whole plant matrices such as tomato and apple (Bot et al., 2018; González-Casado et al., 2018a; Ribas-Agustí et al., 2019b). However, whole products have metabolically active tissues and it has been demonstrated that quality attributes are affected during post-treatment time (González-Casado et al., 2018c; Ribas-Agustí et al., 2019a). In addition, pureed forms of fruits and vegetables can be utilized as baby or elderly foods, that have higher market value for their convenience compared to same fresh products (Al-Ghamdi, Sonar, Patel, Albahr, & Sablani, 2020). Hence, the obtention of derived products would be desirable, not only in terms of quality or consumer preference, but also because of its bioaccessibility, which is generally higher in mechanical processed products (Lemmens et al., 2014; Ribas-Agustí et al., 2018).

This study was aimed at assessing carotenoids and phenolic compounds content and bioaccessibility of oil-added carrot purees after the application of PEF and/or thermal treatment. Additionally, quality attributes, particle size distribution and microstructure were evaluated after treatment application to understand the mechanisms involved in bioaccessibility changes.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium carbonate, magnesium chloride hexahydrate, hexane, acetonitrile and ethanol (HPLC grade), acetic acid, ammonium acetate were obtained from Scharlab (Sentmenat, Spain). Calcium chloride dihydrate was purchased from Merck (Darmstadt, Germany). Butyl hydroxytoluene (BHT) was acquired from Scharlau Chemie S.A. (Barcelona, Spain). Sodium hydrogen carbonate and potassium dihydrogen phosphate were acquired from VWR (Llinars del Vallès, Spain). Potassium chloride was obtained from Panreac (Castellar del Vallès, Spain). Digestive enzymes (porcine α-amylase, porcine pepsin, porcine bile extract, porcine pancreatin and porcine lipase) were acquired from Sigma-Aldrich (Darmstadt, Germany). 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).

Caffeic acid, ferulic acid, *p*-coumaric acid and 5-O-caffeoylquinic acid commercial patterns were obtained from Sigma-Aldrich (St. Louis, MO, USA). B-carotene standard was purchased from Carote-Nature (Ostermundigen, Switzerland), α-carotene was acquired from Supelco-Merck (Darmstadt, Germany) and lutein from Acros Organics (New Jersey, USA).

2.2. Carrot puree preparation

Carrots (*Daucus carota* cv. Nantes) (16 ± 2 cm) were purchased in a local supermarket (Lleida, Spain) and stored at 4 ºC until processing. Carrots were washed with tap water and the excess was removed with a paper cloth before discarding their top and bottom ends. Then, carrots were cut into 1-cm thick slices and puree was prepared by mixing approximately 500 g of carrot slices with water (1:1) (w/w) in a food processor (Taurus Mycook) in crushing function at full power (two intervals of 10 s). Thereafter, extra virgin olive oil (Borges Branded Foods, S.L.U., Tàrrega, Lleida) was added (5 % w/w). Olive oil was selected due to its fatty acid composition and previous results obtained by Colle et al., (2012). Before treatment, purees were stirred for 15 min at 8000 rpm with an Ultra-Turrax IKA equipped with a 3-blade stirring rod at room temperature. Resulting puree was divided into four fractions (~250 mL) to be differently treated: Untreated (U), PEF-treated (PEF), thermal-treated (T) and PEF and thermal treated (PEF/T).

2.3. Pulsed electric fields (PEF) treatments

PEF treatments were carried out using a batch system (Physics International, San Leandro, CA, USA) which delivers monopolar exponential-wave pulses from a capacitor of 0.1 µF at a frequency of 0.1 Hz. The system was 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 rectangular container with two stainless steel parallel electrodes (20 cm × 5 cm) separated by a gap of 5 cm. Puree (~250 mL) was placed into the treatment chamber and was submitted to 5 pulses of 3.5 kV cm^{-1} based on previous results (data not published) in which PEF-treated whole carrots enhanced their carotenoids and phenolic bioaccessibility. The temperature of puree after PEF application did not exceed 20.0 °C.

2.4. Thermal treatments

Carrot purees were packed in re-sealable polyethylene bags (20 × 15 cm) and heated in a water bath for 10 min at 70 °C in order to inactivate pectinmethylesterase and peroxidase activities (Balogh, Smout, Nguyen, Van Loey, & Hendrickx, 2004; Houben, Jamsazzadeh Kermani, Van Buggenhout, Van Loey, & Hendrickx, 2014; Soysal & Söylemez, 2005) thus obtaining stable products. Thereafter, purees were cooled under a constant flow of cold water for 3 min. Aliquots (20 mL) were freeze-dried and stored at -40 ºC until extraction and analysis of phenolic content in non-digested fraction were performed. Both aliquots (20 mL) of PEF- and

thermal-treated purees were subjected to an *in vitro* digestion to determine their carotenoid and phenolic contents in different fractions of digesta.

2.5. Quality attributes

Colour. Colour was assessed by measuring the CIEL*a*b* parameters with a Minolta colorimeter (Minolta CR-400, Konica Minolta Sensing, INC., Osaka, Japan). Total colour difference (ΔE) was calculated according Eq. (1).

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{0.5} \quad (1)$$

where L*, a* and b* refer to data collected after treatments and L^*_0, a^*_0 and b^*_0 refer to untreated carrot purees.

pH. pH was evaluated using a pH-meter (Crison Instruments S.A., Alella, Barcelona, Spain).

Total soluble solids (TSS). TSS was measured with a refractometer (Atago Company LTD., Tokyo, Japan) and expressed as % of total soluble solids.

Viscosity. Viscosity measurements (mPa s) were performed by using a vibro-viscometer (SV-10, A&D Company, Tokyo, Japan) vibrating at 30 Hz, with constant amplitude and working at room temperature.

2.6. Particle size distribution

Particle size distribution of carrot purees was measured by laser diffraction with a Mastersizer 3000™ (Malvern Instruments Ltd., Worcestershire, UK) and was expressed in terms of volume and surface diameter in μm, D [4, 3] and D [3, 2], respectively. The refractive index of water was 1.33 and the particle calculation was set for irregular particles.

2.7. Microstructure

The microstructure of carrot purees was visually analysed using a light microscope (BX41, Olympus, Göttingen, Germany) equipped with UIS2 optical system. All images were

analysed with 10x lens and processed using the instrument software (Olympus CellSense, Barcelona, Spain).

2.8. *In vitro* digestion

The *in vitro* digestion procedure was carried out following the COST Infogest protocol proposed by Minekus et al., (2014). The oral phase was omitted because of the short residence time in the oral cavity that the liquid food spends in it (Minekus et al., 2014). Puree (20 g) was transferred to a glass bottle and the gastric phase started when adding 20 mL of simulated gastric fluid (pH 3 and 37 ºC) and pepsin (Minekus et al., 2014). After 2 h of incubation at 37 °C with agitation, the duodenal phase continued by the addition of a cellulose-membrane dialysis bag (molecular weight cut-off 12,000 Da, Sigma-Aldrich) with simulated intestinal fluid (pH 7 and 37 ºC) inside (Minekus et al., 2014). Dialysis bags simulate the intestinal epithelium and contain the bioaccessible fraction of phenolic compounds released from matrix (Minekus et al., 2014; Ribas-Agustí et al., 2019b). After 30 min of incubation at 37 ºC with agitation, a solution containing simulated intestinal fluid (pH 7 and 37 ºC), bile extract, pancreatin and lipase was added and incubated for 2 h (with agitation and at 37 ºC). Finally, the dialysis bags were rinsed with distilled water until clean and its content was collected. The remaining digesta, which contained carotenoid compounds was separated from the non-digested oil droplets and from the non-digested carrot puree by centrifugation at 5000 g for 15 min at 4 °C (Brodkorb et al., 2019; Eriksen et al., 2017; Svelander et al., 2011). The upper non-digested oil layer was collected with a glass pipette. The supernatant, which corresponded to the aqueous micellar fraction, was divided into two equal volume fractions. One of them was vacuum-filtered through Whatman No.1 paper, corresponding to the released fraction. The other one was microfiltrated across a cellulose filter (pore size 1 – 3 μm; 70 mm diameter; Anoia S.A., Barcelona, Spain) and corresponded to the micellar fraction. Obtained digested fractions were both freeze-dried and stored at -40 ºC until extraction of bioactive compounds and the analysis of bioaccessibility.

In order to prepare the electrolyte and enzymatic solutions, the protocol described by Minekus et al., (2014) was followed. Blank samples containing water instead of carrot were digested in identical conditions. Furthermore, the entire procedure was performed in darkness, in absence of oxygen (bottles were flushed with nitrogen gas) in an orbital incubator (Ovan, Badalona, Spain) at 37 °C and 120 rpm.

Phenolic and carotenoid content of olive oil was determined. It was composed by 0.86 mg/100 g FW of α-carotene, 2.59 mg/100 g FW of β-carotene, 0.18 mg kg^{-1} DW of coumaric acid, 0.02 mg kg^{-1} DW of caffeic acid, 0.02 mg kg^{-1} DW of ferulic and isoferulic acids.

2.9. Carotenoids extraction

2.9.1. Non-digested samples

The method described by Sadler et al., (1990) was followed to extract carotenoids. A solution (50 mL) composed by hexane : acetone : ethanol (50 : 25 : 25) and containing 1 $g \cdot L^{-1}$ BHT was added to carrot puree (2 g) and it was stirred for 20 minutes. Thereafter, NaCl [10 % (w/v)] solution (15 mL) was added to the samples and were stirred for another 10 minutes. Samples were left to stand for ≥ 3 minutes, and the upper organic phase was microfiltrated across a nylon filter (0.45 μm, ø 13 mm, Labbox Labware S.L., Barcelona) and analysed by High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD). Samples were protected from light during extraction and analysis to avoid carotenoid degradation.

2.9.2. Digested samples

To extract the carotenes from the released and micellar digested fractions, 5 mL of the extraction solution was added to 0.2 g of freeze-dried digesta. Non-digested oil was mixed (0.1 g) with 7 mL of the extraction solution. Thereafter, 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 at 4000 g for 5 min (Svelander, 2011). An aliquot of the upper organic phase was microfiltrated across a nylon filter (0.45 μm, ø 13 mm, Labbox Labware S.L., Barcelona) and analysed by HPLC according to the method described subsequently. Samples were protected from light throughout extraction and analysis to avoid carotenoid degradation.

2.10. Identification and quantification of carotenoids by HPLC-DAD

HPLC system 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 quantification was carried out through a procedure validated by Cortés et al., (2004). An aliquot of 20 μL of the extracted samples was injected and carotenoids were separated using a reverse-phase C_{18} Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm x 250 mm). The mobile phase consisted of: (A) methanol/ammonium acetate 0.1 M, (B) milli-Q water, (C) methyl tert-butyl ether and (D) methanol and gradient is described in **Table 6.1**. The flow rate was 1 mL min^{-1} and the total run time was 60 min. The column temperature was set at 30 ºC, while vials were preserved at 4 ºC. Carotenoids were identified by the UV–vis spectral data and their retention times (Cortés et al., 2004; Mouly et al., 1999). Data were compared to calibration curves and quantification of carotenoids was carried out by integration of

the peak areas. Results were expressed on a fresh weigh basis as mg of carotenoid compound in 100 g of carrot puree.

Table 6.1. Chromatographic conditions to identify carotenoid compounds by HPLC-DAD.

Time (min)	Methanol/ ammonium acetate 0.1 M (%)	H_2O (%)	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

2.11. Phenolic compounds extraction

Phenolic compounds were extracted from freeze-dried non-digested or digested carrot samples (0.2 g). Methanol (80:20 v/v) (1.5 mL) was added and samples were vortexed for 1 minute. Then, they were centrifuged (16209 g, 15 min, 4 ºC) and the clear supernatant was microfiltered using polyvinylidene difluoride (PVDF) filters (0.2 µm) (Scharlab, Barcelona, Spain) prior to injection to the chromatographic system.

2.12. Identification and quantification of phenolic compounds by Ultra-Performance™ Liquid Chromatography (UPLC-MS/MS)

AcQuity Ultra-Performance™ liquid chromatography (UPLC) coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford) was used to determine phenolic compounds in methanolic extracts obtained from carrot samples. The analytical column was

an AcQuity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C_{18} (2.1 x 5 mm, 1.7 µm) (Waters, Milford). The column was kept at 30 °C, and the flow rate was 0.3 mL min^{-1}. Mobile phases were acetic acid (0.2 %) and acetonitrile. Elution gradients are shown in **Table 6.2**. 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). The dwell time established for each transition was 30 ms and MassLynx 4.1 software was used for data acquisition. Results were expressed in a dry weight basis as mg of phenolic compound per kg.

Table 6.2. Chromatographic conditions for identifying phenolic compounds in UPLC.

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

2.13. Bioaccessibility calculation

Carotenoids bioaccessibility is referred to the amount of carotenoids released from matrix and incorporated into micelles after *in vitro* digestion. Carotenoids concentration in the released fraction was extracted from the supernatant obtained by centrifugation and vacuum filtration following *in vitro* digestion. The micellar incorporation was estimated as carotenes recovered in the micellar phase isolated by microfiltration.

Phenolic compounds bioaccessibility was calculated in reference to their concentration in the dialyzed fraction of digested samples.

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

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

where $CC_{digested}$ corresponds to the concentration of each compound in the dialyzed (phenolic compounds) or micellar (carotenoid compounds) fraction and $CC_{non\text{-}digested}$ is referred to their concentration in non-digested samples.

2.14. 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). Three different replicates were submitted to each assayed treatment condition and each analysis was conducted twice. Results were reported as the mean ± standard deviation. Results were subjected to an analysis of variance (ANOVA) followed by Tukey *post hoc* test to establish statistical differences among mean values. The statistical significance level was set up at $p < 0.05$.

3. Results and discussion

3.1. Physicochemical characterization of carrot purees

Quality attributes from carrot purees are displayed in **Table 6.3**. Results show that neither pH nor TSS were modified by the applied treatments. Nevertheless, colour was affected in PEF/T-treated purees, since L* (53.16 ± 0.62) and a* (12.88 ± 1.51) values were significantly lower than those of untreated purees (L* = 55.55 ± 0.46; a* = 16.75 ± 1.24). Besides, ΔE values of T- and PEF/T-treated purees ranged between 3.0 and 6.0, which denotes noticeable colour differences for consumer perception (Barba et al., 2012). These results are in agreement with those reported by Aguiló-Aguayo et al., (2014) and García-Parra et al., (2018) in thermal treated carrot purees and pumpkin purees, respectively. Cell decompartmentalization and the action of oxidative enzymes involved in carotenoid degradation (e.g. lipoxygenase) could be the causes of these changes, since decreases in L* and a* indicate reduction in lightness and redness, respectively. In addition, non-enzymatic Maillard reactions could account for the highest ΔE in T-treated purees, since carrots are rich in sugars and high temperatures could favour such reactions (Kebede et al., 2014).

Viscosity increased after treatments that involved temperature application (**Table 6.3**). T-treated purees had a viscosity between 2.08 – 2.2 mPa s, whereas untreated and PEF-treated purees had viscosity values in a range of 1.44 – 1.48 mPa s. Viscosity is highly dependent on pectin content, temperature or pH, as well as the activity of pectinmethylesterase (PME) and polygalacturonase (PG) (Santiago, Jamsazzadeh Kermani, Xu, Van Loey, & Hendrickx, 2017). Several works have previously shown that thermal treatments increase the viscosity of carrot derived products, which has been attributed to the solubilization of pectin and cellulose from cell walls and particle flocculation (Gouma, Álvarez, Condón, & Gayán, 2020). Hence, the observed increase in viscosity was likely related to the formation of a molecular network due to interactions among de-esterified pectin molecules (Anese et al., 2015; Bi, Hemar, Balaban, & Liao, 2015) causing less syneresis in the obtained product (Christiaens et al., 2012).

Carrot purees had different particle distribution and size depending on the applied treatment (**Figure 6.1**). Untreated and T-treated purees were constituted by 29 % of particles smaller than 100 μm, whereas PEF- or PEF/T-treated purees contained a higher amount (61 – 67 %) of particles below 100 μm. These results suggest that untreated and T-treated purees were mostly composed by cell aggregates and just 29 % of single cells or cellular fragments, since carrot cell size is about 125 μm (Lemmens et al., 2010). After the PEF treatment application, either alone or combined with a thermal treatment, the percentage of particles within this range increased, which seems to indicate that cell clusters broke up and decomposed in single cells or fragments (**Figure 6.2**). This is also supported by the reduction of the volume-based diameter and the area-based mean diameter, D [4, 3] and D [3, 2], respectively (Kubo, Augusto, & Cristianini, 2013). D [4, 3] was significantly lower in PEF-treated (305.21 ± 4.67 μm) and PEF/T-treated (281.71 ± 44.22 μm) purees than in those untreated (448.97 ± 40.63 μm) or T-treated (459.63 ± 29.62 μm), which suggests that disruption of the largest particles was triggered. On the other hand, PEF-treated carrot purees exhibited the lowest D [3, 2] values. Indeed, the application of heat did not cause an extra increase in the number of small particles (**Table 6.3**). Scarce information regarding particle size distribution of carrot derived products treated by PEF is available in literature. Nevertheless, similar results on particle reduction have been reported when applying other non-thermal processing techniques such as high pressure processing (Knockaert et al., 2012; Kubo et al., 2013).

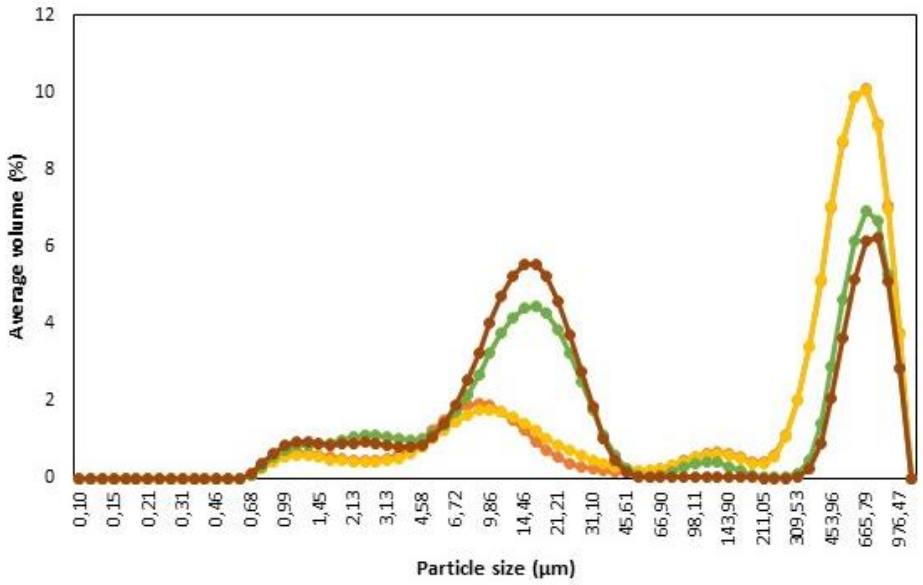


Figure 6.1. Volumetric particle size distribution of U (—), T- (—), PEF- (—) and PEF/T- (—) treated purees.

Table 6.3. Physicochemical characterization of carrot purees just after treatment.

	U	PEF	T	PEF/T
L*	55.55 ± 0.46 a	54.97 ± 0.12 a	53.68 ± 1.45 ab	53.16 ± 0.62 b
a*	16.75 ± 1.24 a	16.6 ± 1.2 a	13.9 ± 1.03 ab	12.88 ± 1.51 b
b*	42.87 ± 0.88 a	42.5 ± 1.27 a	42.97 ± 0.69 a	42.17 ± 1.07 a
ΔE	-	1.7 ± 0.6 a	3.8 ± 0.3 b	4.4 ± 0.8 b
pH	6.14 ± 0.02 a	6.11 ± 0.02 a	6.16 ± 0.02 a	6.11 ± 0.03 a
TSS (%)	4.16 ± 0.24 a	4.48 ± 0.16 a	4.48 ± 0.2 a	4.58 ± 0.08 a
Viscosity (mPa s)	1.44 ± 0.06 a	1.48 ± 0.04 a	2.08 ± 0.1 b	2.2 ± 0.17 b
D [4, 3] (µm)	444.51 ± 25.64 ac	305.21 ± 4.67 b	459.63 ± 29.62 c	281.71 ± 44.22 b
D [3, 2] (µm)	15.3 ± 3.46 ab	10.64 ± 0.55 b	19.9 ± 4.13 a	10.15 ± 1.06 b

Different letters in the same row are referred to significant ($p < 0.05$) differences among treatments. Untreated (U), PEF-treated (PEF), thermally-treated (T) and PEF and thermally-treated purees (PEF/T).

3. Microstructure

Untreated purees (**Figure 6.2A**) contained mostly whole cells with intact membranes and chromoplasts with carotenoids inside. In contrast, purees submitted to thermal treatment (**Figure**

6.2B), PEF (**Figure 6.2C**) or their combination (**Figure 6.2D**) showed cells with irregular shapes and smaller size, which is characteristic of irreversible electropermeabilized cells (Janositz & Knorr, 2010b). PEF- and PEF/T-treated purees also had a different particle size distribution (**Figure 6.1**), probably consisting of cell wall fragments and intracellular components that were released during electroporation. Meanwhile, T-treated purees contained shrunken and irregular cells, but their particle size distribution was similar to that of untreated purees (**Table 6.3** and **Figure 6.1**). These results were likely caused by the action of high temperatures, which weaken cell membranes and cause generally more cell separation instead of membrane breakage (Tydeman, Parker, Wickham, et al., 2010). Likewise, release of negatively charged pectin and positively charged proteins could favour the interactions between particles by van der Waals or electrostatic forces and formation of aggregates (Augusto et al., 2012). Soluble pectin could also promote the aggregation of oil droplets, which would result in an increased particle size (Palmero et al., 2016).

In addition, disruption of cellular barriers by treatments would enable the interchange of intra- and extra-cellular content. This would favour the release of phenolic compounds from matrix and their dialysis. Besides, the incorporation of carotenoids to oil droplets would be also facilitated, which agrees with the obtained bioaccessibility results (**Table 6.4** and **6.5**). Scarce studies about microstructural changes as affected by PEF treatments have been carried out in vegetables. However, the results reached in this study are consistent with those previously obtained in PEF-treated whole products. Jayathunge et al., (2017) observed irregular folds in the cell wall of PEF-treated tomatoes and Lohani and Muthukumarappan, (2016) reported disrupted fibrous structure after treating sorghum flour and apple pomace with PEF.

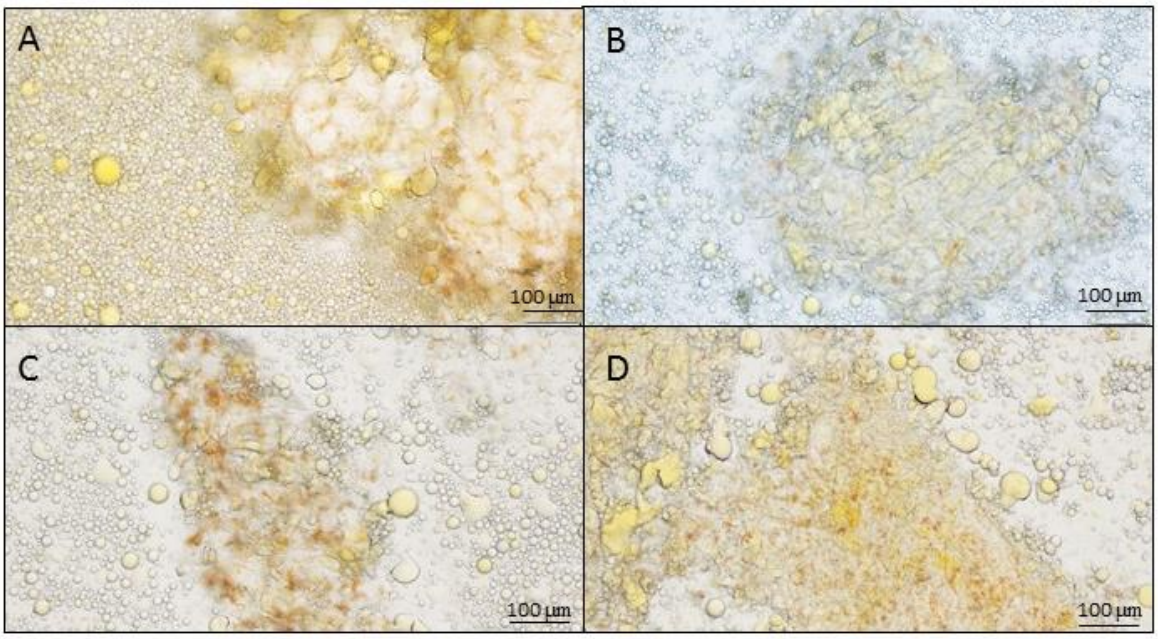


Figure 6.2. Light micrographs of untreated (A), T-treated (B), PEF-treated (C), and PEF/T-treated (D) carrot purees.

3.2. Carotenoid compounds

3.2.1. Carotenoid content in non-digested purees

The main carotenoids found in carrot purees were α-carotene, β-carotene, and lutein. These results are in accordance to those previously reported in literature (Knockaert et al., 2011; Schweiggert et al., 2012), although α-carotene and β-carotene contents were a 49 % and a 33.6 % lower, respectively, than those found in whole carrots (data not published). These compounds are prone to oxidation due to their unsaturation (Rodriguez-Amaya, 2010). Hence, disruption of carrot tissues by blending and decreases in particle size may increase the contact between such carotenoids and lipoxygenase enzyme, leading to their degradation.

On the other hand, carrot purees showed similar carotenoid contents regardless the treatment applied (**Figure 6.3**), whereas lutein content was higher in PEF-treated purees. This could suggest that observed decrease in particle size (**Figure 6.1** and **Table 6.3**) was sufficient to enhance lutein release from matrix. Lutein transfer to oil droplets has been reported to be more efficient than that of β-carotene (Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Blanco-Navarro, et al., 2007; Tyssandier et al., 2001). Hence, lutein may be easily extracted due to its presence in oil fraction. Discordant results have been reported regarding carotenoid content of carrot derived products after being submitted to PEF or thermal treatment. On the one hand, Mannozzi et al., (2018) reported that PEF-treated (0.8 kV cm^{-1}; 0.5 – 765 kJ kg^{-1}) carrot juices

and those untreated had a similar carotenoid content in overall. However, it decreased by half when heating (40 ºC, 60 ºC and 80 ºC) was applied. Additionally, loss in total carotenoids has also been reported in PEF-treated purees (0.5, 0.75 and 1 kV cm^{-1}; 6.8 – 516 kJ kg^{-1}) (Aguiló-Aguayo et al., 2014). On the other hand, an enhancement in carotenoids extraction from carrot purees was obtained when applying PEF (0.3, 0.5 and 0.8 kV cm^{-1}; 35 kJ kg^{-1}) (Leong, Oey, & Burritt, 2016). These differences among studies are probably due to differences in the applied processing parameters (pulses waveform, electric field strength, frequency, among others). In addition, variations in particle size of derived products is an important factor to consider when electropermeabilization takes place, since the larger cells get damaged before the smaller ones (Lebovka & Vorobiev, 2009).

3.2.2. Carotenoid content after *in vitro* digestion

Treatments had a significant effect on carotenoids release and micellarization (**Figure 6.3**). Total carotenoids were easily released from matrix when applying PEF (3.5 mg/100 g FW), heat (3.0 mg/100 g FW) or their combination (3.5 mg/100 g FW) compared to the release from untreated purees (1.7 mg/100 g FW). Individual compounds were also affected by treatments, since α-carotene content in the released fraction was 117.1 % (PEF), 63.4 % (T) and 107.3 % (PEF/T) higher than that found in untreated purees. Meanwhile, β-carotene from treated purees also increased between 91.6 – 93.9 % compared to the content recovered from untreated purees. Lutein content in the released fraction was higher in PEF and PEF/T-treated purees, whereas it could not even be detected in T-treated purees.

Micellar carotenoid content was affected by the applied treatments in a similar way as released content (**Figure 6.3**). PEF- (4.0 mg/100 g FW) and PEF/T-treated (4.4 mg/100 g FW) purees showed higher total content than T-treated (2.3 mg/100 g FW) and untreated purees (1.1 mg/100 g FW). Individual carotenoids followed the same trend as total. Moreover, almost 100 % of released carotenoids were micellarized.

Results show that all treatments were similarly efficient to enhance the α-carotene and β-carotene release from carrot puree, which may be due to changes in cellular permeability by electropermeabilization or weakening of cell walls. However, lutein was absent in the released and micellar fractions of T-treated purees. This could be likely due to its lower presence in non-digested fractions and to its chemical structure, which favours its incorporation to oil (**Figure 6.3**). Besides, the presence of oxygen in its chemical structure makes it more susceptible to degradation (Morales-De La Peña et al., 2011).

Carrot purees submitted to PEF and PEF/T had the highest content of micellarized carotenoids. This could be due to the fact that PEF not only induced changes in cell permeability but also in protein-carotenoid complexes, since α-carotene and β-carotene may be bound to cellular components (Faulks & Southon, 2005) and PEF can modify protein conformation (Perez & Pilosof, 2004). Carotenoids micellarization also depends on compound individual structure and solubility, matrix characteristics and the presence of fibre or dietary fats (Van Buggenhout et al., 2010). Carotenoids incorporation to micelles is inversely proportional to their hydrophobicity (Tyssandier et al., 2001). In PEF- and PEF/T-treated purees, lutein micellarization was higher than in untreated or T-treated purees. Probably, it could be due to their incorporation to oil during PEF treatment, since Granado-Lorencio et al. (2009) reported that its micellarization was enhanced when lutein was dissolved in olive oil. Hence, α-carotene and β-carotene would be located in the core of oil droplets whereas lutein would be found in their surface, thus being more bioaccessible (**Table 6.4**).

Carotenoid content in non-digested oil was investigated to understand whether treatments only favoured the carotenoids release from matrix or also played a role in their micellarization. Carotenoid content in non-digested oil fractions varied upon applied treatment and individual compound (**Figure 6.3**). The highest content of total carotenoids (21.8 mg/100 g FW) was observed for untreated carrot purees, whereas PEF- or PEF/T-treated purees had the lowest content (5.7 – 6.8 mg/100 g FW). Lutein, α-carotene and β-carotene from untreated purees were highly retained in non-digested oil, whereas the application of PEF, heat and their combination led to a decrease. According to these results (**Figure 6.3**), PEF had a beneficial effect in carotenoids transference to oil and their subsequent micellarization, which could be associated to the presence of smaller droplet sizes that could increase the surface area for lipase action and solubilization of carotenoids (Tydeman, Parker, Wickham, et al., 2010). A bigger droplet size would decrease the access of digestive enzymes and also the transfer of carotenoids from the oil phase into the micelles. Therefore, oil presence when PEF is applied may provide an optimum media to harbour released carotenoids, which would be easily micellarized during digestion.

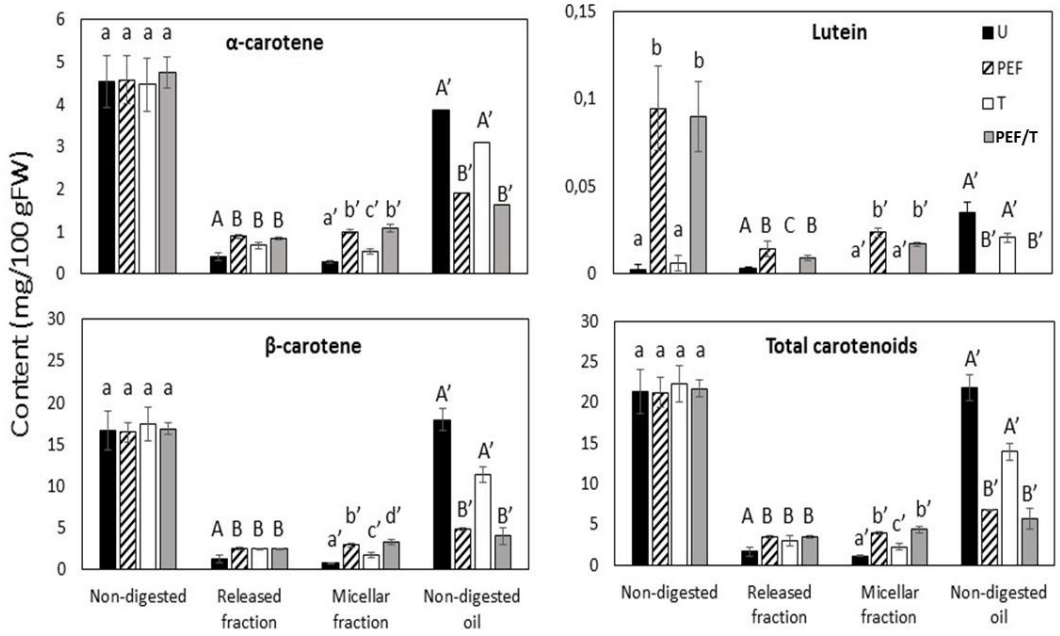


Figure 6.3. Carotenoid content (mean ± SD) in the non-digested, released, micellar and non-digested oil fractions from untreated (■), PEF-treated (▨), T-treated (□) and PEF/T-treated (▩) oil-added carrot purees. Different letters in the same fraction (lowercase letters: non-digested fraction; uppercase letters: released fraction; lowercase' letters: micellar fraction; uppercase' letters: non-digested oil) are referred to significant ($p < 0.05$) differences among treatments.

3.2.3. Carotenoid bioaccessibility

Total carotenoids from untreated purees had a bioaccessibility of 5.3 % whereas PEF- and PEF/T-treated purees showed enhanced bioaccessibility, 18.7 % and 20.4 %, respectively (**Table 6.4**). Regarding individual carotenoids in untreated purees, α-carotene (6.6 %) and β-carotene (5.0 %) had a similar bioaccessibility, although lutein was not bioaccessible under these conditions. T-treated purees doubled β-carotene and total carotenoids bioaccessibility. Besides, α-carotene bioaccessibility was 166 % higher than the obtained in untreated purees. On the other hand, PEF application trebled α-carotene, β-carotene and total bioaccessibility. Likewise, those PEF/T-treated purees quadrupled β-carotene and total bioaccessibility whereas α-carotene bioaccessibility was 345 % higher than in untreated purees. Even lutein became bioaccessible after PEF or PEF/T application, reaching 24 % and 17 %, respectively. **Figure 6.3** shows that lutein was retained in non-digested oil fraction of untreated and T-treated purees. These differences could be due to their different storage in chromoplasts. Whereas α-carotene and β-carotene are stored in crystalline structures, lutein may be stored in plastoglobuli together with *cis* isomers. Therefore, its sequestration in non-digested oil fraction is more favoured than that of carotenes (Vásquez-Caicedo et al., 2006).

On the other hand, carotenoid bioaccessibility and micellar content of T-treated purees were not as high as in PEF-treated purees. Microstructural changes caused by thermal treatments are characterized by cell separation instead of membrane breakage as particle size data seem to indicate (**Figure 6.1** and **Table 6.3**). Probably, thermal treatments caused that more carotenoids were entrapped in cell clusters (Christiaens et al., 2012) than in PEF-treated purees. In addition, pectin could interfere with lipids and bile salts, avoiding micelle formation (Palafox-Carlos et al., 2011) or forming complexes with carotenoids that limit their incorporation to micelles (Palmero et al., 2014).

To the best of our knowledge, the bioaccessibility of carotenoids in PEF-treated vegetable-based products has not been previously studied. In fruit, González-Casado et al., (2018) reported increases in β-carotene and lutein bioaccessibility of purees obtained from PEF-treated tomatoes. It has been reported that cell walls and chromoplasts limit carotenoids release from the food matrix. Hence, the improvement in carotenoids bioaccessibility observed in PEF-treated purees could be mainly caused by electropermeabilization and decrease in particle size (**Figure 6.1** and **Table 6.3**), as has been also reported for HPH-treated purees (Knockaert et al., 2012; Moelants et al., 2012).

Table 6.4. Carotenoid bioaccessibility (%) (mean ± SD) in untreated (U), PEF-treated (PEF), thermally-treated (T) and PEF- and thermally-treated (PEF/T) carrot purees.

Carotenoid compounds	Treatment	Bioaccessibility (%)
α-carotene	U	6.6 ± 0.8 a
	PEF	21.9 ± 0.9 c
	T	11.0 ± 1.4 b
	PEF/T	22.8 ± 2.1 c
β-carotene	U	5.0 ± 0.5 A
	PEF	17.8 ± 1.1 C
	T	10.0 ± 0.7 B
	PEF/T	19.8 ± 1.8 C
Lutein	U	0 ± 0 a’
	PEF	24.0 ± 2.4 b’
	T	0 ± 0 a’
	PEF/T	17.0 ± 1.2 c’
Total carotenoids	U	5.3 ± 0.4 A’
	PEF	18.7 ± 1.0 C’
	T	10.1 ± 1.6 B’

	PEF/T	20.4 ± 1.8 C’

Different letters within the same carotenoid compound (lowercase letters: α-carotene; uppercase letters: β-carotene; lowercase’ letters: lutein; uppercase’ letters: total carotenoids) are referred to significant ($p < 0.05$) differences among treatments.

3.3. Phenolic compounds

3.3.1. Phenolic content in non-digested purees

Changes in phenolic content, their fate through *in vitro* digestion and bioaccessibility are shown in **Table 6.5**. The main phenolic compounds in carrot puree were hydroxycinnamic acids, namely 5-caffeoylquinic acid, coumaric acid, coumaroylquinic acid and 5-feruloylquinic acid (**Table 6.5**). These results are in accordance to previous published studies (Becerra-Moreno et al., 2015; López-Gámez et al., 2020b; Padayachee et al., 2012). Contents were lower than those reported for whole carrots likely as a consequence of mechanical processing, which facilitated contact between oxidative enzymes and phenolic compounds.

Phenolic content was affected by the application of PEF, heat, or their combination. Total phenolic content in untreated (80.8 mg kg^{-1} DW) and T-treated (94.1 mg kg^{-1} DW) purees was higher than in PEF-treated purees (17.6 mg kg^{-1} DW) and those PEF/T-treated (54.27 mg kg^{-1} DW). These results suggest that heat treatment did not cause better phenolic extractability nor degradation by temperature. On the contrary, decrease in phenolic content and particle size (**Table 6.3**) observed after PEF treatment suggest that membrane disruption could favour the contact between released phenolic substances and oxidative enzymes, such as polyphenol oxidase (PPO) or peroxidase (POD).

Phenolic composition could be positively or negatively affected depending on the characteristics of each compound, matrix and treatment applied. On the one hand, PEF application caused a great increase in coumaric acid (117.8 %) and feruloylquinic acid derivatives (79.03 % and 288.9 %) compared to the content found in untreated purees. Ferulic acid and most of their derivatives showed similar content as untreated purees, although certain compounds were more sensitive to PEF, such as 3-feruloylquinic acid, ferulic acid glucoside and ferulic acid coumaroyl glucoside (**Table 6.5**). On the other hand, caffeic acid and their derivatives dramatically decreased (97.7 %) after the application of PEF.

T-treated purees also suffered changes in their phenolic composition. Some compounds showed higher content after treatment (e.g. 4-caffeoylquinic acid, caffeic acid Glu Acetyl

glucoside, 4-feruloylquinic acid and ferulic acid coumaroyl glucoside) (**Table 6.5**). The content of most coumaric and caffeic acid derivatives remained similar to the contents found in untreated purees, although coumaric and caffeic aglycone contents decreased by 61.4 % and 70.3 %, respectively.

Differences in the phenolic content of PEF/T-treated and untreated purees were also noticeable. Most derivatives from caffeic acid were not affected, although caffeic acid and 5-caffeoylquinic acid decreased by 80.8 % and 39.9 %, respectively. In addition, PEF treatment caused a decrease in the content of coumaric acid and some ferulic acid derivatives (e.g. 3-feruloylquinic acid, ferulic acid glucoside, ferulic acid coumaroyl glucoside) (**Table 6.5**). On the other hand, increases in 4-feruloylquinic acid (46.2 %) and feruloylquinic acid derivatives (74.4 % and 188.9 %) were also observed after treatments.

Changes in individual phenolic content are difficult to explain due to the heterogeneity of this chemical group and the complexity of the triggered events after the application of treatments. However, several aspects that may affect their concentration have been identified so far, including molecular structure, interactions with other macromolecules or the matrix structure where they are embedded (Jakobek & Mati, 2019). In general, phenolic compounds with high molecular weight, structural flexibility and more hydroxyl groups in their structure have more capacity to interact with macromolecules within a food matrix (Jakobek, 2015). Caffeic acid and their derivatives were majorly decreased after PEF, likely as a consequence of their oxidizable ortho-diphenol group (Kilmartin et al., 2001), which would be easily accessible by oxidizing enzymes due to decreased particle size (**Table 6.3**). This fact would explain that T-treated purees maintained similar caffeic content compared to untreated purees. Moreover, temperature could have reduced the activity of oxidative enzymes (Anthon & Barrett, 2002). This would explain that, regardless the decrease in particle size, the sum of caffeic acids in PEF/T- treated purees was lower than that found in T-treated purees and higher than in PEF-treated purees.

Decreases in phenolic content from PEF-treated purees could be related to their lower particle size given that polyphenols may be entrapped in hydrophobic pockets formed by cellular fragments (Bourvellec, Bouchet, & Renard, 2005). In addition, it has been suggested that temperature induces the formation of hydrophobic interactions with fibres (Nguela, Poncet-legrand, Sieczkowski, & Vernhet, 2016), such as cellulose or pectin (Phan et al., 2015), which may explain the decrease of some derivatives in T-treated purees.

3.3.2. Phenolic content after *in vitro* digestion

Total and individual phenolic content of dialysed fraction varied among differently treated purees. The highest content was obtained in PEF/T-treated purees (51.99 mg kg^{-1} DW) whereas untreated and PEF-treated purees had the lowest content, 21.48 and 24.92 mg kg^{-1} DW, respectively.

The application of PEF strongly enhanced coumaric (176.2 %) and coumaroylquinic acid (55.9 %) content in the dialysed fraction. Caffeic acid and their derivatives contents remained similar to those of untreated purees, excepting some decreases (**Table 6.5**). However, ferulic derivatives were unevenly affected: increased (e.g. 5-feruloylquinic acid), maintained (e.g. 3-feruloylquinic acid) or decreased (e.g. isoferulic acid).

Heat treatment did not affect the content of coumaric acids and their derivatives in the dialysed fraction. In contrast, it caused an increase in the amount of caffeic acid and most of their derivatives (**Table 6.5**), either when applied alone or in combination with PEF. Besides, ferulic acid derivatives were differently affected depending on their chemical structure. Their content in dialysed fraction decreased in some cases (e.g. isoferulic acid), increased (e.g. 3-, 4-, 5-feruloylquinic acids) or was maintained (e.g. ferulic acid caffeoyl glucoside).

In PEF/T-treated purees, coumaroylquinic acid content strongly increased (436.9 %) in the dialysed fraction, although coumaric acid remained similar to the content found in untreated purees. Some caffeic acid derivatives increased after treatment (e.g. 5-caffeoylquinic acid), but in other cases their content dramatically decreased (e.g. caffeic acid Glu Acetyl glucoside). Finally, most ferulic acid derivatives were affected in a similar way as after applying just PEF treatment.

Phenolic compounds are stored in vacuoles of plant cells and some of them are bound to macromolecules of the cell wall (pectin, cellulose, hemicellulose) or other compounds (carbohydrates, proteins, lipids) (Shahidi & Yeo, 2016; Jakobek, 2015), which hinders their absorption during digestion. The content of some compounds in dialysed fractions was higher than that observed in non-digested fractions, which entails a complete bioaccessibility. These results suggest that phenolics bound to matrix may be detached when submitting purees to gastric pH conditions. PEF could intensify this effect due to particle size reduction, which facilitates the accessibility of digestive enzymes (Saura Calixto, Serrano and Goñi, 2007) and phenolic release during digestion. Meanwhile, increases in bioaccessibility after thermal treatments were likely due to softening of carrot tissues and solubilization of pectin, which cause cell separation instead of their membrane rupture (Tydeman, Parker, Wickham, et al., 2010). This causes an increase in cell wall permeability due to pectin dissolution, which in turn allows gastric juice to easily achieve

intracellular content and enhance bioaccessibility of some compounds (Capuano & Pellegrini, 2019).

On the other hand, some phenolic compounds could be negatively affected by these structural changes because they are unstable in acidic media. They would be more exposed to be degraded than when they were surrounded by cell barriers. In addition, PEF and thermal treatments may also cause other chemical or physical modifications (hydroxylation, methylation, formation of phenolic derivatives, among others) that lead to modify phenolic acids bioaccessibility (Dugo, Paola, Lo Presti, Maria, Öhman, Marcus, Fazio, Alessia, Dugo, Giovanni, Mondello, 2005; Ribas-Agustí et al., 2018).

3.3.3. Phenolic bioaccessibility

Phenolic compounds bioaccessibility was modified according to the treatment applied and individual compound characteristics. Total content bioaccessibility was tripled in PEF- and PEF/T- treated purees, reaching 100 %. On the other hand, T-treated purees maintained similar bioaccessibility as those untreated (**Table 6.5**). Bioaccessibility of coumaric acids and their derivatives reached 100 % in PEF/T-treated purees, whereas it decreased by 34.2 % when just thermal treatment was applied (**Table 6.5**). Caffeic acid and their derivative compounds showed uneven bioaccessibilities among treatments and between compounds. In untreated purees, some of them were totally bioaccessible (e.g. caffeic acid Glu Acetyl glucoside) but other had a lower bioaccessibility (e.g. 5-caffeoylquinic acid, 16.4 %). PEF quintupled bioaccessibility of caffeic acid derivatives, PEF/T quadrupled it, whereas no remarkable changes were triggered in thermally treated purees (**Table 6.5**). Ferulic acid and most of their derivatives were completely bioaccessible regardless the applied treatment, excepting some glycosidic derivatives that showed values between 91.1 % and 34.1 %.

So far, the bioaccessibility of phenolic compounds in carrot purees has been scarcely studied. Consistent results have been obtained by Ribas-Agustí et al., (2019a) in PEF-treated apples (0.01, 1.8, 7.3 kJ kg^{-1}), in PEF-treated (32 and 256 kJ kg^{-1}) fruit juices (Buniowska et al., 2017) and in a PEF-treated (35 kV cm^{-1}) milk-fruit beverage (Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015). Authors attributed the reported increases to changes in matrix integrity that allow a better release of phenols during digestion. Moreover, modifications in phenolic structure would likely lead to modify their bioaccessibility.

Table 6.5. Changes in phenolic content and bioaccessibility in carrot purees and their digested fractions as affected by processing.

Phenolic compounds	Treatments	Content (mg kg^{-1} DW)		Bioaccessibility (%)
		Non-digested fraction	Dialysed fraction	
Coumaric acid	U	4.33 ± 0.58 a	2.73 ± 0.33 a	63.13 ± 4.14 a
	PEF	9.43 ± 3.44 c	7.54 ± 1.23 b	74.71 ± 23.49 ab
	T	1.67 ± 0.04 b	1.33 ± 0.14 a	79.35 ± 6.88 a
	PEF/T	2.03 ± 0.22 b	1.28 ± 0.94 a	95.82 ± 1.43 b
Coumaroylquinic acid	U	3.22 ± 0.6 ab	2.9 ± 0.69 a	91.69 ± 27.7 a
	PEF	2.13 ± 1.28 a	4.52 ± 0.51 b	100 a
	T	6.21 ± 2.28 ab	3.57 ± 0.77 ab	42.73 ± 0.37 b
	PEF/T	6.83 ± 2.43 b	15.57 ± 0.66 c	100 a
Coumaric acid and its derivatives	U	**7.55 ± 0.83 a**	**5.63 ± 0.99 a**	**74.25 ± 7.76 a**
	PEF	**11.56 ± 4.72 a**	**12.06 ± 1.73 b**	**86.44 ± 14.77 ab**
	T	**7.88 ± 2.24 a**	**4.9 ± 0.91 a**	**48.83 ± 0.14 c**
	PEF/T	**8.86 ± 2.54 a**	**16.8 5 ± 0.89 c**	**100 b**
Caffeic acid	U	1.72 ± 0.65 a	nd [1] a	0 a
	PEF	0.3 ± 0.03 b	nd [1] a	0 a
	T	0.51 ± 0.24 b	0.2 ± 0.04 b	42.5 ± 13.0 b
	PEF/T	0.33 ± 0.25 b	0.18 ± 0.08 b	62.48 ± 37.14 b
3-caffeoylquinic acid	U	nd [1] a	0.08 ± 0.05 a	100 a
	PEF	nd [1] a	nd [1] b	0 b
	T	nd [1] a	0.5 ± 0.04 c	100 a
	PEF/T	nd [1] a	0.24 ± 0.03 d	100 a
5-caffeoylquinic acid	U	66.68 ± 35.16 a	8.72 ± 1.46 a	16.45 ± 10.87 a
	PEF	0.92 ± 0.08 b	6.54 ± 3.05 a	100 b
	T	78.71 ± 5.8 a	22.11 ± 0.84 b	28.22 ± 2.79 a
	PEF/T	40.08 ± 17.8 c	27.92 ± 6.32 b	75.01 ± 28.1 b
4-caffeoylquinic acid	U	0.17 ± 0.1 a	0.27 ± 0.07 a	100 a
	PEF	nd [1] a	0.14 ± 0.05 a	100 a
	T	0.8 ± 0.1 b	1.66 ± 0.12 b	100 a
	PEF/T	0.53 ± 0.34 b	1.29 ± 0.21 c	100 a

Caffeoylferuoylquinic acid	U	0.24 ± 0.05 a	0.11 ± 0.03 a	48.24 ± 17.4 a
	PEF	0.12 ± 0.04 b	nd [1] b	0 b
	T	0.23 ± 0.09 ab	0.07 ± 0.01 a	36.12 ± 12 a
	PEF/T	0.16 ± 0.01 ab	0.06 ± 0.04 a	39.99 ± 20.2 a
Caffeic acid arabinoside glucoside	U	0.05 ± 0 a	0.05 ± 0.01 a	85.42 ± 18.94 a
	PEF	0.04 ± 0.02 a	0.03 ± 0 b	73.48 ± 31.55 a
	T	0.05 ± 0.02 a	0.05 ± 0.01 a	84.01 ± 27.7 a
	PEF/T	0.06 ± 0.03 a	0.03 ± 0.01 b	31.79 ± 6.5 b
Caffeic acid Glu Acetyl glucoside	U	0.52 ± 0.04 a	2.02 ± 0.08 a	100 a
	PEF	0.66 ± 0.28 a	0.92 ± 0.18 c	100 a
	T	0.8 ± 0.14 a	2.32 ± 0.13 b	100 a
	PEF/T	0.57 ± 0.44 a	0.63 ± 0.08 d	83.77 ± 28.1 a
Caffeic acid and its derivatives	U	**87.01 ± 26.16 ac**	**11.28 ± 1.63 a**	**20.14 ± 12.68 a**
	PEF	**2.04 ± 0.31 b**	**7.62 ± 2.9 a**	**100 b**
	T	**81.09 ± 5.46 a**	**26.93 ± 0.87 b**	**33.33 ± 2.99 a**
	PEF/T	**41.82 ± 18.41 c**	**30.36 ± 6.51 b**	**77.54 ± 27.6 b**
Ferulic acid	U	0.32 ± 0.03 ab	0.77 ± 0.07 a	100 a
	PEF	0.57 ± 0.17 a	0.78 ± 0.07 a	100 a
	T	0.54 ± 0.18 a	0.67 ± 0.06 b	100 a
	PEF/T	0.26 ± 0.07 b	0.46 ± 0.05 c	100 a
Isoferulic acid	U	0.12 ± 0.04 a	0.23 ± 0.01 a	100 a
	PEF	0.23 ± 0.14 a	0.17 ± 0.02 b	81.15 ± 29.7 a
	T	0.09 ± 0.03 a	0.16 ± 0.01 b	100 a
	PEF/T	0.11 ± 0.03 a	0.11 ± 0.02 c	86.93 ± 23.87 a
3-feruloylquinic acid	U	0.11 ± 0.06 a	0.13 ± 0.02 a	93.78 ± 10.76 a
	PEF	0.03 ± 0 b	0.11 ± 0 a	100 a
	T	0.16 ± 0.03 a	0.3 ± 0.04 b	100 a
	PEF/T	0.04 ± 0.01 b	0.14 ± 0.02 a	100 a
5-feruloylquinic acid	U	1.07 ± 0.16 a	1.36 ± 0.11 a	100 a
	PEF	1.3 ± 0.12 a	2.49 ± 0.32 bc	100 a
	T	1.35 ± 0.27 a	2.21 ± 0.08 b	100 a
	PEF/T	1.61 ± 0.3 a	2.76 ± 0.15 c	100 a
4-feruloylquinic acid	U	0.13 ± 0 a	0.33 ± 0.01 a	100 a
	PEF	0.12 ± 0.01 a	0.4 ± 0.04 c	100 a
	T	0.18 ± 0.04 b	0.61 ± 0.04 b	100 a

	PEF/T	0.19 ± 0.04 b	0.45 ± 0.04 c	100 a
Ferulic acid glucoside	U	0.04 ± 0.01 a	nd [1] a	0 a
	PEF	0.02 ± 0.02 b	0.01 ± 0 b	74.2 ± 65.85 b
	T	0.03 ± 0.01 a	0.02 ± 0.01 b	60.56 ± 35 b
	PEF/T	nd [1] c	nd [1] a	0 a
Ferulic acid coumaroyl glucoside	U	1.52 ± 0.24 a	1.35 ± 0.44 a	81.5 ± 24.446 a
	PEF	0.66 ± 0.12 b	0.29 ± 0.03 b	58.47 ± 40.44 a
	T	2.15 ± 0.48 c	1.79 ± 0.47 a	77.8 ± 22.73 a
	PEF/T	0.36 ± 0.06 b	0.28 ± 0.06 b	80.24 ± 16.9 a
Ferulic acid caffeoyl glucoside	U	0.06 ± 0.03 a	0.04 ± 0.01 a	70.99 ± 31.2 a
	PEF	nd [1] b	0.11 ± 0.04 b	100 a
	T	0.08 ± 0.03 a	0.05 ± 0.02 a	61.62 ± 14.42 b
	PEF/T	nd [1] b	nd [1] c	0 c
Feruloylquinic acid derivative	U	0.43 ± 0.15 a	0.29 ± 0.02 a	73.52 ± 26.73 a
	PEF	0.77 ± 0.19 b	0.68 ± 0.04 b	91.11 ± 17.46 a
	T	0.43 ± 0.01 a	0.29 ± 0.07 a	68.18 ± 14.91 a
	PEF/T	0.75 ± 0.2 b	0.5 ± 0.01 c	70.76 ± 23.47 a
Feruloylquinic acid derivative (2)	U	0.09 ± 0.03 a	0.07 ± 0.01 ac	76.51 ± 21.1 a
	PEF	0.35 ± 0.02 b	0.14 ± 0.02 b	41.52 ± 5.02 b
	T	0.12 ± 0.01 a	0.05 ± 0.02 a	39.35 ± 17.09 b
	PEF/T	0.26 ± 0.07 c	0.09 ± 0.02 c	34.14 ± 10.84 b
Ferulic acid and its derivatives	U	**3.9 ± 0.41 a**	**4.57 ± 0.56 a**	**98.14 ± 3.2 a**
	PEF	**4.04 ± 0.34 ab**	**5.23 ± 0.52 a**	**100 a**
	T	**5.14 ± 0.84 b**	**6.15 ± 0.55 b**	**100 a**
	PEF/T	**3.59 ± 0.59 a**	**4.79 ± 0.25 a**	**100 a**
Total phenolic compounds	U	**80.8 ± 35.61a**	**21.48 ± 2.1 a**	**30.68 ± 15.34 a**
	PEF	**17.64 ± 4.12 b**	**24.92 ± 2.76 a**	**100 b**
	T	**94.11 ± 5.67 a**	**37.97 ± 1.22 b**	**40.5 ± 3.75 a**
	PEF/T	**54.27 ± 19.23 b**	**51.99 ± 7.15 c**	**88.85 ± 19.32 b**

Different letters in the same column are referred to significant ($p < 0.05$) differences among treatments. Untreated (U), PEF-treated (PEF), thermally-treated (T) and PEF and thermally-treated purees (PEF/T). [1] nd: not detected.

4. Conclusions

Results obtained in this study clearly demonstrate that PEF is an effective treatment to enhance the bioaccessibility of carotenoid and phenolic compounds in oil-added carrot purees. Likewise, quality attributes of PEF-treated purees were not negatively affected, whereas thermally–treated purees (T and PEF/T) showed noticeable changes in colour and viscosity. Besides, PEF processing led to important changes in phenolic composition, especially reducing caffeic acid derivatives content (e.g. 5-caffeoylquinic acid), whereas total carotenoid contents remained stable among differently treated purees.

PEF-treated purees suffered microstructural changes characterized by smaller and irregular cells and the presence of cell wall fragments, which was supported by their lower particle size (D [4, 3] = 305.21 ± 4.67 μm). Besides, PEF-treated purees showed the highest carotenoid (18.7 %) and phenolic (100 %) bioaccessibility, improving bioaccessibility of caffeic acid derivatives and ferulic acid derivatives in 80 % and 70 %, respectively. These results suggest that microstructure modifications are likely the main cause of such changes. Probably, electropermeabilization was more effective than thermal treatment in releasing bioactive compounds from the tissue matrix, which would be easily dialyzed in case of phenols or incorporated to micelles in the case of carotenoids. However, further studies are necessary to understand in more depth the relationship between PEF-induced changes and bioaccessibility. Likewise, consumer acceptance, safety and their effects on human health must be investigated.

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GENERAL DISCUSSION

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1. Background

During the past few years, research on food technology and nutrition has been reoriented towards the potential of improving health-related properties of food products in order to meet consumer demands. Increasing attention is given to innovative technologies that help maintaining the nutritional and health-related properties of food products without significantly affecting fresh-like quality attributes. PEF is a promising technology to achieve such purpose since their application could enhance health benefits from two different ways. Firstly, PEF can be applied to induce the accumulation of bioactive compounds in plant tissues by triggering a stress defence response. This could lead to the development of fresh and derived products with greater bioactive content. Secondly, PEF also cause cell membrane disruption and structural modifications, which are essential for releasing bioactive compounds from matrix during digestion. Therefore, PEF is a potential alternative to enhance the health-promoting properties of plant-based food products. The present work was aimed at evaluating the effect of PEF on the content and bioaccessibility of carotenoid and phenolic compounds in whole carrots as well as at studying their influence on quality attributes. Likewise, the main causes under such changes were studied. On the other hand, the influence of processing conditions on phenolic and carotenoid bioaccessibility was assessed. In this regard, microstructure of carrots and derived products was evaluated to elucidate possible causes of bioaccessibility changes.

2. PEF treatments to enhance bioactive content and bioaccessibility in whole carrots

Several studies have previously demonstrated that PEF application could be a potential strategy to activate a stress defence response in plant tissues and to obtain vegetable products with enhanced bioactive content. Electropermeabilization has been reported to cause microstructural changes in plant tissues. Hence, an increase in bioactive compounds could be a consequence of either the triggering of biosynthetic production and/or an enhancement of the extractability due to changes in the structure of the food matrix. In this thesis, the impact of PEF treatments and post-treatment time on the carotenoid and phenolic content as well as on quality attributes of carrots was evaluated. Carrots were submitted to different electric field strengths (0.8, 2.0 and 3.5 kV cm^{-1}) and number of pulses (5, 12 and 30 pulses) and were stored for 48 h at 4 ºC. Additionally, cell viability and permeability were assessed. Based on the obtained results, conditions were selected to determine whether changes in the content of bioactive compounds were caused by

structural changes or by an activation of stress-related biosynthetic pathways. Enzyme activities, respiratory rates and phenolic profile were studied throughout storage in order to provide insights on the induced modifications.

2.1. Effects of PEF on phenolic accumulation in whole carrots

Phenolic content in whole carrots was influenced by the application of PEF treatments. Both electric field strength and number of pulses had a significant impact on total content. Vallverdú-Queralt et al., (2013) reported similar influence of these parameters in phenolic content of PEF-treated tomatoes. In addition, post-treatment time also played an important role in phenolic accumulation. No differences in content were reported immediately after PEF. However, higher increases were observed in carrots treated with 5 pulses of 3.5 kV cm^{-1} (39.5 %) and 30 pulses of 0.8 kV cm^{-1} (40.1 %) and stored for 24 h at 4 ºC. On the other hand, carrots submitted to 2 kV cm^{-1} or 3.5 kV cm^{-1} and stored for 48 h generally showed lower content than untreated carrots. These results are in accordance to those published by Balaša, (2014) and changes during post-treatment time are consistent with the plant stress response mechanism. The first minutes after a stress is applied to a plant tissue, signalling molecules are generated [e.g. oxygen reactive species (ROS)], which activate the biosynthesis of secondary metabolites, whose accumulation is usually given during storage (Schulze, Beck, & Müller-Hohenstain, 2003). Despite this general action pattern, defence response would be different depending on the intensity of the stress, which would explain differences among treatments.

In general, secondary metabolites can be generated or eliminated in several ways. They can be synthesised, used to neutralize ROS, degraded by oxidative enzymes [e.g. polyphenol oxidase (PPO)], utilized for synthesizing other compounds and released from tissues due to structural damage. Therefore, detected phenolic compounds are the surplus of these events. After applying the lowest intensity (0.8 kV cm^{-1}), transient small pores are more likely to be generated than irreversible ones, which is in accordance with the high percentage of cell viability, firmness and media conductivity maintenance. Besides, these results are in agreement to those reported by Wiktor et al., (2015), who determined that 1.85 kV cm^{-1} was not enough to cause irreversible electropermeabilization in carrots. In this case, plant tissues would respond by neutralizing ROS with available antioxidants and by *de novo* biosynthesis of phenolic compounds to repair the inflicted damage. More intense treatments (2 kV cm^{-1} and 3.5 kV cm^{-1}) may cause a strong structural damage (i.e. less intercellular adhesion, permanent pores in membranes, intracellular content release), which is supported by data showing cell viability loss, firmness decrease and media conductivity increase. This intense damage probably involved better extractability of phenolic compounds due to higher membrane damage. However, *de novo* biosynthesis is also

possible since their phenolic content just after treatment was lower than that obtained 24 h after treatment. Hence, if the main cause behind the phenolic increase is a better extractability, the content immediately after and 24 h after treatment is not expected to change.

PEF conditions (5 pulses of 3.5 kV cm^{-1}) were selected based on previous results, showing the highest accumulations of phenolic compounds. With the aim of getting a deeper insight into the effect of such treatment, individual compounds were quantified.

Hydroxybenzoic acids (*p*-hydroxybenzoic and protocatechuic acid) and hydroxycinnamic acids (chlorogenic acid, ferulic acid, and *p*-coumaric acid) were the main phenolic compounds present in carrots. These results are in accordance to those obtained by Becerra-Moreno et al., (2015) and Jacobo-Velázquez, Martínez-Hernández, Del C. Rodríguez, Cao, & Cisneros-Zevallos, (2011). PEF and post-treatment time affected their content depending on the chemical characteristics of each compound. Total content of PEF-treated carrots peaked 24 h (937 ± 63 mg kg^{-1} DW) after PEF, whereas content in untreated carrots was maintained or decreased during storage (514 ± 63 mg kg^{-1} DW at 24 h). Regarding individual compounds, the content of some compounds significantly decreased, e.g. ferulic (56.3 %), protocatechuic (78.1 %) and *p*-coumaric (42.3 %) acids, after PEF application. However, protocatechuic and chlorogenic acid contents increased 12 h after PEF treatment. The highest content of total phenolic content (80.2 %) and some individual compounds such as *p*-hydroxybenzoic (94.7 %), chlorogenic (74.9 %) and ferulic (52.2 %) acids was reached 24 h after treatment application. Eventually, most compounds dramatically decreased 36 h after PEF.

Antioxidant potential of phenolic compounds depends on their molecular structure and number of hydroxyl groups (Heo, Kim, Chung, & Kim, 2007). For instance, chlorogenic and ferulic acids are effective scavenging ROS and are also formed as a consequence of either biotic or abiotic stresses (Dixon & Paiva, 1995; Grace & Logan, 2000; Rice-Evans, Miller, Paganga, 1996). Initial decreases in content could be related to their release throughout formed pores or their utilization to scavenge generated ROS by electropermeabilization. The phenolic increase observed over the first 24 h following PEF treatment seems to be indicative of the triggering of a defence stress response. In contrast, losses at 36 h could be related to cell metabolic exhaustion due to major membrane damage (Balaša, 2014; Schulze et al., 2003). Further studies are needed to evaluate the effect of different PEF intensities on phenolic accumulation. However, some trends can be discerned considering the adaptive plant stress response (Dixon & Paiva, 1995). Excessive intensities could cause irreparable cell membrane ruptures that increase phenolic extractability instead of their biosynthesis. On the other hand, lower intensities could induce the formation of reversible pores, leading to a progressive accumulation of phenolic compounds during storage.

PEF intensity and storage time should be therefore optimized to achieve the greatest content in the shortest time.

Phenolic compounds were also determined by UPLC prior to *in vitro* digestion. The most abundant phenolic compounds (e.g. coumaroylquinic or 5-caffeoylquinic acids) in carrots were especially sensitive to PEF. Hence, phenolic content of PEF-treated carrots (97.9 mg kg^{-1} DW) was much lower than that of untreated carrots (254.1 mg kg^{-1} DW). Nonetheless, some compounds also increased after PEF, such as caffeoylferuloylquinic (437.5 %), ferulic (114.6 %) and coumaric acid (161.5 %). Likewise, some other phenolic compounds remained unaltered (e.g. 5-feruloylquinic acid or caffeic acid Glu Acetyl glucoside). These results were different to those previously determined spectrophotometrically or after hydrolytic extraction. The spectrophotometric determination is likely to overestimate the overall content due to interferences with other compounds such as ascorbic acid or reducing sugars (Vallverdú-Queralt et al., 2012). Hydrolysis extraction was applied to identify and quantify free phenolic compounds and those bound to matrix by HPLC-DAD in their aglycone form. Therefore, results obtained by UPLC allow quantifying phenolic compounds as they are naturally found in carrots without hydrolysing such compounds, which means that UPLC quantification was the most accurate.

Major changes in phenolic content can be explained by electropermeabilization. On the one hand, modifications of membrane permeability probably enhance the extraction of some phenolic compounds (Wang, He, & Chen, 2014). Besides, pores formation may trigger a stress defence response leading to the accumulation of phenolic compounds over 24 h of post-treatment time (López-Gámez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, 2020b; Ribas-Agustí, Martín-Belloso, Soliva-Fortuny, & Elez-Martínez, 2019a). On the other hand, decreases could be explained by structural decompartmentalization, which facilitates the contact between oxidative enzymes (PPO or POD) and phenols (Kilmartin, Zou, & Waterhouse, 2001) or their diffusion throughout pores.

2.2. Effects of PEF on carotenoids accumulation in whole carrots

As for phenolic compounds, total and individual carotenoid content in whole carrots were mainly affected by electric field strength and post-treatment time. Total content increased just after applying 2 kV cm^{-1} or 3.5 kV cm^{-1}, reaching the maximum content (83.8 %) when 5 pulses of 3.5 kV cm^{-1} were applied. After 24 h, PEF-treated carrots generally had a similar content than untreated carrots. Besides, individual compounds were differently affected. Phytoene (59.1 – 95.4 %) and β-carotene (36.5 – 91.8 %) increased just after applying treatments of 2 kV cm^{-1} and 3.5 kV cm^{-1} whereas α-carotene content was generally similar to that found in untreated carrots. On

the other hand, lutein content decreased (15.1 – 96.4 %) after treatments of 0.8 kV cm^{-1} and 2 kV cm^{-1}.

Results concerning total content are in accordance to those reported by Wiktor et al. (2015), which suggest that extractability is enhanced by electropermeabilization and other structural changes. Nonetheless, differences in the content of individual carotenoids after PEF could result from the modulation of the carotenoid biosynthesis pathway triggered by pores formation and subsequent generation of ROS. The carotenoid biosynthetic pathway has a bifurcation step after lycopene and two branches are differentiated: one of them accounting for the synthesis of β-carotene and its derivatives and a different one through which α-carotene and its derivatives are formed. The presence of lycopene β-cyclase (βLCY) and lycopene ε-cyclase (εLCY) enzymes is necessary for modulating this process. Hence, PEF could act regulating the activity of such enzymes, as has been reported by Vallverdú-Queralt, et al., (2013b) in PEF-treated tomatoes and for other non-thermal technologies such as high pressure processing (Ramos-Parra et al., 2019). This hypothesis is supported by correlations found between the applied specific energy and individual carotenoids, namely phytoene ($r = 0.761$; $p < 0.001$), lutein ($r = 0.648$; $p < 0.001$) and α-carotene ($r = 0.402$; $p < 0.05$).

Considering all the above, the most probable cause of the increases in carotenoid content could be a combination of a better extraction and an enzyme modulation [e.g. phytoene synthase (PSY), βLCY or εLCY] of the carotenoids biosynthetic pathway. Further studies on gene expression of involved enzymes would be required to support this hypothesis.

2.3. Effects of PEF on quality attributes of whole carrots

Food processing usually impacts some quality attributes such as colour, texture, pH or total soluble solids. These differences will directly affect consumer acceptance. Hence, evaluating some of the most important quality parameters of PEF-treated carrots would provide information about the feasibility of applying such technology to obtain carrots with enhanced bioactive content and fresh-like properties.

Some colour parameters were affected by PEF and post-treatment time. Specifically, electric field strength and its interaction with pulse number were the most influential factors. L* of carrots subjected to 2 kV cm^{-1} and 3.5 kV cm^{-1} progressively decreased during post-treatment time, whereas those submitted to 0.8 kV cm^{-1} generally maintained their colour. These results seem to point out that carrots treated with 0.8 kV cm^{-1} did not suffer irreversible structural modifications. Similar decreases in L* and h values were reported for PEF-treated tomatoes and

apples, which was associated to decompartmentalization and increased contact between phenolic substances and oxidative enzymes (González-Casado, Martín-Belloso, Elez-Martínez, & Soliva-Fortuny, 2018a; Ribas-Agustí et al., 2019a). A downward relationship was observed between L* (r = -0.697; $p < 0.01$), C* (r = - 0.467; $p < 0.05$) and h (r = - 0.434; $p < 0.05$) values of carrot peel and the applied specific energy. Changes in h could be attributed to modifications in redness and carotenoids content. As previously discussed, electropermeabilization could promote darkening of tissues, which would explain decreases in L* when increasing energy input.

PEF-treated carrots subjected to 12 pulses of 3.5 kV cm^{-1} (0 h) and 30 pulses of 2 kV cm^{-1} (24 h) exhibited higher ΔE values than untreated carrots, reaching ΔE = 10 and ΔE = 8.9, respectively. Likewise, carrots subjected to treatments of 2 kV cm^{-1} and 3.5 kV cm^{-1} that were stored for 48 h had ΔE values between 7.1 and 16. Therefore, PEF caused modifications in carrot appearance since ΔE higher than 2 indicates that colour differences can be noticed by consumers (Tiwari, Patras, Brunton, Cullen, & O'Donnell, 2010). Lightness decrease is likely related to the electropermeabilization of cellular structures, which favours contact between phenolic substances and oxidative enzymes, which in turn leads to colour changes. On the other hand, cortical BI values could be associated to carotenoid content since they are stored in secondary phloem (Baranska, Baranski, Schulz, & Nothnagel, 2006). Correlations between carotenoid content and BI have been found, phytoene (r = 0.638; $p < 0.05$) and β-carotene (r = 0.683; $p < 0.05$) contents. Besides, a correlation was also noticed between cortical h and lutein contents (r = 0.701; $p < 0.05$). Different effects of PEF on food colour have been reported depending on the matrix and processing parameters (García-Parra, González-Cebrino, Delgado-Adámez, Cava, & Martín-Belloso, 2018; Jin, Yu, & Gurtler, 2017; Wiktor et al., 2015). Probably, higher intensities lead to greater colour changes due to formation of irreversible pores. Besides, degradation of coloured pigments or the activation of oxidative enzymes also contribute to colour modification.

Hardness of vascular cylinder of carrots was softer (8.8 – 26.5 %) than cortical tissues. Both were also affected by electric field strength and its interaction with the applied number of pulses. Just after treatments, neither hardness of cortical tissues nor vascular cylinder were affected. However, hardness of carrots subjected to 3.5 kV cm^{-1} treatments significantly decreased during post-treatment time. Meanwhile, hardness of carrots treated at 2 kV cm^{-1} slightly increased over 48 h, which could be related to the induction of a lignification process. This is a plant defence mechanism to prevent water loss, which was observed in wounded carrots under water stress by Becerra-Moreno et al., (2015). Decreases in carrot firmness were also reported by Lebovka, Praporscic, & Vorobiev, (2004). They are likely related to disruption of cell membranes in carrots treated with the most intense treatments. This decompartmentalization leads to a redistribution of water in tissues and leakage of intracellular content, which causes softening. These results are in

accordance with the correlations found between hardness and weight loss (r = -0.590; p < 0.01), lightness (r = 0.579; p < 0.01) and media conductivity (r = -0.667; p < 0.01). Additionally, percentage of cell viability decreased when 3.5 kV cm^{-1} treatments were applied, being epidermis and cortical tissues the most affected areas.

In general, pH and TSS were neither significantly affected by PEF treatment parameters nor by post-treatment time. Nevertheless, other authors have reported increases in TSS of PEF-treated fruit, which has been associated to faster ripening and the accumulation of sugars (Amami, Vorobiev, & Kechaou, 2005; González-Casado et al., 2018a). Regarding pH, similar results to those reported in this thesis were obtained by Balaša, (2014) and Leong & Oey, (2014) in cell culture and carrot purees, respectively. Conversely, González-Casado, Martín-Belloso, Elez-Martínez, & Soliva-Fortuny, (2018b) reported increases immediately after treatment and during storage of PEF-treated tomatoes (0.02 and 0.38 kJ kg^{-1}). Such increases in TSS and pH were likely the consequence of tissue softening and the triggering of higher respiration rates, which are related to metabolism acceleration and climacteric ripening.

2.4. Effects of PEF on enzyme and respiratory activities of whole carrots

As previously mentioned, changes in bioactive content may result from either the activation of their biosynthesis pathways triggered by stress response and/or their enhanced extractability favoured by structural modifications. In order to understand the main causes behind such changes, the influence of PEF on the physiological response of whole carrots was investigated. Conditions were selected based on previous results: carrots were subjected to PEF (5 pulses of 3.5 kV cm^{-1}) and stored for 12, 24 and 36 h at 4 ºC. Hence, the influence of PEF on pectinolytic [pectinmethylesterase (PME) and polygalacturonase (PG)], oxidative [polyphenol oxidase (PPO) and peroxidase (POD)], and biosynthetic [phenylalanine ammonia lyase (PAL)] enzyme activities, phenolic profile and respiration was evaluated during storage.

PAL is the key enzyme in the phenylpropanoid biosynthesis pathway. Determination of PAL activity can provide relevant information about the causes of previously reported phenolic accumulation. The applied PEF treatment influenced PAL activity of carrots, which progressively increased during post-treatment time, whereas that of untreated carrots remained unaltered. The accumulation of phenolic compounds match with the enhancement in PAL activity over 24 h after treatment. Thereafter, phenolic content decreased regardless PAL activity. These results suggest that the phenolic utilization rate to scavenge ROS was higher than their biosynthesis rate. The impact of PEF treatments on the PAL activity of plant products has been scarcely studied, but similar results to those reported in this thesis have been observed by other authors in apple and

tomato cell cultures (Balaša, 2014; Gürsul, Gueven, Grohmann, & Knorr, 2016). Under the applied conditions, the phenolic content from untreated and just treated carrots was similar, which suggests that extraction was not immediately enhanced. Moreover, PAL activity at 24 h after PEF treatment was higher than that of untreated carrots, which is in accordance with the highest phenolic accumulation. Besides, firmness was maintained after such treatment conditions. These results are consistent with the statement that the phenolic content enhancement at 12 h and 24 h after PEF treatment is mainly due to the induction of a plant defence response. Nevertheless, better extractability caused by cell membrane disruption cannot be totally dismissed (Fincan, DeVito, & Dejmek, 2004; Jaeger, Schulz, Lu, & Knorr, 2012; Lebovka, Praporscic, Ghnimi, & Vorobiev, 2005).

PG and PME are considered as main hydrolysis enzymes related to loss of cellular integrity. Hence, their activities were studied in order to understand structural changes after PEF and their relationship with the enhancement in bioactive compounds. To the best of our knowledge, the effect of PEF treatments on pectinolytic enzymes activity in a whole commodity has not been previously evaluated. PME activity was affected by PEF application and post-treatment time. PEF-treated carrots showed an instant increase in PME activity (164 %) compared to untreated carrots. Thereafter, both remained rather stable during storage. PME activity is usually activated to strengthen tissues through cross-linking between pectin when cell disruption occurs. Therefore, results suggest that PEF induce immediate PME activation, most likely as a consequence of pore formation and cell disruption. Besides, PEF also had an impact on PG activity. PG activity values were lower (31 – 32 %) than those observed in untreated carrots just after PEF and throughout storage time. Softening has been usually associated to high PG activity, although, under stress conditions, PME can act linearly instead of randomly, which causes the strengthening of cell walls and the reduction of PG activity (Kader & Lindberg, 2010; Micheli, 2001).

Respiration rate and volatile organic compounds production can provide information about metabolic activity in vegetable products (Sheshadri, Nishanth, & Simon, 2016) submitted to abiotic stress factors. Both were considerably affected by PEF treatments given that carrots produced 123–164 % more CO_2 than those untreated from 12 h to 36 h of storage. Additionally, higher amounts of ethylene (50 ng kg^{-1} s^{-1}), ethanol (68 ng kg^{-1} s^{-1}) and acetaldehyde (7 pg kg^{-1} s^{-1}) were detected in PEF-treated carrots, whereas these metabolites were not found in untreated carrots. This presence has also been reported in PEF-treated tomatoes and apples (Dellarosa et al., 2016; González-Casado et al., 2018a). The increase in respiration may account for the generation of ROS and ethylene, which act as signals triggering the biosynthesis of antioxidant compounds (e.g. carotenoids and phenolic compounds). Likewise, the presence of acetaldehyde

and ethanol may suggest that anaerobic metabolism could have been triggered due to structural damage and intracellular content leakage caused by PEF.

Plants have developed non-enzymatic (i.e. biosynthesis of phenolic and carotenoid compounds) and enzymatic (i.e. PPO, POD) mechanisms to scavenge ROS and avoid their excess, which would cause severe cellular damage (Smith-Becker et al., 1998). After PEF application, PPO activity was not significantly affected. On the other hand, POD activity of carrots was influenced by PEF. POD activity showed a 12 h delay in the peak of maximum activity compared to the trend observed for untreated carrots. According to our results, electropermeabilization can favour the contact between substrates and corresponding enzymes, enhancing their activity. However, such contact can also be difficulted by substrates relocation (Balaša, 2014). Furthermore, reversible changes in protein conformation could lead to a decrease in their biological activity (Yeoh & Ali, 2017). Likewise, enzymes such as catalase, superoxide dismutase or glutathione peroxidase (Gill & Tuteja, 2010) could be acting as ROS scavengers. Therefore, in-depth studies about gene expression and additional enzyme activities are necessary to fully understand defence response against PEF abiotic stress.

2.5. Effects of PEF on the bioaccessibility of bioactive compounds in whole carrots

A higher content of bioactive compounds does not necessarily entail a greater bioaccessibility. Hence, it is important to evaluate the bioaccessible fraction to estimate the potential effect that consumption of whole carrots or their derived products on health. The effect of a selected PEF treatment on carotenoid and phenolic bioaccessibility of whole carrots was investigated. Applied conditions (5 pulses of 3.5 kV cm^{-1} and post-treatment time of 24 h at 4 ºC) were based on previous results in which one of the highest phenolic contents was obtained while keeping the original carotenoid content without significantly altering their quality attributes.

2.5.1. Effects on carotenoids bioaccessibility

Bioaccessibility of carotenoids was influenced by PEF application. Besides, bioaccessibility was differently affected depending on the individual compound. While carotenoids from untreated carrots were differently released from the carrot matrix [phytoene (22.4 %) > lutein (16.7 %) > β-carotene (11.2 %) > α-carotene (9.1 %)], the application of PEF caused an increase in such rates, reaching 20 – 26.8 % in any of them. Such release from the carrot tissue involved an increase in carotenoids bioaccessibility. Hence, the bioaccessibility of

carotenoids in PEF-treated carrots was higher (11.9 %) than that in untreated carrots (6.6 %). Phytoene (17 %) and lutein (23.6 %) were the most bioaccessible compounds, although their bioaccessibility ratio was not affected by PEF treatments. On the other hand, the bioaccessibility of α-carotene and β-carotene outstandingly improved by 65 % and 58.7 %, respectively. In concomitance with our results, some authors have reported that less hydrophobic carotenoids generally are highly bioaccessible (e.g. lutein), since they are easily incorporated into micelles (Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Pérez-Sacristán, et al., 2007; Tyssandier, Lyan, & Borel, 2001). Carotenoid bioaccessibility is strongly influenced by factors related to matrix structure. Hence, cell wall degradation and changes in intracellular carotenoids location observed in PEF-treated carrot tissues may suggest that electropermeabilization improves the release of carotenoids during digestion. Moreover, pectin may also interact with bile salts or digestive enzymes involved in the formation of micelles, which comprises carotenoids bioaccessibility (Cervantes-Paz, Ornelas Paz, Pérez-Martínez, Reyes-Hernández, Zamudio-flores, & Rios-velasco, Ibarra-Junquera, Ruiz-Cruz, 2016; Jeffery, Holzenburg, & King, 2012). In this regard, PEF could also activate some pectic enzymes related to tissue softening, which would be helpful for a better transference of carotenoids to micelles.

2.5.2. Effects on phenolic compounds bioaccessibility

The application of PEF led to a significant increase in the overall bioaccessibility of phenolic compounds (20.8 %), compared to 13.6 % observed for untreated carrots. Bioaccessibility also varied between chemical groups: coumaric acid derivatives (85.2 %) > ferulic acid derivatives (40.1 %) > caffeic acid derivatives (4.4 %). Most caffeic acid derivatives from PEF-treated carrots had lower bioaccessibility than untreated carrots whereas that of ferulic acid derivatives was generally maintained. However, the bioaccessibility of some individual compounds, e.g. isoferulic acid (62.2 %), was enhanced. Bioaccessibility of phenolic compounds is highly dependent on their initial concentration, matrix structure, chemical structure and interactions during digestion (Ribas-Agustí, Martín-Belloso, Soliva-Fortuny, & Elez-Martínez, 2018). The content of some phenolic compounds in the non-digested fraction varied after PEF, which directly led to bioaccessibility changes. However, the most abundant compounds were not necessarily the most bioaccessible. The changes in phenolic bioaccessibility could depend on the decrease in carrot firmness, which in turn would be related to microstructural rearrangements and cell wall degradation. In agreement to our results, some authors have found an inverse correlation between bioaccessibility of some compounds and firmness of PEF-treated apples (Ribas-Agustí, Martín-Belloso, Soliva-Fortuny, & Elez-Martínez, 2019b), although some compounds were independent from the matrix integrity. Interactions also play an important role in bioaccessibility. Hence, larger molecules with many hydroxyl groups would be more propense to interact and form

complexes with other macromolecules, which make them less bioaccessible than low molecular weight compounds (Jakobek, 2015). Likewise, some of them would be more exposed to be degraded or suffer modifications (e.g. isomerization, glycosylation), which would affect bioaccessibility (Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, 2015).

3. Enhancing content and bioaccessibility of bioactive compounds in carrot derived products throughout PEF application

Once we ascertained the feasibility of applying PEF treatments to increase the bioaccessibility of carotenoids and phenolic compounds in whole carrots, we aimed at evaluating how the treatments impact on their bioaccessibility in carrot derived products. A first experiment was carried out in order to evaluate the bioaccessibility of carotenoids and phenolic compounds in three carrot derived products (juices, purees and oil-added purees) obtained from PEF-treated carrots. Based on the obtained highest bioaccessibility results, carotenoid and phenolic bioaccessibility was also assessed after directly applying PEF to oil-added purees.

3.1. Application of PEF treatments to whole carrots before processing into derived products

3.1.1. Effects of PEF on content and bioaccessibility of carotenoids in carrot-derived products

The application of PEF to whole carrots before obtaining derived products did not affect carotenoid content in any of the matrices. These results were in accordance with those previously reported in which PEF-treated whole carrots had similar content as those untreated when 24 h post-treatment was elapsed. Conversely, some authors have reported increases in total carotenoid content of juices obtained from PEF-treated peppers (1 kJ kg^{-1}) and tomatoes (1 pulse of 1 kV cm^{-1}) (Jayathunge et al., 2017; Rybak et al., 2020), which were attributed to better extractability caused by membrane disruption. Differences among carotenoid content from different products were mainly due to mechanical processing since neither further thermal treatment nor oil addition caused significant changes in carotenoid amount. Hence, the highest content was obtained in carrot juices (43.4 mg/100 g FW), which was similar to that previously determined in whole carrots. Nevertheless, both types of puree showed lower total content (21.3 – 23.2 mg/100 g FW). Besides, α-carotene and β-carotene contents decreased approximately by 63.4 % and 53.4 % in both purees compared to those obtained in whole carrots, whereas in juices α-carotene was

reduced by 40.7 %. Mechanical processing has been widely related with enhanced carotenoids extractability (Lemmens, Colle, Buggenhout, Loey, & Hendrickx, 2014). However, these compounds are also prone to oxidation due to their unsaturation (Rodriguez-Amaya, 2010). Therefore, disruption of carrot tissues by blending and decreases in particle size may have increased the contact between such carotenoids and lipoxygenase enzymes, thus promoting their degradation.

Total carotenoid bioaccessibility was not significantly affected by PEF treatment. However, mechanical and thermal processing as well as oil addition exerted a strong influence on carotenoids bioaccessibility. Carotenoids in oil-added purees obtained from PEF-treated carrots (5.3 %) were the most bioaccessible, followed by those in oil free purees (3.1 %) and juices (0.3 %). Oil addition and thermal treatment led to further enhancement of the carotenoids bioaccessibility, reaching 7.8 %. Regarding individual compounds, PEF only affected bioaccessibility of β-carotene in juices, which was decreased by 42.3 %. Similar decrease in β-carotene bioaccessibility of PEF-treated tomato chromoplast fraction was reported by Bot et al., (2018), which was attributed to induced modifications in the carotenoids-protein complexes limiting their bioaccessibility.

Previous studies have demonstrated that matrix disruption is critical for enhancing carotenoids bioaccessibility since they are stored in chromoplasts or bound to membrane (Faulks & Southon, 2005; Parada & Aguilera, 2007). Our results are in total agreement with these observations since bioaccessibility was enhanced in both thermally-treated purees and juices, which was probably related to their reduced particle size. However, carotenoids bioaccessibility in juices was the lowest one, although juices had a lower particle size than purees. This suggests that other factors such as pectin, interactions with other macromolecules or individual carotenoid structures are involved in bioaccessibility changes. The presence of pectin seemed to increase the size of micelles and consequently carotenoid bioaccessibility decrease (Gence, Servent, Poucheret, Hiol, & Dhuique-Mayer, 2018), which is in agreement with obtained results of particle size in the intestinal phase of those juices obtained from PEF-treated carrots (data not published). Thermal processing could lead to a greater depolymerisation of pectin from cell walls or to the triggering of the conversion of *trans* isomers to *cis* isomers, which would enhance bioaccessibility (Lemmens, Van Buggenhout, Oey, Van Loey, & Hendrickx, 2009; Marx, Stuparic, Schieber, & Carle, 2003; Ribas-Agustí, Buggenhout, Palmero, Hendrickx, & Loey, 2014). In addition, incorporation to micelles is another essential requirement for carotenoids absorption. Therefore, the absence of lipids probably prevented carotenoids micellarization in juices and oil free purees. According to Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, (2012) and Huo, Ferruzzi, Schwartz, & Failla, (2007) the incorporation of oils rich in long-chain polyunsaturated

fatty acids (e.g. olive oil) lead to increase carotenoids bioaccessibility, which is in agreement to our results.

3.1.2. Effects of PEF on content and bioaccessibility of phenolic compounds in carrot-derived products

Phenolic content was affected by both PEF treatment and further thermal and mechanical processing, whereas oil addition did not cause significant changes. Hence, total phenolic content of purees and oil-added purees obtained from PEF-treated carrots was maintained, whereas juices obtained from PEF-treated carrots showed a significant reduction (38.5 %) of the overall phenolic content compared to those untreated. Phenolic content was also affected by mechanical processing since juices (124 ± 8 mg kg^{-1} DW) had greater content than oil-added purees (77 ± 20 mg kg^{-1} DW) and purees (37 ± 5 mg kg^{-1} DW). Otherwise, the effect of thermal processing on phenolic concentration depended on the type of derived product. While phenolics from juices were not affected, content was doubled in purees without oil. Regarding phenolic profile, purees were mainly composed by 5-caffeoylquinic acid, coumaroylquinic acid, 5-feruloylquinic acid and ferulic acid caffeoyl glucoside. Juices had a similar phenolic composition, although caffeoylshikimic acid or caffeic acid arab/xiloside were only found in this product matrix. Probably, phenolic compounds from juices are easily released given that their low dietary fibre content (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011) and their lower particle size compared to purees.

The effect of PEF on phenolic composition was dependent on each individual compound and on the type of derived product. In general, phenols from purees were not strongly affected by PEF, excepting coumaric acid that increased by 41.2 % and caffeic acid that decreased by 64.7 % in oil-added purees. On the other hand, the amount of coumaric [e.g. coumaroylquinic acid (60 %)], caffeic [e.g. 5-caffeoylquinic (68.5 %)] and ferulic acid [e.g. 5-feruloylquinic acid (32.6 %)] derivatives generally decreased in juices. Previous studies reported a phenolic accumulation in PEF-treated carrots (López-Gámez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, 2020), which was not observed in derived products. These results could be explained by degradation of some compounds during juicing and blending, the formation of new interactions that hinder their complete extraction (Ribas-Agustí et al., 2018) or their amount remaining in pomace (Rybak et al., 2020).

Further thermal treatment generally caused increases in caffeic acid and their derivatives content from both juices and purees. This has also been reported by He et al., (2016) and Rodríguez-Roque, Ancos, Sánchez-Moreno, Cano, Elez-Martínez, Martín-Belloso, (2015) in

thermally-treated fruit juices, which was probably related to reduction of oxidative enzyme activities.

Bioaccessibility of total phenolic compounds was affected by PEF treatment and further mechanical processing. PEF caused the highest increase in phenolic bioaccessibility of purees, which reached 100 %. Nevertheless, that of juices (27.9 %) and oil-added purees (24 %) was not significantly affected. On the other hand, neither a further thermal treatment nor oil addition influenced total bioaccessibility of any derived product. Regarding individual compounds, most caffeic and ferulic acid derivatives in purees obtained from PEF-treated carrots had increased bioaccessibility. In oil-added purees, bioaccessibility of coumaric acid derivatives increased whereas that of some caffeic derivatives (e.g. caffeic acid arabinoside glucoside) decreased. In juices, PEF did not cause significant effects in phenolic bioaccessibility compared to untreated juices. On the other hand, further thermal treatment increased caffeic and ferulic acid derivatives bioaccessibility in all derived products, which was probably related to their lower particle size and modifications in pectin state.

Bioaccessibility was dependent on the product matrix and individual compound. Juices had lower particle size (D [4, 3] = 487 μm) than purees (D [4, 3] = 596 μm), which favoured their leakage in non-digested fraction. Nevertheless, phenols were also more available to be entrapped by water soluble pectin or be degraded during digestion, which would explain their lower bioaccessibility in juices. Results suggest that PEF was not enough to break the bonds that link phenols to cell wall and some of them may remain in the insoluble fraction. On the other hand, phenolic bioaccessibility in oil free purees was strongly increased. Despite no correlation was found with particle size, cell permeability changes could have caused their better dialysis. Limited information about phenolic bioaccessibility in juices and purees as well as the influence of oil addition are available in literature. Therefore, further studies about pectin content and characteristics would be necessary to delve into the causes that produce discussed changes in bioaccessibility.

3.2. Application of PEF treatments to processed derived products

The application of PEF to whole carrots before obtaining purees and the further oil-addition led to boost phenolic and carotenoid bioaccessibility, respectively. Hence, based on such results and considering the strong relationship between bioaccessibility and matrix structure, the influence of applying PEF directly to oil-added carrot purees on carotenoid and phenolic bioaccessibility was investigated.

3.2.1 Effects on content and bioaccessibility of carotenoids

As previously mentioned, main carotenoids present in purees were α-carotene, β-carotene and lutein. The application of PEF did not cause significant changes in total carotenoid content. Likewise, further thermal processing was not enough to cause neither degradation nor better extractability of carotenoid compounds. Regarding individual compounds, only lutein contents were increased after PEF treatment. Decreases in particle size of purees could be related to their better extractability. Likewise, lutein could be transfer more efficiently to oil than β-carotene (Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Blanco-Navarro, et al., 2007; Tyssandier et al., 2001).

Despite total carotenoid contents remained unaltered, the direct application of PEF to purees led to a dramatic enhancement of total carotenoid bioaccessibility by 252.8 %. This reached 18.7 %, which was similar to that obtained in further thermally-treated purees (20.4 %) and higher than those of untreated (5.3 %) and just thermally-treated purees (10.1 %). Likewise, α-carotene and β-carotene in just PEF-treated purees were increased by 231.8 % and 256 %, respectively. Besides, bioaccessibility of lutein reached 24 %, whereas it was not bioaccessible in untreated purees. Similar increments were obtained in purees treated with PEF and further thermal treatment. Increased bioaccessibility and carotenoid contents found in digested fractions (released, micellar and non-digested oil) corroborate that carotenoids from PEF-treated oil-added puree were better released from matrix and solubilized into micelles than those from untreated or just thermally-treated purees. These results could be explained by microstructural and particle size distribution changes caused by electropermeabilization. As far as we know, scarce information about particle size distribution from PEF-treated products and its relationship with bioaccessibility is available. However, it has been demonstrated that a decrease in particle size is highly correlated to an enhanced carotenoid bioaccessibility in high-pressure homogenized carrot and tomato purees (Knockaert, Lemmens, Van Buggenhout, Hendrickx, & Van Loey, 2012; Moelants et al., 2012). Untreated purees were mainly composed by cell clusters with a mean particle size of 448.97 μm and 29 % of particles below 100 μm, which corresponds to single cells or fragments of them. Meanwhile, PEF-treated purees and those further thermally-treated showed 61 % of smaller particles (< 100 μm) with lower mean size (305.21 μm). These results, together with microscopy images, allow concluding that intracellular components such as carotenoids were better released after PEF and were more available to be incorporated into micelles during digestion. On the other hand, thermal treatment may cause the weakening of cell membranes and pectin release, which led to increase bioaccessibility to a lesser extent than PEF. Additionally, carotenoids from untreated purees were retained in the non-digested oil fraction, whereas carotenoid content in such fraction dropped by 68.8 % in PEF-treated purees. This suggests that

PEF had a beneficial effect in carotenoids transference to oil and their subsequent micellarization, which could be associated to the presence of smaller droplet sizes that could increase the surface area for lipase action and solubilization of carotenoids (Tydeman et al., 2010).

3.2.2. Effects on content and bioaccessibility of phenolic compounds

PEF treatment dramatically decreased total phenolic content of purees (78.2 %). Further thermal processing led to a similar reduction. However, phenolic content was maintained just after the application of thermal treatment. Individual compounds were differently affected depending on their chemical structure. In this line, caffeic acid and their derivatives as well as some ferulic derivatives were extremely sensitive to PEF. For instance, 5-caffeoylquinic acid and ferulic acid coumaroyl glucoside decreased by 98.6 % and 56.6. %, respectively. Nevertheless, some increments were also observed in coumaric acid (117.8 %) and feruloylquinic acid derivatives (79.03 % and 288.9 %). After applying PEF and further thermal treatment most of caffeic acid derivatives were not affected and the most abundant compound, 5-caffeoylquinic acid, decreased by 39.9 %, which was not as much as in PEF-treated puree (98.6 %). Besides, ferulic glucosides derivatives and coumaric acid were also negatively affected. On the other hand, some compounds increased after treatments, such as coumaroylquinic acid (112.1 %) or 4-feruloylquinic acid (46.2 %). Changes in phenolic content are difficult to explain due to their heterogeneity, although cell disruption and particle size decrease could be involved. On the one hand, release of intracellular content could improve their extractability. On the other hand, this also would facilitate their degradation due to greater contact between phenolic compounds (e.g. caffeic acid derivatives) and oxidative enzymes. Additionally, interactions between compounds and cell wall fragments are more probable when the number of small particles is high (Nguela, Poncet-legrand, Sieczkowski, & Vernhet, 2016), which could entrap phenolic compounds in hydrophobic pockets (Bourvellec, Bouchet, & Renard, 2005). Differences between phenolic content of PEF-treated purees and those further thermally-treated could be due to membrane disruption caused by the treatment, which increased contact of oxidative enzyme (e.g. PPO and POD) with phenolic substances, whereas thermal treatment reduce their activities (Anthon & Barrett, 2002; Soysal & Söylemez, 2005).

Despite the decrease in phenolic content after PEF, their total bioaccessibility reached 100 %, whereas that of untreated and just thermally-treated purees exhibited a much lower bioaccessibility (30.7 % and 40.5 %, respectively). Phenolic compounds bioaccessibility was also affected by their chemical structure. Thus, untreated purees showed different bioaccessibilities: ferulic acid derivatives (98.1 %) > coumaric acid derivatives (74.3 %) > caffeic acid derivatives (20.1 %). On the other hand, those of PEF-treated purees, regardless the further thermal treatment,

suffered some modifications: caffeic acid derivatives (100 %) = ferulic acid derivatives (100 %) > coumaric acid derivatives (86.4 %). Bioaccessibility was generally enhanced after PEF, although it decreased for some compounds, e.g. caffeoylferuloylquinic acid or feruloylquinic acid derivatives. Some authors have proposed that phenolic structure may suffer modifications during digestion, which would improve or hinder their bioaccessibility (Buniowska, Carbonell-Capella, Frigola, & Esteve, 2017; Ribas-Agustí et al., 2018). Our results could be related to changes in puree microstructure and decreases in particle size, as previously discussed. Phenolic compounds can be stored in vacuoles or bound to cell wall macromolecules (Shahidi & Yeo, 2016). In general, those bound phenolics are difficultly absorbed due to digestive enzymes are unable to reach them. However, cell disruption and breakage of cell clusters by PEF application could facilitate the access of digestive fluids, leading to improve their release.

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CONCLUSIONS

CONCLUSIONS

The results obtained from the different studies performed in this Doctoral Thesis led to the following conclusions:

- PEF is a feasible technology for improving the phenolic content of whole carrots without altering their physicochemical characteristics. Total phenolic content and quality attributes from whole carrots were influenced by PEF intensity and post-treatment time. The highest increases were obtained in carrots subjected to 5 pulses of 3.5 kV cm^{-1} (39.5 %) and 30 pulses of 0.8 kV cm^{-1} (40.1 %) and stored for 24 h at 4 ºC. At such conditions, hardness and colour were not significantly affected, but cell disruption promoted the leakage of intracellular content and a decrease in weight loss and cell viability (83 – 84 %).

- Post-treatment time influenced phenolic compounds content, respiration and enzyme activities of PEF-treated carrots. Considerable increases in *p*-hydroxybenzoic acid (94.7 %), chlorogenic acid (74.9 %) and ferulic acid (52.2 %) were observed after 24 h of applying PEF. In concomitance, some indicatives of stress induction were detected: larger CO_2 production, the presence of acetaldehyde and ethanol, the enhancement of 50 % in PAL activity and the alteration of POD, PME and PG activities during storage.

- PEF treatments were effective in enhancing carotenoid content in whole carrots. Total and individual carotenoids were affected by electric field strength and post-treatment time. Total carotenoids increased by 39.3 – 81.3 % just after 2 kV cm^{-1} and 3.5 kV cm^{-1} treatments. At such conditions, phytoene (59.1 – 95.5 %) and β-carotene (36.5 – 91.8 %) increased, whereas lutein decreased (15.1 – 96.4 %) for treatments of 0.8 kV cm^{-1} or 2 kV cm^{-1}. Positive correlations were found between specific energy input and individual carotenoids: phytoene (r = 0.761), lutein (r = 0.648) and α-carotene (r = 0.402). Likewise, significant correlations were found between the hue angle of the cortex and lutein (r = 0.701) as well as between browning index and phytoene (r = 0.638) and β-carotene (r = 0.683). Total soluble solids and pH were not affected by the PEF treatments.

- The application of PEF (5 pulses of 3.5 kV cm^{-1}) to whole carrots affected their phenolic content, and improved carotenoids and phenolic bioaccessibility. Additionally, PEF caused microstructural modifications in cell walls and promoted changes in the location of intracellular carotenoids. Carotenoid content was unaltered after PEF, whereas their total bioaccessibility rose to 11.9 %. Likewise, the main carotenoids, α-carotene and β-

carotene, were bioaccessible by 9.9 % and 11.9 %, respectively. Phenolic content and bioaccessibility were differently affected depending on their chemical structure. While coumaric acid content was enhanced by 163.1 %, bioaccessibility of caffeic acid derivatives decreased by 70.2 %. Likewise, total phenolic bioaccessibility was enhanced (20.8 %), whereas individual compounds were differently affected following this sequence: coumaric acid derivatives (74.6 %) > ferulic acid derivatives (40.6 %) > caffeic acid derivatives (4.7 %).

- Content and bioaccessibility of phenolic and carotenoid compounds in derived products were affected by the application of PEF (5 pulses of 3.5 kV cm^{-1}) before further mechanical and thermal processing (70 ºC for 10 min). Likewise, bioaccessibility was also affected by oil addition. The highest phenolic bioaccessibility was reported in purees obtained from PEF-treated carrots (100 %), whereas carotenoids were more bioaccessible in oil-added purees after a subsequent thermal treatment (7.8 %). On the other hand, PEF neither affected carotenoid content in any derived product nor the phenolic content in purees. However, phenolic content of juices obtained from PEF-treated carrots decreased by half. Particle size and microstructure of derived products obtained from PEF-treated carrots remained similar to those untreated. However, further thermal treatment caused decreases in D [3, 2] of juices. Conversely, D [3, 2] was increased in thermally-treated purees.

- Bioaccessibility of carotenoids and phenolic compounds was enhanced in PEF-treated (5 pulses of 3.5 kV cm^{-1}) oil-added carrot purees as well as in those subjected to a thermal treatment. These purees were composed by smaller cells (D [3, 2]) with irregular shapes. Besides, the application of PEF led to the highest carotenoid (18.7 %) and phenolic (100 %) bioaccessibility. Conversely, total carotenoid content was not affected by PEF, whereas phenolic content markedly decreased. Individual phenols were differently affected depending on their chemical structure. Caffeic acid derivatives dramatically decreased whereas the contents of coumaric and ferulic derivatives remained similar to those found in untreated oil-added purees.