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

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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*-OH-benzoic (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

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 et al., 2020). 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 (10 - 500 kV m^{-1}; 500 - 20000 J 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 (185 kV m^{-1}) to carrot slices increased

77 carotenoid extraction up to 11 %. Aguiló-Aguayo et al., (2014) demonstrated that PEF application
78 (100 – 400 kV m^{-1}) enhanced the extractability of sugars from carrot. In addition, changes in cell
79 permeability caused by PEF has been used to improve osmotic dehydration (Amami et al., 2007),
80 drying efficiency (Wiktor et al., 2019) or reduce cutting force (Leong et al., 2014) of carrots.

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

89 The induced stress response after PEF in plant tissues has not been completely discerned. An
90 increase in the phenylalanine ammonia lyase (PAL) activity, key enzyme in the phenylpropanoid
91 metabolism, and in the phenolic content has been reported after applying some non-thermal
92 technologies such as ultrasound (Cuéllar-Villarreal et al., 2016a; Yu et al., 2016). However,
93 information regarding the effects of PEF is yet scarce. Balaša (2014) reported increases of 54 %
94 in PAL activity and 20 % in total phenolic content 9 h after applying PEF (4.1 J kg^{-1}) to apple cell
95 cultures. In addition, Vallverdú-Queralt et al., (2013) reported that PEF-treated tomatoes (30
96 pulses of 120 kV m^{-1}) exhibited an increase in total polyphenols and in some individual compound
97 contents (39 % - 170 %) 24 h after treatment. Similar results were obtained by López-Gámez et
98 al., (2020), who reported an increase in total phenolic content (40 %) at 24 h after applying PEF
99 (5 pulses of 350 kV m^{-1}) to whole carrots. On the other hand, González-Casado et al., (2018)
100 reported changes in respiration rate and production of some volatile compounds (e.g. ethylene) in
101 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 dried 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 350 kV m^{-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 200 and 400 kV m^{-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 analyzer (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

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

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

164 **2.6. Polygalacturonase (PG) activity**

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

173 **2.7. Polyphenol oxidase (PPO) activity**

174 PPO extraction was based on the procedure described by Alegria et al. (2016) with slight
175 modifications. In this case, PPO was extracted from carrot tissues (4 g) adding 15 mL of cold
176 phosphate buffer (0.1 M; pH 6.5) and 0.4 g of polyvinylpyrrolidone. Then, samples were vortexed
177 for 1 min and centrifuged at 20000 × g for 15 min at 4 ºC. The resulting supernatant was filtered
178 across Whatman No. 1 filter. During the whole procedure, samples were maintained in an ice-
179 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).

206 **2.10. Extraction of phenolic compounds**

207 Free and bound phenolic compounds were extracted following the methodology proposed by
208 Mattila and Kumpulainen (2002) with slight modifications, in which acid and alkaline hydrolysis
209 were performed. The modification consisted in adding a centrifugation step (8784 × g for 5 min)
210 before filtrating samples through a polytetrafluoroethylene (PTFE) membrane filter (0.45 μm, ø
211 13mm) (Labbox Labware S.L., Barcelona) for the High-Performance Liquid Chromatography
212 (HPLC) analysis. After quantification, the results from alkali and acid hydrolysates were
213 calculated to represent total phenolic acids expressed on a dry weight basis (mg kg^{-1}).

214 **2.11. Identification and quantification of phenolic compounds by High-Performance Liquid**
215 **Chromatography (HPLC)**

216 Phenolic compounds were separated following the procedure described by Morales-de la Peña,
217 Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2011) using a reverse-phase C18 Spherisorb
218 ODS2 (5 μm) stainless steel column (4.6 mm x 250 mm) at room temperature. The HPLC system
219 comprised of a 600 Controller, a 486 Absorbance Detector programmed to scan from 200 to
220 350 nm, a thermostatic column compartment, and a 717 Plus Auto Sampler with cooling system
221 (Waters, Milford, MA).

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

227 **2.12. Statistical analysis**

228 Statistical analyses were carried out using the SigmaPlot software (version 11.0, Systat Software
229 Inc, Chicago, IL, USA). Three different replicates were assayed for every treatment condition.
230 Each replicate consisted of 6 carrots. Enzymes and phenolic extractions were carried out twice
231 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 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 et al., 2005). Some authors have previously reported a decrease in O_2 consumption and CO_2 production in PEF-treated apple cylinders subjected to 25 and 40 kV m^{-1} (Dellarosa et al., 2016), 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 et al., 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 10 to 40 kV m^{-1}.

Ethylene synthesis is enhanced by stress (Chervin et al., 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.

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 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 et al., 2014). PPO activity of PEF-treated carrots trended upwards while they remained constant in untreated carrots (**Figure 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 and Elez-Martínez, 2005) due to lose of conformation, but after applying moderate electric fields (50 - 500 kV m^{-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 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 et al., 2017). A similar mechanism may

311 be proposed to explain the PME activation after PEF application, given that electroporation causes
312 cell wall damage (Janositz and Knorr, 2010). In fact, some authors have previously reported an
313 increase of PME activity after the application of PEF. Samaranayake and Sastry (2016) displayed
314 that PME activity of tomato homogenates was enhanced after applying 0.008 kV m^{-1} combined
315 to thermal treatment (69.5 ºC), while at higher temperatures inactivation occurred, probably due
316 to enzyme denaturation.

317 Besides, PG activity was also significantly affected by PEF application (**Figure 1D**) as PEF-
318 treated carrots showed a decrease between 31 % - 32 % in PG activity compared with those
319 untreated at the same storage time. High intensity pulsed electric fields technology has been
320 widely applied to inactivate or reduce enzyme activity (e.g. PME and PG) in liquid products
321 (Martín-Belloso et al., 2014). However, to the best of our knowledge, this is the first study in
322 which PG activity is evaluated after PEF application to whole commodities. An increase in PG
323 activity is associated with the solubilization of pectic substances and softening in many fruit and
324 vegetables. However, PME and PG enzymes act differently depending on pH, ionic strength and
325 methyl esterification grade of pectin (Micheli, 2001). PME may act randomly,
326 demethylesterifying pectin chains, causing the release of protons and promoting PG activity,
327 hence reducing cell wall rigidity. On the other hand, PME may act linearly allowing the
328 interaction of free carboxyl groups with Ca^{2+}. In this situation, PG activity is reduced, and cell
329 wall structure is maintained. This mechanism may be triggered by increases in intracellular pH,
330 considered as signals of stress detection (Kader and Lindberg, 2010), which have been reported
331 in some PEF-treated fruit (González-Casado et al., 2018). This suggests the second alternative as
332 the most likely to occur.

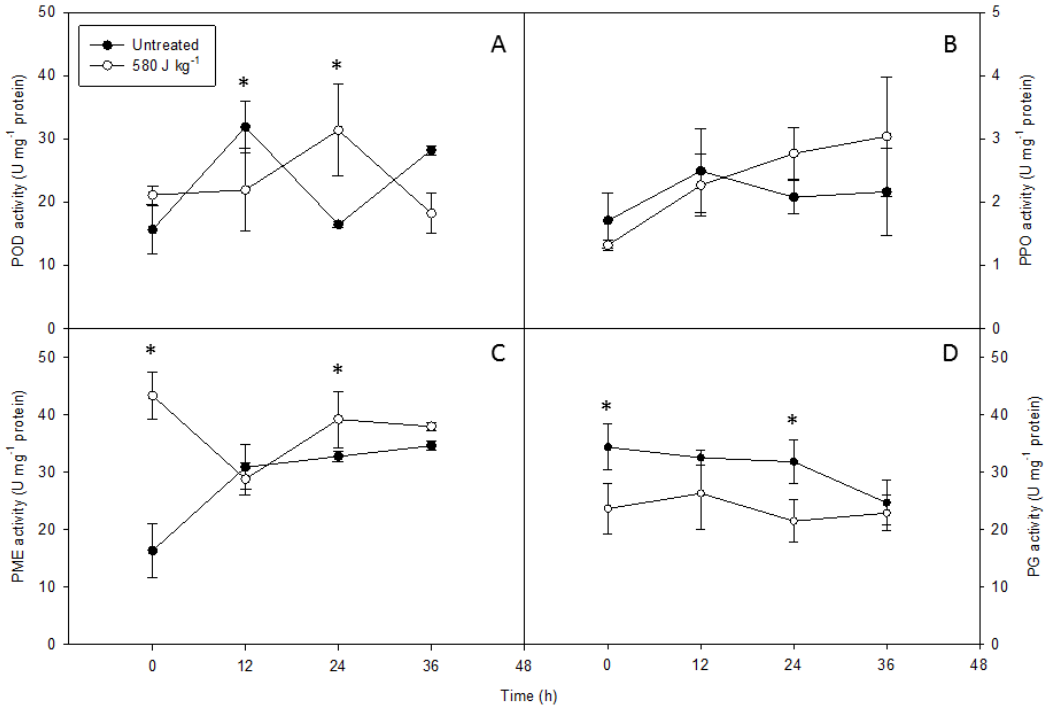

Fig 1. Effects of PEF and post-treatment time on the enzyme activity of (A) POD; (B) PPO; (C) PME and (D) PG in carrots stored at 4 ºC. Values represent the mean of 3 replicates with their standard error bars. Data points with an asterisk (*) indicate statistical difference ($p < 0.05$) between the untreated 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 2A**). Main phenolic compounds found in this study include hydroxybenzoic acids (*p*-OH-benzoic 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**). 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 and 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)**. These results may indicate their release through the formed pores, which is likely due to their high affinity to water (Sánchez-Rangel et al., 2014). Protocatechuic acid, chlorogenic acid and total phenolic content **(Table 2** and **Figure 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*-OH-benzoic (94.7 %), chlorogenic acid (74.9 %) and ferulic acid (52.2 %) were observed in comparison to those found in untreated carrots **(Table 2)**. Later on, 36 h after PEF treatment, *p*-OH-benzoic acid, chlorogenic acid, ferulic acid and total phenolic content decreased. These results are in accordance to 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*-OH-benzoic acids (**Table 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 and 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 and 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., 2016b).

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 2B**).

The enhancement in PAL activity at 24 h was in accordance with the accumulation of phenolic compounds at that time (**Figure 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 et al., 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 (200 kV m^{-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 120 kV m^{-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 and 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).

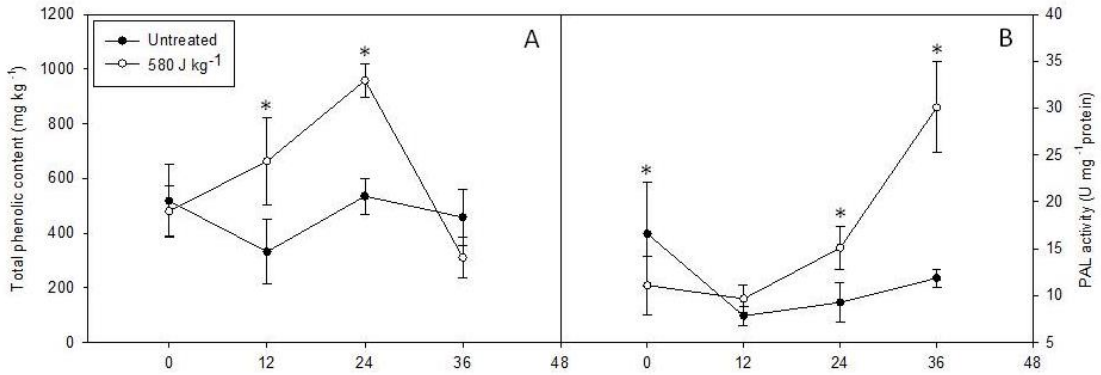

Fig 2. Effects of PEF and post-treatment time on (A) total phenolic content and (B) PAL activity of carrots stored at 4 ºC. Values represent the mean of 3 replicates with their standard error bars. Data points with an asterisk (*) indicate statistical difference ($p < 0.05$) between the untreated and PEF-treated carrots.

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*-OH-benzoic (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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CRediT authorship contribution statement

Gloria López-Gámez: Conceptualization, methodology, investigation, formal analysis, visualization, writing- original draft. **Pedro Elez-Martínez:** Visualization, writing- review & editing. **Olga Martín-Belloso:** Visualization, writing- review & editing. **Robert Soliva-Fortuny:** Conceptualization, supervision, validation, writing- review & editing.

Declaration of Competing Interest

Authors of this article declare that there is no financial/personal interest. Conflict of interest does not exist.

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643 Table 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		nd		nd	
	580	8 ± 0.9		nd		nd		nd	
12	**Untreated**	1.3 ± 0.3		nd		nd		nd	
	580	3.3 ± 0.7	*	50 ± 28	*	7 ± 6	*	68 ± 46	*
24	**Untreated**	0.7 ± 0.1		nd		nd		nd	
	580	1.6 ± 0.4	*	34 ± 7	*	4 ± 2	*	43 ± 13	*
36	**Untreated**	0.4 ± 0.2		nd		nd		nd	
	580	1.0 ± 0.3	*	24 ± 7	*	2 ± 1	*	46 ± 5	*

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

647 Table 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*-OH-benzoic 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

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