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Pulsed electric fields as a green technology for the extraction of bioactive compounds from thinned peach by-products

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Abstract

Thinned fruits are agricultural by-products which nowadays have few economic or environmental benefits. However, previous studies have revealed that these immature fruits have a large amount of antioxidant compounds. The aim of this study was to evaluate whether pulsed electric fields (PEF) might be a suitable green technology for enhancing the extraction of phenols, flavonoids and antioxidant compounds from fresh thinned peaches, thus reducing the use of methanol. Moreover, response surface methodology has been used to determine the optimal PEF treatment conditions, observing that the solvent is the main factor. The highest amounts of bioactive compounds were extracted using 80% methanol and no PEF. Methanol combined with PEF produced a negative effect on the extraction yield. However, the use of water as a solvent increased the amount of total bioactive compounds and individual phenols (chlorogenic acid, coumaric acid and neochlorogenic acid). Thus, PEF-assisted extraction of bioactive compounds from thinned peach fruits using water as a solvent is an alternative to conventional extraction methods which require dried products, large amounts of organic solvents and long extraction times.

Keywords: thinned peach, pulsed electric field, antioxidant, phenolic compounds

1. Introduction

Thinning is an agricultural practice carried out in fruit trees to reduce the total number of fruits with the advantage of increasing both their final size and the value of the crop. The small thinned fruits are usually abandoned in the fields generating large quantities of waste and, in some cases, being incinerated, with the environmental problems which that entail (Nuncio-Jáuregui et al., 2015). Moreover, thinning has both economic and time costs which have been calculated at 3.43-4.11 euro per tree and 200-300 hours per hectare (Martin et al., 2010).

It has been demonstrated that thinned fruits might be considered as a rich source of bioactive compounds due to the high concentrations of phenols, proanthocyanidins and antioxidant compounds. For example, a high concentration of these compounds have been found in thinned fruits of apples (Zheng et al., 2012), pomegranates (Nuncio-Jáuregui et al., 2015), peaches, nectarines, (Redondo et al., 2017), etc. There is already enough evidence that these bioactive compounds found in thinned fruits play a vital role in protection against many human diseases due to their antioxidant properties (Anagnostopoulou et al., 2006). In addition, they may play technological roles in foods, for example in the control of oxidative deterioration (Ahn et al., 2008). Therefore, thinned fruit extracts may be used as supplements in the pharmaceutical, cosmetics and food industries with considerable economic and environmental benefits. For instance, extracts obtained from these thinned fruits have been successfully employed to prevent enzymatic browning in minimally processed peaches (Redondo et al., 2016).

In general, the extraction of bioactive compounds from vegetable tissues is a mass transfer process. In most situations, these compounds are found inside the cell, so their extraction requires them to pass through the cell membranes. The extraction rate

depends on the phase gradient and the resistance of the molecule to migrating from one phase to the other (Aguilera and Stanley, 1999). Fundamentally, this resistance depends on the characteristics of the medium through which the component is transferred, as well as on any type of interaction between the component and the medium itself (De Dios Alvarado and Aguilera, 2001). Traditional techniques employed for the extraction of antioxidant compounds in fruits involve the use of organic solvents (Li et al., 2006). However, the use of these kinds of solvents increases the cost of the process, may cause important environmental problems and the extraction may be negligible if the cells are intact. Therefore, it is becoming important improve on these techniques to reduce the amount of organic solvents, and increase the extraction yield. An emerging technology that has gained increasing interest in recent years for improving mass transfer operations in the food industry is Pulsed Electric Field (PEF) (Donsi et al., 2010; Knorr et al., 2011; Puértolas et al., 2012). PEF consists of the application of high external electric fields (1-50 kV/cm) for a short time (microseconds to milliseconds) to cell material inducing the electroporation of the cell membranes. Electroporation means an increase in the permeability of the cell membrane to the transport of ions and macromolecules. Therefore, the decrease of the resistance to diffusion through cell membrane makes the extraction of the bioactive compound from the cell easier, promoting the extraction yield. The application of PEF has many advantages over other techniques such as high temperatures and enzymatic treatments. PEF process has lower energy costs, the release of undesirable substances into the extraction liquid is avoided because slight denaturation of the cell membranes is caused, and the loss of thermosensitive bioactive compounds is reduced because no temperature increase is generated (Cacace and Mazza, 2003; Eshtiagi and Knorr, 2002). PEF has already been used to improve the extraction of sucrose from sugar beet (Almohammed et al., 2016, Loginova et al., 2011,

López et al., 2009a), natural colorants such as betaine (E-162) from red beet (López et al., 2009b) or carotenoids from tomato (Bot et al., 2018), phenolic compounds from red wine (El Darra et al., 2016, Yang et al., 2016, López et al., 2008) and oil from maize, olive and rapeseed (Guderjan et al., 2005).

The aim of this study is to verify whether the use of PEF increase the extraction of bioactive compounds from thinned peach fruits with low energy costs and avoiding the use of organic solvents.

2. Materials and methods

2.1. Raw materials

Fresh peaches (*P. persica* (L.) Batsch var. 'Royal Glory') were hand-thinned 48 days after blooming on 26 April 2014 at an orchard in Nonaspe (Zaragoza, Spain). The experiment involved 20 randomly located trees with the same growth vigour and age. 400 fruits (20 samples per tree) of similar size (1.5 x 2.0 cm, approximately) and colour and with an absence of any defect were randomly and manually picked, transferred immediately to the laboratory and stored at 1 ºC until analysis.

2.2. PEF equipment

The PEF apparatus used in this investigation was supplied by ScandiNova (Modulator PG; ScandiNova, Uppsala, Sweden). The equipment generates square waveform pulses of a width of 3 μs with a frequency up to 300 Hz. The maximum output voltage and current are 30 kV and 200 A, respectively. The equipment has a direct current power supply which converts the 3-phase line voltage to a regulated DC voltage. It charges up 6 IGBT switching modules (high-power solid-state switches) to a primary voltage of around 1000 V. An external trigger pulse gates all the modules and

controls their discharge to a primary pulsed signal of around 1000 V. Finally, a pulse transformer converts this primary 1000 V pulse to the desired high-voltage pulse.

The treatment chamber consists of a cylindrical methacrylate tube closed with two polished stainless steel cylinders. The gap between the electrodes was 3 cm and the diameter of the treatment chamber was 2.5 cm.

The actual voltage and current intensity applied were measured with a high-voltage probe (P6015A; Tektronix, Wilsonville, OR) and a current probe (Stangenes Industries Inc., Palo Alto, CA), respectively, connected to an oscilloscope (TDS 220, Tektronix).

2.3. Experimental design

2.3.1 Selection of the optimal PEF pre-treatment

The first step when seeking to apply PEF to food is to determine the permeability achieved in the cells during treatment. For this purpose, the intracellular liquid released from the PEF-treated tissue extracted by centrifugation was used. The treatments of the thinned peach samples were carried out placing 6 whole fruits in the treatment chamber containing McIlvain buffer of 1.5 mS/cm^2. PEF treatments ranging from 10 to 50 pulses of 3 μs (30–150 μs) were applied, set at electric field strengths ranging from 0 to 5 kV/cm. The specific energy of these treatments ranged from 0.61 to 9.98 kJ/kg (Table 1). A pulse frequency of 1 Hz was used.

Table 1. Specific energy of PEF treatments as a function of electric field strength, intensity, treatment time and number of pulses.

Electric field strength (kV/cm)	Gap (cm)	Intensity (A)	Treatment time (μs)	Pulses	Specific energy (kJ/kg)
3	3	68	3	10	0.61
				30	1.84
				50	3.06
4	3	107	3	10	1.28
				30	3.85
				50	6.42
5	3	133	3	10	2.00
				30	5.98
				50	9.98

The permeabilization of the thinned peach cells by PEF was determined through the quantification of the liquid released by centrifugation. This is a well-established technique to evaluate the degree of permeabilization caused by PEF. Because the presence of an intact cell membrane that acts like a barrier limits the release of intracellular components such as water, the permeabilization of this membrane by PEF improves the release of water from inside the cell when it is submitted to centrifugation. After PEF treatment, approximately 20 g of fruit was centrifuged at 2300 g for 10 min. The results were expressed as an increment of the grams of liquid released compared to the amount released from the untreated sample per 100 g of thinned peach fruits. Depending on the degree of permeabilization, different volumes of liquid are released from the thinned peaches.

2.3.2. Experimental design for selection of optimal extraction conditions

Once the time, number and intensity of pulses, obtained in the previous section, were selected, response surface methodology (RSM) was used to determine optimal PEF treatment conditions for the thinned peach fruits before the bioactive compound extraction. A central composite design (CCD) approach was used to investigate the

effects of the electric field strength (from 0 to 5 kV/cm), temperature (from 15 to 35 ºC) and solvent concentration (methanol from 0 to 80%) on the total phenol (TPC) and total flavonoid (TFC) contents and on the antioxidant activity by DPPH scavenging. For this, whole fruits inside the treatment chamber were treated with three different electric field strengths, 0.0, 2.5 and 5 kV/cm. After that, the fruits were cut into 4 pieces by both transverse and horizontal cuts, and placed in 250 mL volumetric flasks to which 200 mL of the corresponding water:methanol solvent was added. These flasks were stirred at the selected temperature for 10 hours. Preliminary studies indicated that neither higher volume of solvent nor longer times increased the phenol extraction yields. Each point of the CCD was carried out in triplicate. The data obtained were modelled with the following second-order polynomial equation 1:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{3} \beta_{ij} X_i X_j \quad \text{(Eq. 1)}$$

where Y is the response variable to be modelled, X_i and X_j are independent factors, β_0 is the intercept, β_i the linear coefficients, β_{ij} the quadratic coefficients and β_{ij} the cross-product coefficients. A backward regression procedure was used to determine the parameters of the models. This procedure systematically removed the effects that were not significantly associated (p>0.05) with the response until a model with only a significant effect was obtained.

The CCD and the corresponding analysis of the data were carried out using the software package Design-Expert 6.0.6 (Stat-Ease Inc., Minneapolis, MN).

2.4. Bioactive compound analysis

The total phenol content (TPC) was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965) with some modifications. Briefly, an aliquot (1 mL) of the

extract or of a standard solution of gallic acid (0-250 mg/L) was added to a 10 mL volumetric flask and mixed with 1 mL of Folin-Ciocalteu reagent. After 5 min, 1 mL of 7.5% Na_2CO_3 water solution was added and the solution was diluted to 10 mL with deionized water. After 1 hour of incubation at room temperature in darkness, the absorbance was determined at 760 nm with a spectrophotometer (Unicam UV 500, England). The TPC was expressed as mg gallic acid equivalents (GAE) per 100 g of fresh weight.

The total flavonoid content (TFC) of each extract was determined by a colorimetric spectrophotometric assay using the method developed by Iacopini et al. (2010) with some modifications. Briefly, at time zero 0.1 mL of a water solution containing 5% $NaNO_2$ was added to 0.5 mL of the extract. After 5 min, 0.1 mL of 10% $AlCl_3$ water solution was added and after 6 min, 0.6 mL of 1M NaOH was also added and immediately diluted with 1.7 mL of distilled water. A calibration curve was constructed with different concentrations of catechin (0-100 mg catechin/L) as the standard. Absorbance of the pink mixture samples was measured with a spectrophotometer at 510 nm against a water blank, and the flavonoid content was expressed at mg catechin equivalents (CE) per 100 g of fresh weight.

The antioxidant radical scavenging of extracts was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) nitrogen free radical. Its colour changes from violet to yellow when it is reduced by the electron donation process. The DPPH assay is based on the method used by Llorach, et al. (2008) with modifications. Briefly, 200 µL of extract was mixed with 3 mL of DPPH (133 µM in methanol). The free radical scavenging activity was evaluated by measuring the variation in absorbance at 515 nm after 2 h and 30 min of reaction and the results were expressed as a percentage of reduction according to the formula (Eq. 2):

$$\%\, reduction = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control}\ x\ 100 \quad (\text{Eq. } 2)$$

2.5. Identification of individual phenols by HPLC/DAD

The individual phenols in the extracts were determined by HPLC/DAD using the method described by Tomas-Barberán et al. (2001). A Hewlett-Packard Series 1100 chromatograph (Agilent Technologies; Santa Clara, USA) coupled with a photodiode array detector (DAD) (Series 1100) and an autosampler (Series 1100) operated by HP ChemStation 3365 software was used for the sample analysis. The column was a Zorbax SB-C18 (3.5 µm, 150 x 4.6 mm I.D., Agilent Technologies). The temperature of the column was maintained at 30 °C.

The elution solvents used were 5 % formic acid in methanol HPLC grade (A) and 5% formic acid in milli-Q water (B). Formic acid was added to increase peak resolution. A linear gradient method was used: 0-5 min, 95% B; 5-10 min, 95-88% B; 10-35 min, 88-75 %; 35-50 min, 75-50 %; 50-52 min, 50-20 % B; 52-60 min, 20-0 % B, followed by washing and reconditioning of the column. The flow rate was 1 mL/min and chromatograms were recorded at 510, 340 and 280 nm. The thinned peach extracts were filtered (0.45 µm nylon syringe filter, VWR, West Chester, PA, USA) and then directly injected (20 µL) into the chromatograph. Three HPLC analyses were carried out for each sample.

The individual phenols analysed (catechin, coumaric acid, chlorogenic acid, neochlorogenic acid and quercetin) were selected based on the results obtained by Redondo et al. (2017) and were identified according to their retention time in comparison to the commercial standard. Quantification was carried out using the external standard method and the final concentrations were expressed in mg per 100 g of fresh weight.

2.6. Statistical analysis

All the samples were analysed in triplicate and the results were presented as mean values ± standard deviation. The statistical analyses were performed using a one-way ANOVA test and the significance of the difference between means was determined by Duncan's multiple range test ($p<0.05$). The Statistical Package for the Social Science (SPSS) software version 22.0 (SPSS, Chicago, IL, USA) was used.

3. Results and discussion.

3.1. Selection of PEF initial conditions

The permeability achieved in the cells during PEF pre-treatments was evaluated as the percentage of intracellular liquid extracted by centrifugation. This technique provides simply and economically a reflection of the degree of permeabilization of cell membranes in vegetables. Since the permeabilization of the cell membrane facilitates the release of intracellular liquid, higher liquid extraction is correlated with a higher degree of electroporation. Liquid release by centrifugation has been used for orange peel (Luengo et al., 2013) showing a high correlation (R=0.92) with the cell disintegration index (CDI) another common technique used to analyse the permeabilization of vegetables (Ade-Omowaye et al., 2003, Asavasanti et al., 2010, Eshtiagi and Knorr, 2002). Figure 1 shows the influence of the electric field strength and treatment time on the percentage of liquid released per 100 grams of fruit by centrifugation. The percentage released in an untreated sample (control) is also included. The amount of extracted intracellular fluid increased with the electric field intensity and treatment time. However, no significant statistical differences ($p>0.05$) were observed between treatments of 4 and 5 kV/cm and no clear positive effect on the amount of extracted liquid was detected with treatment times exceeding 90

microseconds (30 pulses of 3 μs). Therefore this was the optimal treatment time selected for further experiments. The treatments with the greatest amount of extracellular fluid extracted were those with electric fields of 4 and 5 kV/cm and specific energies of 3.85 kJ/kg and 5.98 kJ/kg, respectively. Both intensities, especially 4 kV/cm, can be considered as low energy consumption, their values being similar to the energy required to raise the temperature of one kilogram of water one degree centigrade (4.18 kJ/kg).

3.2. Optimisation of treatment conditions

A central composite design was used to optimize the conditions for the extraction of bioactive compounds from thinned fruits. The PEF treatment time for different electric field strengths was set at 90 μs (30 pulses of 3 μs) in accordance with the results of the previous study on the influence of the treatment time on the release of intracellular liquid. The electric field (0, 2.5 and 5 kV/cm), the extraction temperature (15, 25 and 35 ºC) and the methanol concentration (0, 40 and 80%) were established as independent variables. Maximum electric field (5 kV/cm) was selected based on the high intracellular liquid extraction of previous experiments, and maximum temperature (35 ºC) based on studies that have reported a sharp decrease in phenol concentrations at extraction temperatures higher than 45 ºC (Cacace and Mazza, 2003). The TPC, TFC and DPPH scavenging resulting from the experimental conditions investigated for the samples treated by PEF is shown in Table 2.

Table 2. Experimental design and responses of the dependent variables to extraction conditions. Values based on fresh weight.

Independent variables			Dependent variables		
X_1	X_2	X_3	Y_1	Y_2	Y_3
0	0	15	6.4±1.9	0.6±0.1	1.0±0.1
0	0	35	17.6±3.7	5.8±0.3	9.4±0.5
0	40	25	51.3±3.7	30.7±4.4	46.3±2.2
0	80	15	58.5±0.6	31.6±1.7	50.6±5.2
0	80	35	83.3±7.2	54.3±4.9	57.8±2.4
2.5	0	25	14.5±6.2	4.3±2.1	6.0±2.0
2.5	40	15	26.4±7.4	12.4±3.2	19.3±6.7
2.5	40	25	37.2±4.6	20.3±2.5	27.8±2.8
2.5	40	25	42.1±1.9	25.2±0.2	35.6±1.5
2.5	40	25	47.2±4.3	27.3±0.8	41.3±4.4
2.5	40	35	42.7±2.4	30.8±2.7	52.7±1.2
2.5	80	25	53.3±4.8	33.3±2.9	43.5±0.5
5	0	15	25.0±2.0	6.3±0.1	9.2±0.4
5	0	35	47.8±5.4	12.4±4.2	17.7±3.0
5	40	25	34.4±0.4	20.3±0.2	29.4±5.6
5	80	15	49.0±8.2	31.1±8.1	35.6±2.5
5	80	35	57.1±8.0	37.8±8.5	43.0±1.3

X_1: electric field strength (kV/cm); X_2: solvent (% methanol); X_3: temperature (ºC); Y_1: TPC (mg GAE/100 g); Y_2: TFC (mg CE/100 g); Y_3: DPPH scavenging (%).

The TPC, TFC and DPPH scavenging varied from 6.4 mg GAE/100 g, 0.6 mg CE/100 g and 1.0% for 0 kV/cm, 0% methanol and 15 min, to 83.3 mg GAE/100 g, 54.3 CE/100 g and 57.8%, respectively, for 0 kV/cm, 80% methanol and 35 min. The concentrations obtained using 80% methanol are similar or higher than found in other by-products such as industrial sour cherry pomace (66.1 mg GAE/100 g and 35.1 mg CE/100 g) (Kołodziejczyk et al., 2013), peel (105 mg GAE/100 g) or seed (100 mg GAE/100 g) of papaya (Ayala-Zavala et al., 2010). These authors concluded that industrial fruit byproducts represent a potential source of natural food ingredients; therefore, our results might indicate that thinned peaches can be used with the same purpose.

RSM was used to determine and quantify the potential advantages or disadvantages of the application of a PEF treatment, further including the effect of

temperature and solvent, for bioactive compound extraction from thinned peach. This approach enables the evaluation of the effect of several factors and their interactions on response variables. RSM has been used successfully by several researchers for optimising the extraction by PEF of bioactive compounds such as anthocyanins from black currants and purple-fleshed potato (Cacace and Mazza, 2003, Puértolas et al., 2013). The application of a multiple regression analysis to the independent and response variables shown in Table 2 resulted in the following equations for TPC (Eq. 3), TFC (Eq. 4) and DPPH scavenging (Eq. 5) after removing non-significant terms (p>0.05):

$$\mathbf{Y_1} = -9.16 + 0.74*S + 0.83*T - 0.11*E*S \quad \text{(Eq. 3)}$$

$$\mathbf{Y_2} = -10.17 + 0.49*S + 0.59*T - 0.04*E*S \quad \text{(Eq. 4)}$$

$$\mathbf{Y_3} = -10.28 + 1.05*S + 0.65*T - 0.0005*S^2 - 0.06*E*S \quad \text{(Eq. 5)}$$

where Y_1 is the TPC (mg GAE/100 g), Y_2 is the TFC (mg CE/100 g), Y_3 is the DPPH scavenging (%), E is the electric field (kV/cm), S is the solvent concentration (% methanol), and T the extraction temperature (ºC). Table 3 shows the results of the ANOVA for the quadratic model developed for the equations below. The determination coefficient (R^2) of the models of 0.90, 0.92 and 0.91 for the TPC, TFC, and DPPH scavenging, respectively, the no significance of the lack of fit (p>0.05), and the high F-values indicate that the models were significant (p<0.0001) and could be used to predict the response. According to the F-values for the model parameters, the solvent was the most important. This means that changes in this factor have the highest influence on the independent variables. The temperature and the interaction electric field cross solvent terms were also significant (p<0.05), but with lower F-values. Moreover, the presence of the square of the solvent factor in the DPPH scavenging equation was also significant

(p<0.05). The presence of these square terms in the equation means that when the percentage of the solvent changed, its effect on the DPPH scavenging was non-linear.

Table 3. Regression coefficients of the predicted second-order polynomial models for TPC, TFC and DPPH scavenging for fresh thinned peach fruits.

Assay	Y_1		Y_2		Y_3	
	F-value	***p-value***	***F-value***	***p-value***	***F-value***	***p-value***
Model	28.55	<0.0001	35.56	<0.0001	22.19	<0.0001
E	-	-	-	-	-	-
S	79.29	<0.0001	119.50	<0.0001	84.56	<0.0001
T	15.21	0.0021	16.56	0.0016	10.11	0.0088
E^2	-	-	-	-	-	-
S^2	-	-	-	-	7.46	0.0195
T^2	-	-	-	-	-	-
E*S	19.65	0.0008	5.09	0.0435	6.47	0.0273
E*T	-	-	-	-	-	-
T*S	-	-	-	-	-	-
Mean	40.81		22.62		30.96	
Standard deviation	6.75		4.59		6.44	
R^2	0.905		0.922		0.910	
Adjusted R^2	0.873		0.896		0.869	
Coefficient of variation	16.53		20.30		20.79	
Lack of fit	1.97	0.3827	1.74	0.4190	0.87	0.6394

E. electric field strength; S: solvent; T: temperature; Y_1: TPC (mg GAE/100 g); Y_2: TFC (mg CE/100 g); Y_3: DPPH scavenging (%).

To illustrate the influence of the electric field strength, temperature and solvent on the TPC, various surface plots were generated that show combinations of these three parameters to obtain different yields of phenol extraction (Fig. 2). The percentage of solvent had a linear positive effect on the extraction of polyphenols. The higher the concentration of methanol in the extraction media, the higher the polyphenol yield. The increase in the TPC with the presence of methanol in the extraction medium and with the increment of the temperature is consistent with mass transfer principles. The presence of methanol facilitated extraction by enhancing solubility and diffusivity. Moreover, phenol extraction is higher in methanol than in water, possibly due to the methanol effects on cell permeability by acting on the phospholipid bilayer of biological membranes (Bridgers et al., 2010; Goldstein & Chin, 1981). The temperature also

facilitated extraction by increasing both the diffusion coefficient and solubility of phenols. The application of PEF increased the extraction yield in comparison with the control when extraction was performed with methanol up to a 25 %. When extractions were performed with methanol concentrations higher than 25%, the application of PEF did not improve the extraction of bioactive compounds and the yields obtained were lower than the ones obtained from the untreated samples. This effect is reflected in the negative term of the interaction between electric field and percentage of solvent, meaning that the influence of the solvent in the extraction yield was lower when the electric field increased.

Figure 3 shows the surface plots obtained to illustrate the influence of the independent variables on the TFC. It was observed that with an increase in the methanol percentage, the yield of flavonoids also increased linearly. However, the slope of the lines that describe this relationship decreases when increasing the electric field. It was observed again that the application of electric fields is very useful when water is used as solvent and counterproductive when methanol percentages above 20% were used. As in the previous case, the temperature had a slight positive linear effect on the extraction of the compounds of interest.

Finally, Figure 4 shows a graphical representation of the modelling of the extraction of compounds with antioxidant capacity, determined by the DPPH scavenging method. The increase in the percentage of methanol resulted in a higher extraction of compounds with antioxidant activity, although in this case the relationship was not linear. An increase in the percentage of methanol from 0 to 40% shows a greater effect than an increase of 40 to 80%. As for the TPC and TFC, the electric field again produced a linear relationship, the application of electric fields in the absence of methanol being positive whereas with a high percentage of methanol the effect is the

opposite. The temperature, as in the previous cases, has a positive linear effect on the extraction.

In summary, in all cases (TPC, TFC and DPPH scavenging), the highest yield was observed using 80% methanol. This greater efficiency for extracting phenolic compounds using a mixture of organic solvent and water instead of pure solvent has been demonstrated by other authors for various different products (Pinelo et al., 2005, Shi et al., 2003, Spingo et al., 2007, Yilmaz and Toledo, 2005). This could be because certain polyphenols (such as anthocyanidins, proanthocyanidins, etc.) are more soluble in water, while others are more soluble in organic solvents (catechin, epicatechin, etc.) (Shi et al., 2003).

The effect of the temperature has also been observed by other authors. Boussetta et al. (2012) obtained higher concentrations of polyphenols from grape seeds when increasing the temperature from 20 to 50 °C. Puértolas et al. (2013) extracted more anthocyanins from PEF treated purple potato, with values from 28.9 to 61.5 mg anthocyanin/100 g, when the temperature rose from 10 to 40 °C. These authors stated that the higher temperature allows the removal of compounds because it increases both the diffusion coefficient and the solubility in the extraction medium.

In the present study, the use of methanol as a solvent decreased the extraction of bioactive compounds as the electric field strength increased. This result does not coincide with those obtained by other authors who have detected an improvement in the extraction yield applying PEF and using solvents other than water. Boussetta et al. (2014) and Corrales et al. (2008) in the case of flaxseed hulls and grape by-products, respectively, increased the extraction of total phenols by 50% with PEF and using ethanol rather than water. Puértolas et al. (2013) also observed an increase in the

extraction of bioactive compounds, in this case anthocyanins from purple potato, by increasing both the temperature and the ethanol concentration. However, as in the present study, the use of higher percentages of ethanol (80%) was more effective for untreated PEF samples than for treated ones regardless of the extraction temperature. These authors concluded that the decrease in the efficacy of ethanol compared with water as an extraction solvent when samples were pre-treated by PEF could be because the increase in the diffusivity as a consequence of the ethanolic denaturation of the phospholipid bilayer is less significant when the cells have been previously permeabilised by PEF.

In our opinion, this behaviour may be due also to the promoting effect of the PEF on the enzymatic oxidation of polyphenols. While in the untreated sample little or no contact between the enzyme and phenolic compounds was produced, in the PEF sample the disruption of the membranes favoured their contact producing brown colours in the pulp (Figure 5), reducing the total amount of polyphenols present in the thinned fruits, and thus, reducing the amount of phenolic compounds that could be extracted. Grimi et al. (2011), studying the impact of apple processing modes on the quality of extracted juice also showed that PEF treatment with 400 V/cm accelerated the browning of apple pulp although they were not subjected to long contact with external air. Therefore, treating fruits with high enzymatic activity by PEF require a different approach for reducing the reaction rate. The authors recommend a series of strategies for future studies such as reducing the treatment temperature, or reducing the pH of the treatment media.

Although higher concentrations of bioactive compounds can be obtained with a high methanol concentration, the future use of thinned fruits as a source of bioactive compounds relies in the development of an environmentally friendly and cheap

technique to obtain them. Results obtained in this research show a good potential of PEF for improving a solvent free process. A benefit of the PEF permeabilization of thinned peach before extraction is the possibility of using water instead of organic solvents to obtain significant amounts of interesting compounds. To illustrate this potential, figure 6 was obtained using the corresponding regression models. We observed that with the use of 5 kV/cm, it could be obtained the same TPC using 35 ºC and 0% methanol (40 mg GAE/100 g) that untreated PEF samples with 35 ºC and 27% methanol or with 15 ºC and 48% methanol. Moreover, with water, the use of PEF permits us to reduce the temperature from 35 to 15 ºC to obtain the same values as control without PEF (20 mg GAE/100 g). This behaviour can also be observed with TFC and DPPH scavenging. Therefore, in the range of the values of the variables assayed, the application of a PEF treatment to thinned peaches before extraction could permit the use of water, a more environmentally friendly solvent than methanol.

3.3. Individual phenols extracted from thinned peach fruits treated by PEF

Redondo et al. (2016) identified by HPLC-DAD-MS^n/ESI a total of 12 individual phenols in lyophilized thinned peach fruits. These included 3 flavonols (quercetin 3-rutinoside, kaempherol-3-hexoside and kaempherol-3-rutinoside), 7 hydroxycinnamic acids (neochlorogenic acid, chlorogenic acid, isochlorogenic acid, 4-p-coumaroylquinic acid, 4-caffeoylquinic acid, 3-feruloylquinic acid, 3-p-coumaroylquinic acid) and 2 flavan-3-ols (catechin and epicatechin), the most important being catechin, neochlorogenic acid, chlorogenic acid and 3-p-coumaroylquinic acid.

Based on these previous results, Table 4 shows the concentrations of five phenolic compounds identified by HPLC in thinned peach fruits untreated and treated by PEF: catechin, chlorogenic acid, coumaric acid, neochlorogenic acid and quercetin.

It compares the concentration of the individual phenols at the highest temperature tested (35 °C) in untreated samples and in samples treated with PEF at an electric field of 5 kV/cm, using methanol and water as solvent. Similar to the concentration of the bulk of polyphenols, smaller amount of the main individual polyphenols were extracted with 80% methanol in comparison with the control. However, with water, a positive effect on the application of electric pulses was observed, increasing the extraction of coumaric, chlorogenic and neochlorogenic acids.

Table 4. Concentration of individual phenols from thinned peach fruits treated with PEF. PEF treatment conditions: 30 pulses of 3 µs.

Electric field (kV/cm)	Solvent (% methanol)	Temperature (ºC)	Phenolic compounds (mg/100 g)				
			Catechin	Coumaric acid	Chlorogenic acid	Neochlorogenic acid	Quercetin
0	80	35	2.5±0.2 a	0.5±0.2 ab	29.7±4.2 a	38.1±6.3 a	0.3±0.2 a
5	80	35	2.1±0.3 a	0.8±0.1 a	16.5±2.8 b	27.4±3.9 b	0.2±0.1 a
0	0	35	1.7±0.2 ab	0.2±0.1 b	2.5±0.6 d	4.5±1.1 d	0.0±0.0 b
5	0	35	2.0±0.2 a	0.8±0.2 a	9.8±1.0 c	16.3±2.6 c	0.0±0.0 b

Different letters in the same column indicate significate differences (p<0.05).

Using 80% methanol, almost twice of the amount of chlorogenic (29.7 mg/100 g compared to 16.5 mg/100 g) and neochlorogenic (38.1 mg/100 g compared to 27.4 mg/100 g) acid were extracted without applying PEF than when applying an electric field of 5 kV/cm. However, for the rest of the identified phenolic compounds, no statistically significant differences (p>0.05) were observed. Therefore, the use of PEF for the extraction of phenolic compounds with 80% methanol would not be of interest. In the case of extraction with water, the application of PEF resulted in an increase of coumaric acid (0.8 mg/100 g), chlorogenic acid (9.8 mg/100 g) and neocholorgenic acid (16.3 mg/100 g) compared to the amounts extracted without the application of electric fields (0.2, 2.5 and 4.5 mg/100 g, respectively). No statistically significant differences (p>0.05) were observed for epicatechin, while quercetin was not detected.

4. Conclusions

The results of this study have demonstrated that the extraction yield of bioactive compounds from thinned fruits using PEF is dependent on the electric field strength, extraction temperature and methanol percentage, the solvent being the most important factor. The highest yields were achieved using 80% methanol and without applying PEF. However, the application of PEF is very useful when water is used. For this reason, the PEF-assisted extraction of bioactive compounds from by-products such as thinned peach fruits stands as an alternative to conventional extraction methods which require dried products, large amounts of organic solvents and long extraction times. However, comparative studies at preindustrial scale are required in order to evaluate the advantages of PEF assisted extraction against current extraction procedures from an economic and environmental point of view.

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Figure captions

Figure 1. Influence of treatment time (μs) on the intracellular liquid extracted by centrifugation for 100 g of fresh thinned peaches at different electric fields (x: 0 kV/cm; ●:3 kV/cm; □:4kV/cm; ▲: 5 kV/cm).

Figure 2. Response surface plots of total phenol content (TPC) (mg GAE/100 g) as a function of solvent and electric field (a), temperature and electric field (b), and temperature and solvent (c).

Figure 3. Response surface plots of total flavonoid content (TFC) (mg CE/100 g) as a function of solvent and electric field (a), temperature and electric field (b), and temperature and solvent (c).

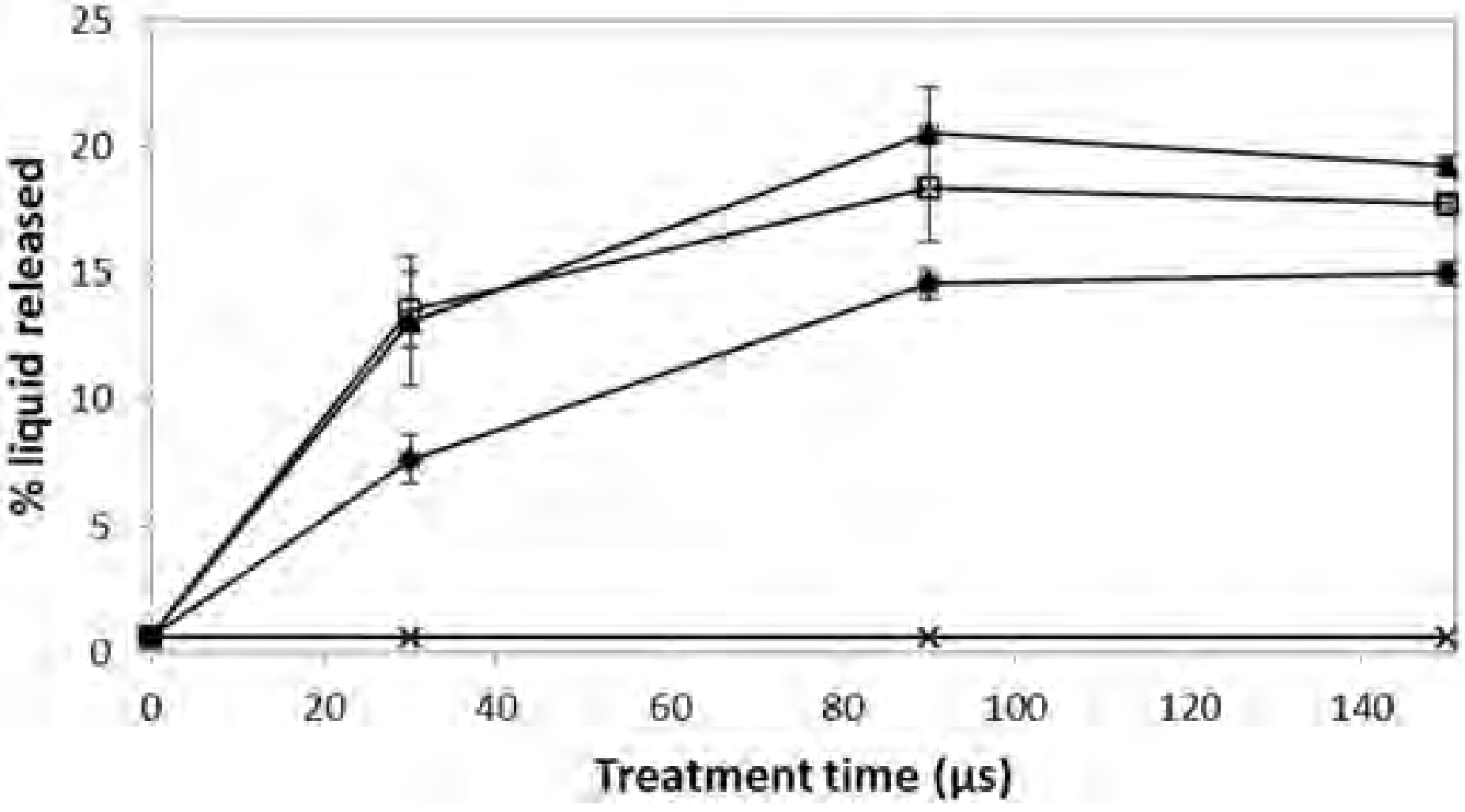
Figure 4. Response surface plots of DPPH scavenging (%) as a function of solvent and electric field (a), temperature and electric field (b), and temperature and solvent (c).

Figure 5. Visual aspect of thinned peach fruits for untreated (A) and treated (B) samples with PEF (5 kV/cm, 15ºC). The fruits were cut from the whole thinned peaches immediately after the treatment, and the pictures were taken after 5 minutes of contact with external air.

Figure 6. Relationship between TPC, TFC and DPPH scavenging with methanol concentration (0-80%) when modifying electric field (0-5 kV/cm) and temperature (15-35ºC).

Highlights

- Solvent is the main factor for extraction of bioactive compounds from thinned fruits
- Application of PEF is very effective when water is used as solvent
- PEF extraction of phenol compounds stands as a green alternative to conventional ones


25
20
15
10
5
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% liquid released
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20
40
60
80
100
120
140
Treatment time (µs)

Figure 1

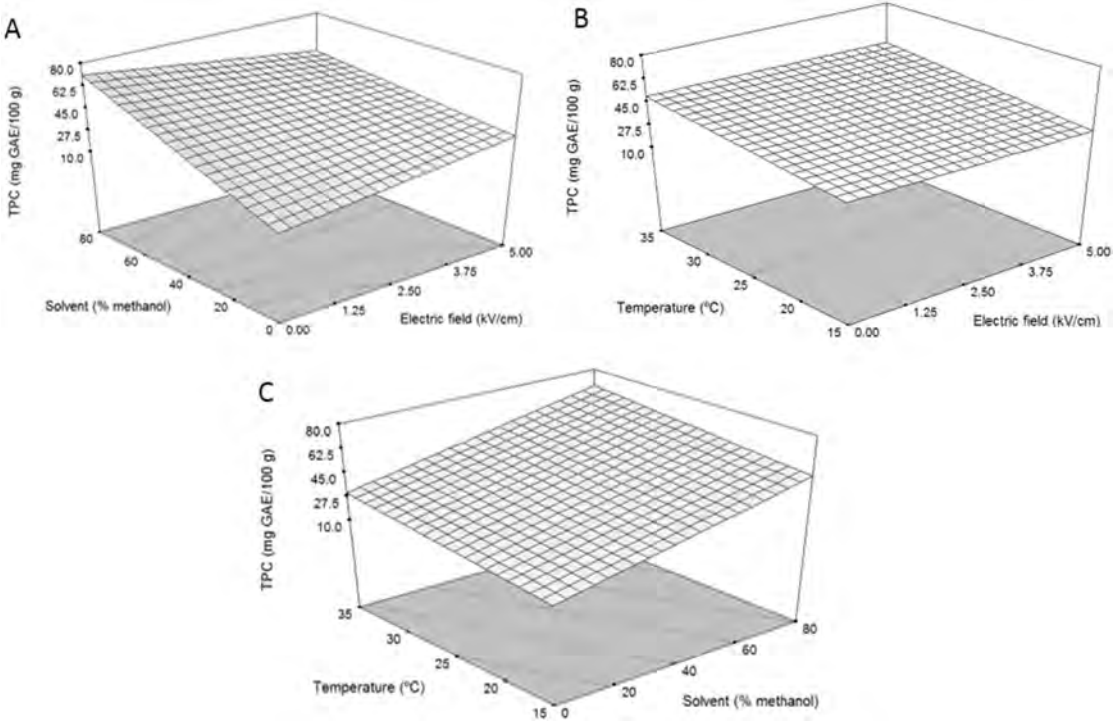
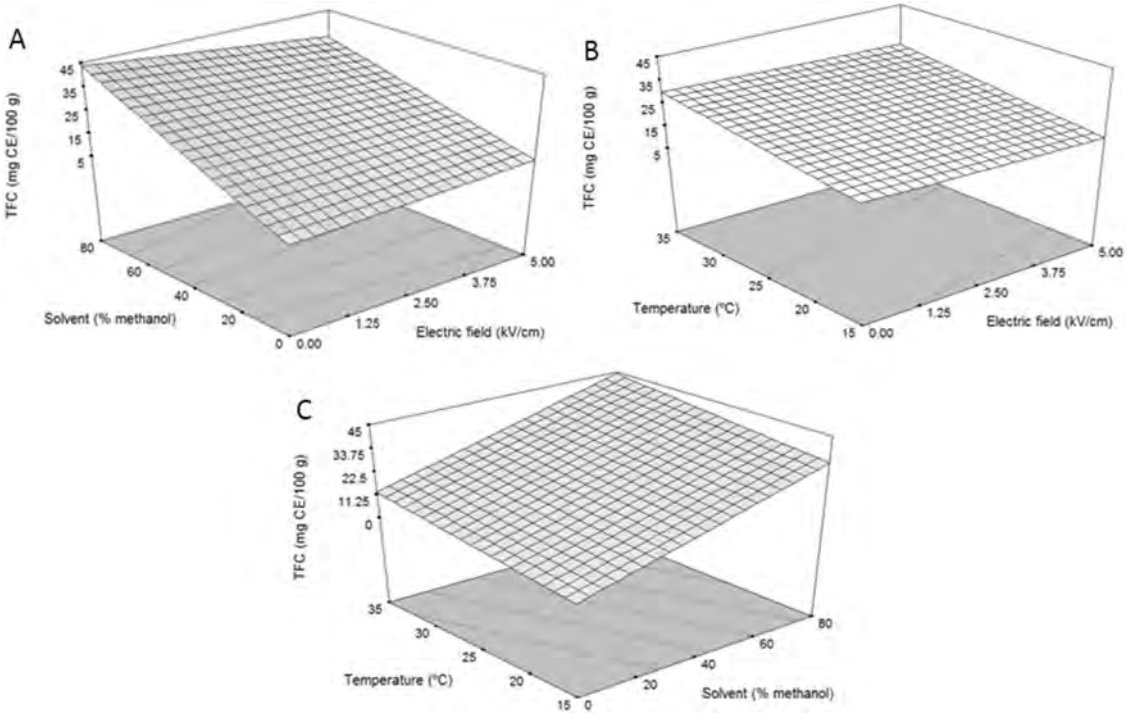

A
TPC (mg GAE/100 g)
80.0
62.5
45.0
27.5
10.0
Solvent (% methanol)
80
60
40
20
0
Electric field (kV/cm)
0.00
1.25
2.50
3.75
5.00
B
TPC (mg GAE/100 g)
80.0
62.5
45.0
27.5
10.0
Temperature (°C)
35
30
25
20
15
Electric field (kV/cm)
0.00
1.25
2.50
3.75
5.00
C
TPC (mg GAE/100 g)
80.0
62.5
45.0
27.5
10.0
Temperature (°C)
35
30
25
20
15
Solvent (% methanol)
0
20
40
60
80

Figure 2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TFC (mg CE/100 g)
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35
25
15
5
80
60
40
20
0
Solvent (% methanol)
0.00
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2.50
3.75
5.00
Electric field (kV/cm)
B
TFC (mg CE/100 g)
45
35
25
15
5
35
30
25
20
15
Temperature (°C)
0.00
1.25
2.50
3.75
5.00
Electric field (kV/cm)
C
TFC (mg CE/100 g)
45
33.75
22.5
11.25
0
35
30
25
20
15
Temperature (°C)
0
20
40
60
80
Solvent (% methanol)

Figure 3

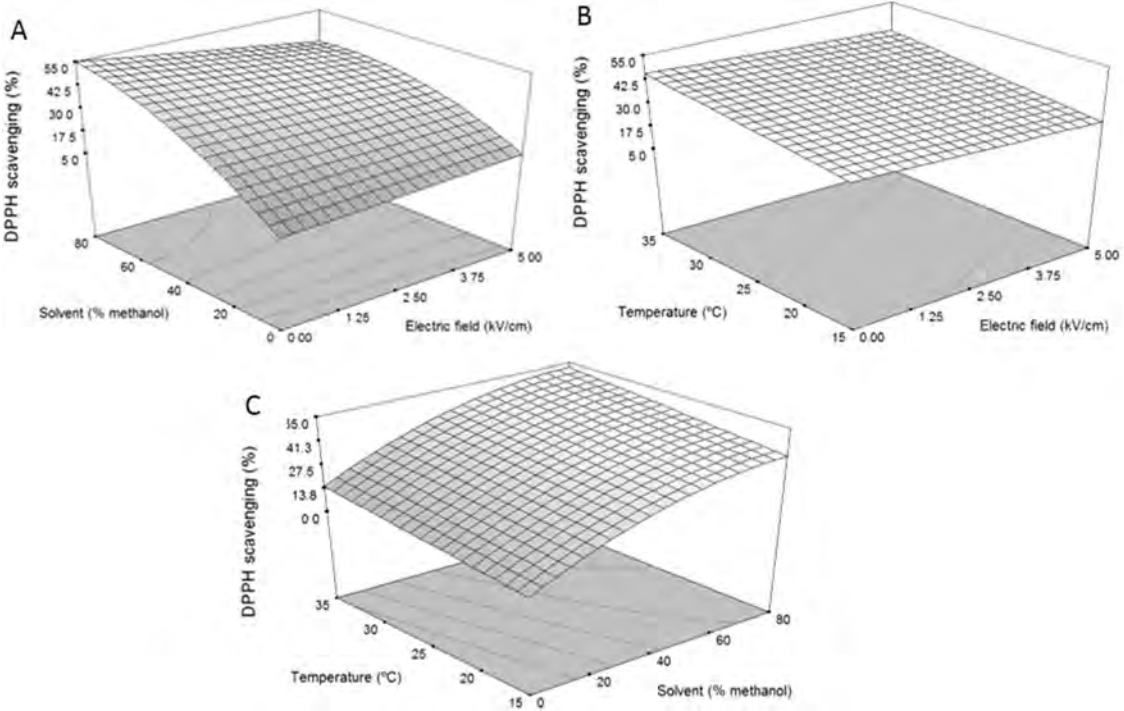
A
DPPH scavenging (%)
55.0
42.5
30.0
17.5
5.0
80
60
40
20
0
Solvent (% methanol)
0.00
1.25
2.50
3.75
5.00
Electric field (kV/cm)
B
DPPH scavenging (%)
55.0
42.5
30.0
17.5
5.0
35
30
25
20
15
Temperature (°C)
0.00
1.25
2.50
3.75
5.00
Electric field (kV/cm)
C
DPPH scavenging (%)
55.0
41.3
27.5
13.8
0.0
35
30
25
20
15
Temperature (°C)
0
20
40
60
80
Solvent (% methanol)

Figure 4

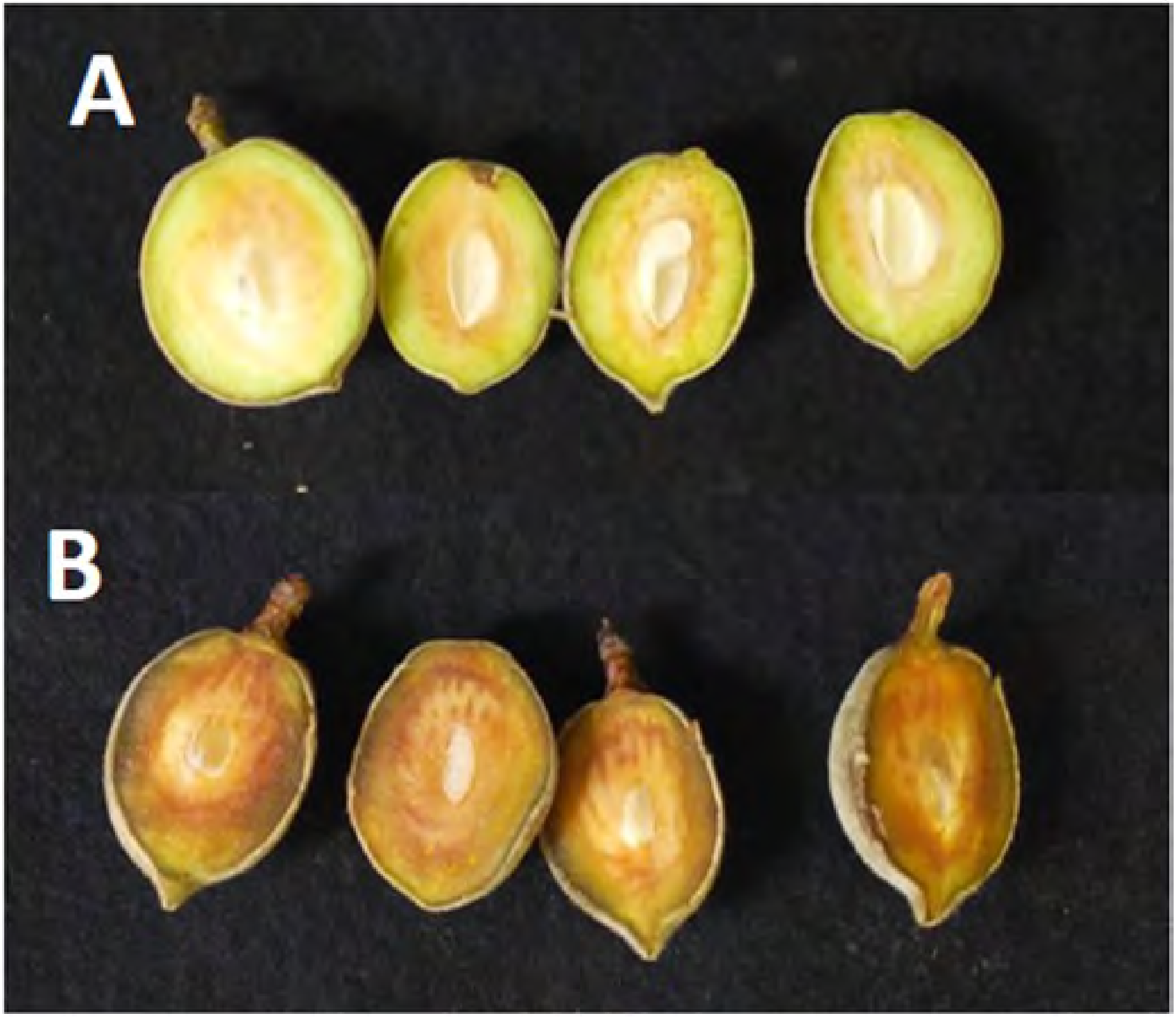

Figure 5

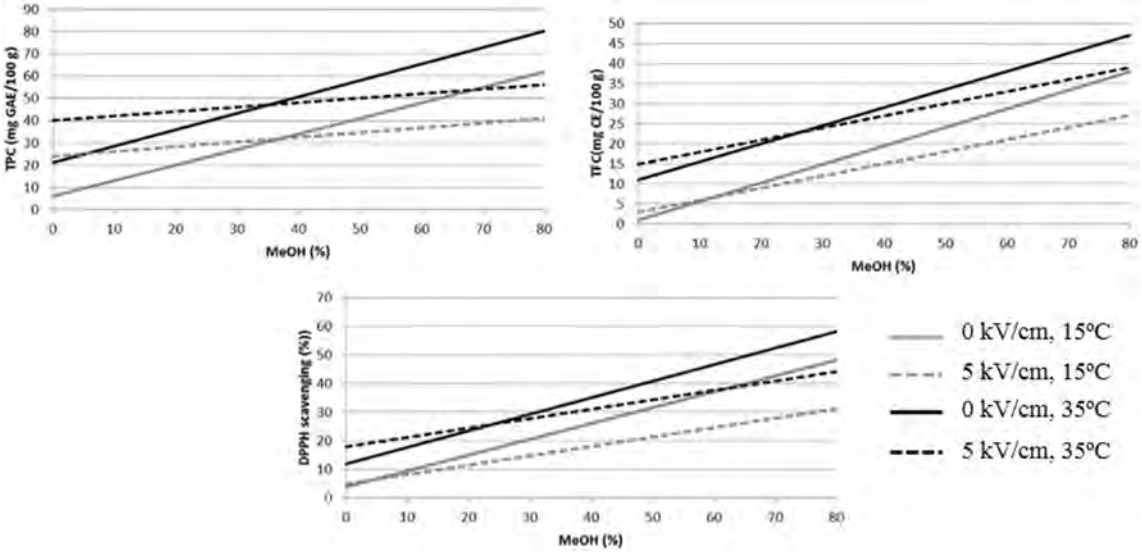
TPC (mg GAE/100 g)
MeOH (%)
TFC(mg CE/100 g)
MeOH (%)
DPPH scavenging (%))
MeOH (%)
0 kV/cm, 15°C
5 kV/cm, 15°C
0 kV/cm, 35°C
5 kV/cm, 35°C

Figure 6