



The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products

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ABSTRACT

Emerging extraction techniques, including pulsed electric field (PEF) and ultrasound (US), are attracting considerable interest in the recovery of bioactives. Though, limited work has focused on PEF application as pre-treatment for US assisted extraction to enhance the release of phenolics from herbs. Hence, the present study investigated the use of an optimized PEF pre-treatment to enhance the recovery of phenolics from fresh rosemary and thyme by-products in a subsequent US assisted extraction step. Total phenolic content (TPC), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP) were assessed as an index of extraction efficacy. Qualitative and quantitative analyses were performed through liquid chromatography-mass spectrometry analyses to evaluate the influence of the methods on individual phenolic compounds and the formation of potential derivatives. The results indicated that in a number of cases PEF pre-treatment enhanced ($p < 0.05$) the recovery of phenolic compounds and antioxidant capacity compared to US individually.

1. Introduction

Rosmarinus officinalis L. (rosemary) and *Thymus vulgaris* L. (thyme) constitute two important species of *Lamiaceae* family with several purported medicinal properties owing to their high content in secondary metabolites (Alvi, Ahmad, Ishrat, Iqbal, & Khan, 2019; Bistgani et al., 2019). These metabolites, including phenolic compounds, are traditionally recovered from their respective sources with conventional maceration and various extraction solvents (Milevskaya, Temerdashev, Butyl'skaya, & Kiseleva, 2017). The use of fresh herbs for the extraction of phenolics can be a convenient process for herb producers (Albu, Joyce, Paniwnyk, Lorimer, & Mason, 2004), particularly when large volumes of herbal by-products have accumulated prior to retail. However, the extractability of phenolics from fresh herbs is partly a function

of the permeability of their plant tissues, as intact cellular membranes impede the diffusivity of intracellular compounds (Fincan, 2015) such as polyphenols that are located in vacuoles, chloroplasts, and apoplasts of plant cells (Parada & Aguilera, 2007).

Nowadays, non-thermal processing technologies including PEF are attracting considerable scientific interest (Fincan, 2015). PEF is broadly acknowledged as an energy efficient method to induce permeabilization of cellular membranes, while it has been also proposed as a novel stress promoter capable of inducing the production of bioactive compounds (Fincan, 2015; Jacobo-Velázquez et al., 2017). Due to its ability to electroporate cell envelopes PEF could be employed as a pre-treatment to facilitate improved recoveries of bioactive compounds followed by a subsequent traditional or novel extraction step (Tiwari, 2015). Aqueous EtOH mixtures are primarily employed in the recovery of

Abbreviations: PEF, Pulsed electric field; US, Ultrasound; TPC, Total phenolic content; FRAP, Ferric reducing antioxidant power; TPTZ, 2,4,6-Tri(2-pyridyl)-s-triazine; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; Na₂CO₃, Sodium carbonate; K₂S₂O₈, Potassium persulfate; EtOH, Ethanol; NaCl, Sodium chloride; HCl, Hydrochloric acid; ACN, Acetonitrile; MeOH, Methanol; FeCl₃•6H₂O, Ferric chloride hexahydrate; CV, Coefficient of variation; F-C, Folin-Ciocalteu's; UPLC-MS/MS, Ultra-high-performance liquid chromatography-tandem mass spectrometry; HPLC, High-performance liquid chromatography; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UAE, Ultrasound assisted extraction; TE, Trolox equivalents; GAE, Gallic acid equivalents; FW, Fresh Weight; DW, Dry Weight.

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phenolic compounds as their medium polarity is compatible with the spectrum of polarity, which phenolic compounds possess, whereas they are also food-friendly (Siddeeg et al., 2019). In fact, the use of EtOH mixtures in combination with some novel extraction technologies, such as ultrasound assisted extraction (UAE), has given promising results in some cases (Hosseini, Bolourian, Yaghoubi Hamgini, & Ghanuni Mahababadi, 2018; Jovanović et al., 2017).

The ability of UAE to enhance recovery of target metabolites has been attributed to its ability to disrupt cell-membranes through the transmission of US pressure waves and the subsequent phenomenon of cavitation (Baysal & Demirdoven, 2011; Bellumori et al., 2016). UAE is regarded as a clean and environmentally friendly method (Tiwari, 2015), and may limit the degradation of thermolabile compounds due to its ability to aid in the recovery of metabolites at low temperatures and reduced extraction times (Bellumori et al., 2016). Similar to PEF the technology can also be upscaled thus facilitating industrial scale extraction of target compounds (Vinatoru, 2001). Novel technology assisted extraction techniques have received considerable attention in recent times (Wiktor et al., 2018), however, very little scientific data on the combined effect of PEF and US with respect to their influence on the recovery of phenolic compounds is currently available (Aadil et al., 2018; Manzoor et al., 2019). Taking into consideration the effects of US and PEF independently on various herbal materials (Jovanović et al., 2017; Segovia, Luengo, Corral-Pérez, Raso, & Almajano, 2015; Upadhyay, Nachiappan, & Mishra, 2015; Zderic & Zondervan, 2016), it could be anticipated that their combination could lead to an enhanced extraction efficiency of the treated plant materials.

Hence, in this study, fresh rosemary and thyme by-products were initially treated with PEF to achieve cellular permeabilization and were subsequently exposed to US treatment with aqueous EtOH. Through cell disintegration index ($Z\sigma$), the effect of a low energy PEF-induced permeabilization on the extraction of targeted intracellular phenolic compounds was assessed as a pre-step for further optimization. Total phenolic content (TPC) and antioxidant capacity (DPPH and FRAP) assays, as well as UPLC-MS/MS analyses were employed to assess the effect of the two methods in combination or separately. To the best of our knowledge, there is no other published study so far concerning the effect of PEF application as a pre-treatment step prior to UAE on fresh rosemary and thyme in the recovery of antioxidant phenolic compounds.

2. Materials and methods

2.1. Samples

Fresh herbal by-products of rosemary (originating from Kenya, Ethiopia and Morocco) and thyme (Ireland and Spain) consisting of plants with visual defects and/or unsuitable shape, size and colour for retail purposes were provided by McCormack Farms (Grange, Ireland), following authentication by experienced personnel. After collection, each herbal by-product (leaves and stems) was cut manually into approximately 0.5–2 cm pieces, placed in a vacuum-bag, and after proper mixing (pooled sample from different origins), was vacuum-packed and stored in dark at 4 °C for up to 5 days before processing. All the extraction experiments for each species were conducted from the same pooled sample. The dry weight (DW) of rosemary and thyme plants was $65.4 \pm 0.3\%$ and $73.8 \pm 0.6\%$, respectively.

2.2. Reagents

Folin–Ciocalteu's (F–C) phenol reagent, gallic acid, sodium acetate (anhydrous), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), DPPH, sodium carbonate (Na_2CO_3), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), formic acid, EtOH, sodium chloride (NaCl) and 37% hydrochloric acid (HCl) and the standards cryptochlorogenic acid, 4-hydroxybenzoic acid, phlorizin, luteolin-7-O-glucuronide, and

rosmanol, were obtained from Sigma-Aldrich (now Merck, Wicklow, Ireland). Acetonitrile (ACN) and methanol (MeOH) were purchased from Romil (Lennox Laboratory Supplies LTD, Dublin, Ireland) and acetic acid was acquired from AppliChem GmbH (Darmstadt, Germany). The standards of phenolic compounds, namely rosmarinic acid, carnosic acid, protocatechuic acid, chlorogenic acid, caffeic acid, carnosol, hesperidin, naringenin, naringenin-7-O-glucoside, luteolin, luteolin-4-O-glucoside, luteolin-7-O-glucoside, apigenin, narirutin, and apigenin-7-O-glucoside were obtained from Extrasynthese (Extrasynthese Co., Genay Cedex, France). Milli-Q® (18 mΩ) (Merck Millipore, Molsheim, France) water was used throughout, except for PEF pre-treatment where distilled water was used.

2.3. Preparation of extracts

2.3.1. PEF pre-treatment

PEF pre-treatment of rosemary and thyme by-products was conducted with a 5 kW HVP ELCRACK 5 (DIL, Quakenbrück, Germany) PEF generator operating in batch mode. The applied conditions were based on previously optimized conditions for a similar plant material (Zderic & Zondervan, 2017) and were further optimized for the present equipment. The PEF apparatus was set to generate electric pulses of near-rectangular shape at 10 Hz frequency in burst mode. The electric pulses were applied in a parallel plate treatment chamber of 16 cm^2 , consisting of two stainless steel parallel electrodes ($4 \times 4 \text{ cm}$). Each treatment, which included a series of 167 bipolar pulses of 30 μs pulse duration at $1.1 \pm 0.2 \text{ kV cm}^{-1}$ was applied to a fixed sample size of 33 g and 36 g of rosemary and thyme respectively, in the chamber filled up with 24 g of 0.1% aqueous NaCl (1: 1.4 w/v for rosemary, and 1: 1.5 w/v for thyme). The applied conditions did not result in a temperature increase greater than 10 °C. Directly after treatment the plant material was vacuum-filtered and subsequently transferred into a beaker, covered with parafilm and stored at 4 °C in the dark for a short time until further US extraction. The liquid fraction was collected and analyzed separately but the obtained results were taken into account for the US treated samples. PEF treatment was performed in triplicate for each of the two species.

The energy per pulse (W_{PEF}) was estimated according to Luengo, Condón-Abanto, Álvarez, and Raso (2014) from the Eq. (1):

$$W_{\text{PEF}} = \int_0^i \sigma^* E(t)^2 dt \quad (1)$$

Where: σ is the conductivity measured in S m^{-1} , E is the electric field strength in V m^{-1} , and t is the pulse duration in s. The total applied energy in kJ was estimated after multiplying the energy per pulse by the number of pulses. Subsequently, the total specific energy in kJ kg^{-1} , was estimated after dividing the total applied energy by the mass of the PEF treated sample. Therefore, as determined, the specific PEF energy input was 0.36 and 0.46 kJ kg^{-1} for rosemary and thyme, respectively.

2.3.2. Characterization of the ultrasonic field

Various amplitudes corresponding to different acoustic powers were selected, while the output power (W) was determined by calorimetric measurements for a specific volume of water (100 mL) according to the equations as defined by Milne, Stewart, and Bremner (2013), and Tiwari (2015). Thermocouples were used to monitor the temperature for 60 s in duplicate, and the power output (mode of 0.5 s on, 0.5 s off) was determined based on the Eq. (2):

$$P = m^* C_p [dT/dt]_{t=0} \quad (2)$$

Where: P is the ultrasonic power in Watt, C_p is specific heat of water that equals $4.18 \text{ J g}^{-1} \text{ °C}^{-1}$, m is the mass of water used in g, dT/dt is the temperature change in °C s^{-1} . (Milne et al., 2013; Tiwari, 2015). Based on the calorimetric measurements, the efficiency of the energy transformation when supplying 50% amplitude (200 W) was 53 W.

The specific US energy input (W_{us}) in kJ kg^{-1} was estimated from the Eq. (3) according to the equation of Barba, Galanakis, Esteve, Frigola, and Vorobiev (2015) with the use of the actual power of the generator (calorimetric measurement):

$$W_{us} = P \cdot t_{us} / m \quad (3)$$

Where: P is the actual generator power at 50% amplitude (53 W), t_{us} is the treatment time in s and m is the mass of sample in kg (after determining the density of 55.19% aqueous EtOH solution and adding 5 g of the used plant material (1: 20 w/v)). As it was estimated, the US specific energy was 136.44, 204.65 and 409.31 kJ kg^{-1} for 4.16, 6.24 and 12.48 min, respectively.

2.3.3. Ultrasound assisted extraction (UAE)

UAE was conducted with or without PEF pre-treatment based on optimized conditions for dried rosemary (Hosseini et al., 2018). An ultrasonic processor model UP 400S (Dr. Hielscher GmbH, Germany) with a maximum input power of 400 W and an ultrasonic transducer that transforms the applied electrical into US waves at a frequency of 24 kHz was employed. US was applied via a cylindrical 22 mm titanium sonotrode horn tip model H22 (Dr. Hielscher GmbH, Germany), which was submerged 2.5 cm under the surface of the sample. The treatment was carried out with 5 g of sample submerged in 100 mL of 55.19% aqueous EtOH (1: 20 w/v) using an input power of 200 W (50% amplitude based on the available equipment) for a period of 4.16, 6.24 or 12.48 min (optimal extraction time). The probe was operated in non-continuous mode (0.5 s on, 0.5 s off). The extraction temperature was kept constant at 40 ± 2 °C through a cytostatic water-bath that was circulating through the US glass jacket. The temperature was monitored using thermocouples connected to a data logger. Non-PEF pre-treated samples were compared with PEF pre-treated. The quantity of plant material due to water absorption after PEF pre-treatment was evaluated before further UAE. The water uptake for rosemary and thyme was $36.5 \pm 3.5\%$

and $46.8 \pm 7.5\%$, respectively. All UAE experiments were performed in triplicates. The experimental steps involved are shown schematically in Fig. 1.

2.4. Disintegration index (Z_σ)

For estimating the induced cell damage degree (P) the electrical conductivity disintegration index (Z_σ) was calculated according to Lebovka, Shynkaryk, and Vorobiev (2007) by measuring the impedance of the herbal materials (rosemary and thyme by-products and 0.1% aqueous NaCl) placed inside a 16 cm^2 PEF chamber and after application of 0–4000 pulses. The frequency signals were generated by a function generator DG 1022 (Rigol, Germany) which provides a 1 V peak to peak sinusoidal signal of 1 kHz. The voltage-drop after the wave passed through the samples was measured by an oscilloscope TDS 2012 (Tektronix, UK) before and after each treatment at ambient temperature.

The conductivity (σ) of the samples was estimated by measuring the impedance with the oscilloscope with Eq. (4):

$$\sigma(\omega) = l_s / A_s |Z(j\omega)| \quad (4)$$

Where: σ is the electrical conductivity measured in S m^{-1} , l_s is the length of the chamber in m, A_s is the area perpendicular to the electric field in m^2 , and $|Z(j\omega)|$ is the absolute value of the complex impedance in Ω .

The Z_σ index was calculated based on the Eq. (5):

$$Z_\sigma = \sigma - \sigma_i / \sigma_d - \sigma_i \quad (5)$$

Where: σ is the measured conductivity in S m^{-1} , whereas the subscripts i and d refer to the values for intact (before application of pulses) and totally destroyed plant tissues (after application of 4000 pulses), respectively. Each measurement was performed in duplicate for each herbal by-product species.

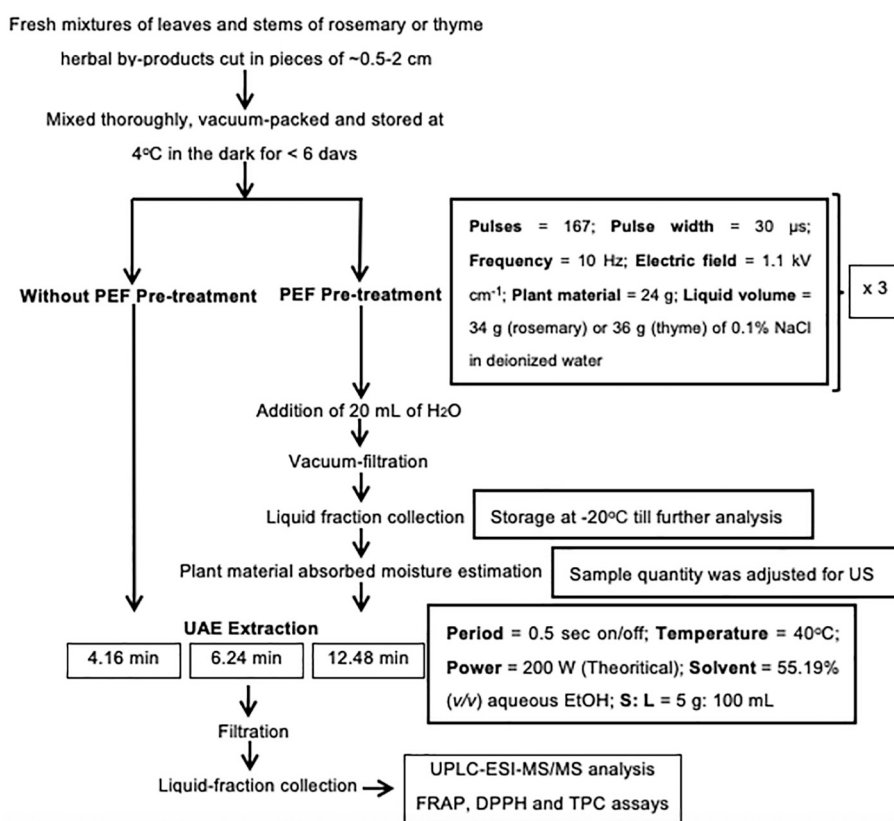


Fig. 1. Schematic flow chart illustrating the extraction process and analysis of rosemary and thyme by-products (S = solid, L = liquid).

2.5. F—C assay

TPC was estimated following the F—C phenol method, as described by Singleton, Orthofer, and Lamuela-Raventos (Singleton, Orthofer, & Lamuela-Raventos, 1999) with slight modifications. Gallic acid solutions were used as standards. Aliquots of 100 μL of the appropriately diluted extract/standard/blank, 100 μL of water, 100 μL of F—C reagent and 700 μL of Na_2CO_3 (20%) were added together and vortexed. Soon after vortexing, the reaction mixture was placed for 20 min in dark and subsequently centrifuged at 13,000 rpm for 3 min. The absorbance of 200 μL aliquots of the supernatant was measured at 735 nm by a SPECTROstar Omega microplate reader (BMG Labtech, Offenburg, Germany) in a 96-polystyrene microplate. All measurements were performed three times for each sample and standard solution, and the samples were corrected after subtraction of the reagent blank (in the position of samples the extraction solvent). The TPC was expressed as mg gallic acid equivalents (GAE) 100 g^{-1} fresh weight (FW) of sample.

2.6. FRAP assay

FRAP assay was conducted based on the method of Stratil, Klejduš, and Kubáň (Stratil, Klejduš, & Kubáň, 2006) with slight modifications. The FRAP reagent was freshly prepared each day of analysis after mixing 38 mM of anhydrous sodium acetate buffer (pH 3.6), 20 mM of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in Milli-Q water and 10 mM of TPTZ in 40 mM HCl, in a ratio of 10:1:1. After incubation for 5 min at 37 °C and till further use, 180 μL of FRAP reagent was added in 20 μL of appropriately diluted sample/standard/blank in a polystyrene microplate, mixed and incubated for 40 min at 37 °C. After incubation, the absorbance was measured directly by microplate reader at 593 nm in a 96-polystyrene plate and each extraction solvent was used as blank. Trolox solutions were used as standards and the FRAP values were expressed as mg trolox equivalents (TE) 100 g^{-1} FW of sample.

2.7. DPPH assay

DPPH radical-scavenging activity was evaluated as described by Goupy et al. (Goupy, Hugues, Boivin, & Amiot, 1999) with slight modifications. DPPH stock solution in MeOH (0.238 mg/mL) was freshly prepared each day of analysis, sonicated for 30 min to get completely solubilized and stored for 2 h in the dark under refrigeration after. A further 1:5 dilution of the stock solution in MeOH was prepared of which 100 μL was mixed with 100 μL of appropriately diluted sample/standard in a 96-polystyrene microplate. Standards were prepared with trolox, whereas the extraction solvent of each extract was used for blanks. After incubation at room temperature and in the dark for 30 min, the absorbance was measured at 515 nm on a microplate reader and the results were expressed as mg TE/100 g FW of sample.

2.8. Identification and quantification of major phenolic compounds through UPLC-ESI-MS/MS analysis

UPLC-ESI-MS/MS analysis was performed for the qualitative and quantitative analysis of phenolic compounds in all extracts of rosemary and thyme by-products on a Waters Acquity UPLC (Waters Corporation, Milford, MA, USA) coupled to tandem quadrupole detector (TQD) mass spectrometry adapting the method of Gangopadhyay, Rai, Brunton, Gallagher, and Hossain (Gangopadhyay, Rai, Brunton, Gallagher, & Hossain, 2016). Briefly, an Acquity UPLC HSS T3 (2.1 mm \times 100 mm; 1.8 μm particle size) column was utilized with a binary mobile phase of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in ACN (solvent B), while the flow rate was set to 0.5 mL/min for 10 min. A multiple reaction monitoring (MRM) method was used for the identification and quantification of the different phenolic compounds. The MRM transitions (Table S1) for each compound were obtained by Waters Intellistart™ software (Waters Corp., Milford, MA, USA) when standards

were available, whereas the studies of Mena et al. (2016) and Vallverdú-Queralt et al. (2014) were used to obtain the fragmentation patterns when the standards were absent. The MRM transitions for each phenolic compound are given in Table S1, whereas in Fig. S1, the UPLC-ESI-MS/MS chromatograms for one of the examined compounds, namely rosmarinic acid are additionally shown. The UPLC-ESI-MS/MS data were obtained in negative ESI mode for all the phenolic compounds. The quantification of phenolic compounds in the samples and standards was carried out using the TargetLynx™ software (Waters Corporation, Milford, MA, USA).

2.9. Statistical analysis

Three samples of each plant material were evaluated for each treatment and time. For the antioxidant indices, each extract ($n = 3$) was analyzed independently three times (three technical replicates) and the results were reported as mean \pm standard deviation (SD). A coefficient of variation (CV) lower than 15% was considered acceptable between the technical replicates of spectrophotometric measurements. For UPLC-ESI-MS/MS analysis, the concentration of analyzed compounds was measured triplicates and was reported as mean \pm SD ($n = 3$). Normality and homogeneity of variance were assessed with Shapiro-Wilk and Levene's tests, respectively. One-way ANOVA and Tukey's honesty significant difference (HSD) post hoc test were performed in cases of normal distribution, whereas Kruskal-Wallis stepwise comparison was used for non-normal data ($p < 0.05$). When homogeneity of variance was violated, Welch analysis followed by Games-Howell post hoc test was applied. Pearson correlation (r) coefficients were used for the datasets of all the extracts to reveal any relationship between the results of the individual antioxidant assays. The significance level of all tests was specified to be 0.05. Statistical analysis was carried out using the SPSS Statistics, Version 24 (IBM Corp.) and heat maps were generated through the conditional formatting of Excel 16.31 (Microsoft Corp.).

3. Results and discussion

3.1. Z_σ evaluation

Z_σ was measured to gain a deeper understanding of the extent to which PEF induced microstructural changes in the target material and to identify optimal applied conditions that maximized cell permeabilization. This index has a range from 0 to 1, describing a totally intact and total disintegrated plant material, respectively (De Vito, Ferrari, Lebovka, Shynkaryk, & Vorobiev, 2008).

Figs. 2a and b illustrate the influence of the applied pulses under the same field strength (E) on the Z_σ of rosemary and thyme by-products, respectively. Results indicated that the extent of cell membrane permeabilization was increased by increasing the number of pulses up to a saturation point at 4000 pulses, above which no further cellular damages could be observed. As it has been shown, more intense treatments lead to an augmented conductivity due to the release of greater amounts of conductive intracellular components that is theoretically connected to the formation of irreversible pores (López-Gómez, Elez-Martínez, Martín-Belloso, & Soliva-Fortuny, 2020).

For the highest pulse number (4000 pulses), Z_σ reached a maximum value of 1. As it was indicated, the same optimal conditions (167 pulses) induced a lower cell permeabilization in rosemary (0.28) compared to thyme (0.42), indicating a lower rupture of tissues for the former. This effect may be attributable to the fibrous nature of rosemary leaves and their apparent rubbery texture (Mulinacci et al., 2011). Support to this assumption is a previous study by Hosni et al. (2013), who suggested that the differences in the morphological and structural characteristics of the secretory glands among rosemary and thyme, can result in lower yield of essential oil in rosemary compared to thyme, after enzyme-assisted pre-treatment with cellulase, hemicellulase and their combination. As the authors suggested based on a number of additional

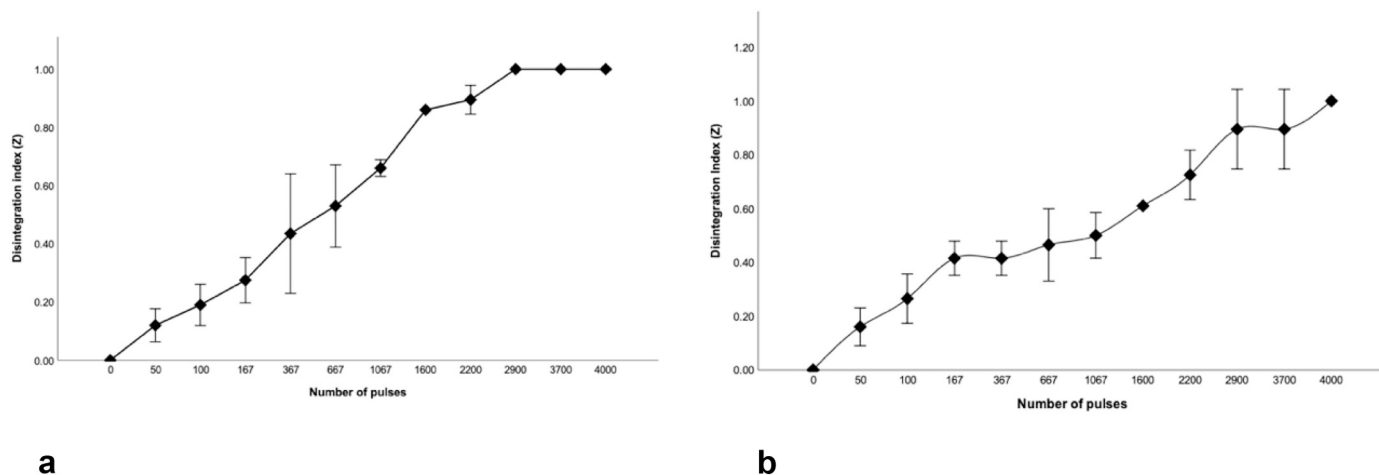


Fig. 2. a Plot of a rosemary and, b thyme Z_{50} , after application of different numbers of pulses. Results are expressed as mean \pm standard deviation, based on two replicates.

studies, the presence of toughened cuticle on the whole surface area of glandular trichome in rosemary constituted the main extraction barrier. Conversely, thyme glandular trichomes were characterized by a porous and thin cuticle that enabled a more efficient recovery (Hosni et al., 2013). Optimal PEF treatment conditions should enable not only an adequate cell disintegration level, but also efficiently reduce the specific energy output (Rooijnejad, Everett, & Oey, 2014). Electric field strengths between 0.5 and 3 kV cm⁻¹, pulse width between 1 and 100 μ s and at frequencies between 1 and 100 Hz have been shown to be effective for the irreversible electroporation of plant materials (Eugene Vorobiev & Lebovka, 2008). As it has been additionally suggested by Puértolas, Cregenzán, Luengo, Álvarez, and Raso (2013), permeabilization of cellular membranes can be attained at low specific energies (<10 kJ kg⁻¹) (Puértolas et al., 2013), while these conditions are in accordance with those applied in this study. As it was shown in the same study, PEF treatments for the recovery of anthocyanins from purple-fleshed potato, with a treatment time in the range of 45–105 μ s and electric field strengths between 1 and 5 kV cm⁻¹, led to a specific energy output among 0.54 and 13.50 kJ kg⁻¹. As it was indicated, an electric field strength of 1 kV cm⁻¹ and treatment time of 75 μ s resulted in an electric field strength of 0.54 kJ kg⁻¹ (Puértolas et al., 2013) that is considerably close to the specific energy outputs of our study, when similar conditions were applied. In an additional study, a specific energy of 15.63 kJ kg⁻¹ was estimated after employing PEF for the extraction of anthocyanins from mashed cabbage at an electric field strength of 2.5 kV cm⁻¹ and 50 pulses of 15 μ s pulse width (Gachovska et al., 2010), indicating the need of higher specific energy output from cell membrane permeabilization from this recommended by Puértolas et al. (2013). Wiktor et al. (2015) also utilized PEF to extract polyphenols from apple and carrot tissues at a range between 0 and 5 kV cm⁻¹ and 0–100 pulses that resulted in a specific energy range from 0 to 80 kJ kg⁻¹ (Wiktor et al., 2015). An important observation was that the highest specific energy inputs of 40 and 80 kJ kg⁻¹ led to significant ($p < 0.05$) degradation of phenolics, equal to 23 and 36%, respectively. Nonetheless, the lowest specific energy of 1.13 kJ kg⁻¹ was indicated as potentially effective for the recovery of phenolic compounds (Wiktor et al., 2015). In the case of US, considerably higher specific energies were estimated, whereas values of that range (121–363 kJ kg⁻¹) have been found during US application to increase the phenolic extractability during red wine fermentation, using a similar probe system (El Darra, Grimi, Maroun, Louka, & Vorobiev, 2013). In this study, we aimed to assess how a low cell membrane permeabilization degree could amplify the recovery of targeted intracellular phenolic compounds in the subsequent solvent extraction step and assess its potential for further optimization. Hence, a higher number of pulses was not applied. The application of identical

conditions for the two different herbs used in this study, which resulted in a different disintegration level would potentially enable additional assumptions about the efficiency of PEF as an extraction pre-treatment step.

3.2. Antioxidant capacity and phenolic content estimation

Different processes including freezing and drying, which encompass high energy inputs are often employed for the disruption of cellular structures of raw materials (Wiktor & Witrowa-Rajchert, 2016) in other studies. However, the application of often unfeasibly high energy inputs would make a process cost inefficient to apply. Therefore, this was not the case in our study. So far, a limited number of studies have evaluated the effect of the combined application of US and PEF treatment in food systems. For instance, as it has been stated the combination of US and PEF treatment led to enhanced recovery of bioactive compounds (anthocyanins and total phenols) from raspberry and blueberry purees in comparison to US or PEF independently in a number of cases (Medina-Meza, Boioli, & Barbosa-Cánovas, 2016). The influence of PEF pre-treatment on the phenolic content and antioxidant capacities of rosemary and thyme after application of US was evaluated with three tests, namely DPPH, FRAP and TPC. F–C assay is used for the estimation of TPC, but it is based on a similar electron-transfer reaction to that of DPPH and FRAP assays. According to Wong and Kitts (2006) several studies have reported that phenolic compounds are the major components that are responsible for the antioxidant capacity in herbs (Wong & Kitts, 2006). TPC assay constitutes a method that results in an estimate of the total polyphenolic content (Iswaldi et al., 2011), whereas DPPH is a relatively common assay for the evaluation of natural antioxidants (Katalinic, Milos, Kulisic, & Jukic, 2006; MacDonald-Wicks, Wood, & Garg, 2006; Pyrzyńska & Pękal, 2013) by assessing their capacity to act as hydrogen donors or free radical scavengers (Pyrzyńska & Pękal, 2013). Whilst the FRAP assay has of the analogous redox mechanisms with DPPH, there is a difference in reagents and their products (Stratil et al., 2006), and the lipophilic antioxidants tend to be more active in the FRAP assay and phenolic compounds superior DPPH scavengers (Bendif et al., 2018). Previous studies showing that DPPH and FRAP assays (Clarke, Ting, Wiart, & Fry, 2013; Katalinic et al., 2006) as well as FRAP (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009; Katalinic et al., 2006; Wong & Kitts, 2006) and DPPH (Clarke et al., 2013) with TPC, are well correlated to each other, which was also evident in this study. The obtained values were further compared to those after application of only US. As it is outlined in Table 1 and the correlation plot of Fig. S2, the different extracts of rosemary and thyme yielded significantly ($p < 0.01$) strong Pearson correlation coefficients (r).

Table 1

Coefficients r between the antioxidant activity assays (DPPH, FRAP, TPC) for the extracts of US treated rosemary and thyme by-products with or without PEF pre-treatment¹.

	DPPH ²	FRAP ²	TPC ³
DPPH ²	1.000		
FRAP ²	0.736	1.000	
TPC ³	0.838	0.972	1.000

¹ $p < 0.01$.

² mg TE 100 g⁻¹ FW.

³ mg GAE 100 g⁻¹ FW.

In respect to the extraction time, the same initial samples that were PEF pre-treated were subjected to the same US extraction time as the non-PEF pre-treated samples in each case. Therefore, for each specific extraction time, the observed differences could only be a result of the prior application of PEF. The purpose of using three different extraction times was to evaluate if a sample that was initially subjected to PEF for a shorter period of time, could result in a higher or comparable extractability of phenolic compounds to this of a non-PEF pre-treated sample after higher duration of US application. The results showed that compared to the untreated samples, PEF pre-treatment increased the TPC and antioxidant capacities of rosemary extracts significantly in several cases (Fig. 3a, Table S2). DPPH values of rosemary extracts were enhanced through PEF application and more interestingly, a specific pattern was observed in respect to the subsequent US extraction time. As it can be seen the highest DPPH activity was observed in the rosemary extracts after 12.48 min of PEF + US (593 mg TE 100 g⁻¹ FW) and this was significantly higher compared to that of US at the same treatment time (445 mg TE 100 g⁻¹ FW), resulting in a 1.3-fold increase of DPPH radical scavenging capacity. However, rosemary extracts after PEF + US for 6.24 min did not exhibit significantly lower DPPH values (512 mg TE 100 g⁻¹ FW) in comparison to those obtained after PEF + US for 12.48 min. In all cases, PEF treated samples yielded significantly higher DPPH values compared to only US for the same time. Phenolics are not uniformly dispersed throughout diverse parts of plants with the outer plant layers regularly contain higher concentrations in comparison to the interior sections (Brown, 1998). Therefore, the elevated antioxidant capacity of the extracts could be attributed to the utilization of electrical fields and the subsequent formation of irreversible pores. After the

application of electrical fields, irreversible permeabilization can be attained through the permanent disruption of a number of pores of the plasma membrane (Barba et al., 2015). Therefore, by eliminating this extraction barrier, a higher extraction efficiency can be achieved (Barba et al., 2015). However, a further improvement was achieved with the application of ultrasonic irradiation that according to Vinatoru (2001) can lead to additional diffusion and release of the intracellular compounds of interest in the extraction medium (Vinatoru, 2001). Therefore, the increased DPPH activity that was observed in PEF + US could have been resulted from the synergistic effect of both treatments.

However, as it can be seen, even if the applied PEF pre-treatment increased the antioxidant activity and extractability of phenolics in rosemary based on their FRAP and TPC values, these increases were not significant in most cases. The values obtained from FRAP assay indicated a tendency of increased antioxidant potential after PEF pre-treatment compared to the untreated samples. A significant effect was observed only for the extracts after 4.16 min of subsequent US extraction. In respect to the extraction time, no significant effect was observed between the US-treated samples or those that had been previously subjected to PEF pre-treatment, indicating that phenolics that affect the values of FRAP assay are potentially recovered in a shorter time compared to the optimal. As it was shown, PEF pre-treatment did not significantly affect the TPC levels of rosemary. However, TPC values after US application alone (196 mg GAE 100 g⁻¹ FW) as compared to application of US+PEF after 12.48 min (297 mg GAE 100 g⁻¹ FW), had significantly higher phenolic content (1.51-fold increase). In this study, we aimed to mimic conditions that would be realistic during an industrial extraction process. Therefore, pooled randomized samples of mixed leaves and stems from different origins were utilized as opposed to single origin homogeneous materials frequently used by other authors. Other studies have indicated that when soft plant materials are subjected to electric field strengths in the range of 0.1–10 kV cm⁻¹ this is sufficient to augment cell membrane permeabilization but these levels may have no influence on hard materials with high contents of lignin, including stalks, where electric fields up to 20 kV cm⁻¹ are required to induce noticeable cell destruction (Puértolas, Saldaña, & Raso, 2016). Therefore, the mixed nature of the starting material (mixed leaves and stems of different sizes) may have contributed to the high variability in the content of secondary metabolites that are measured in these assays. Furthermore another significant aspect that has been neglected in other

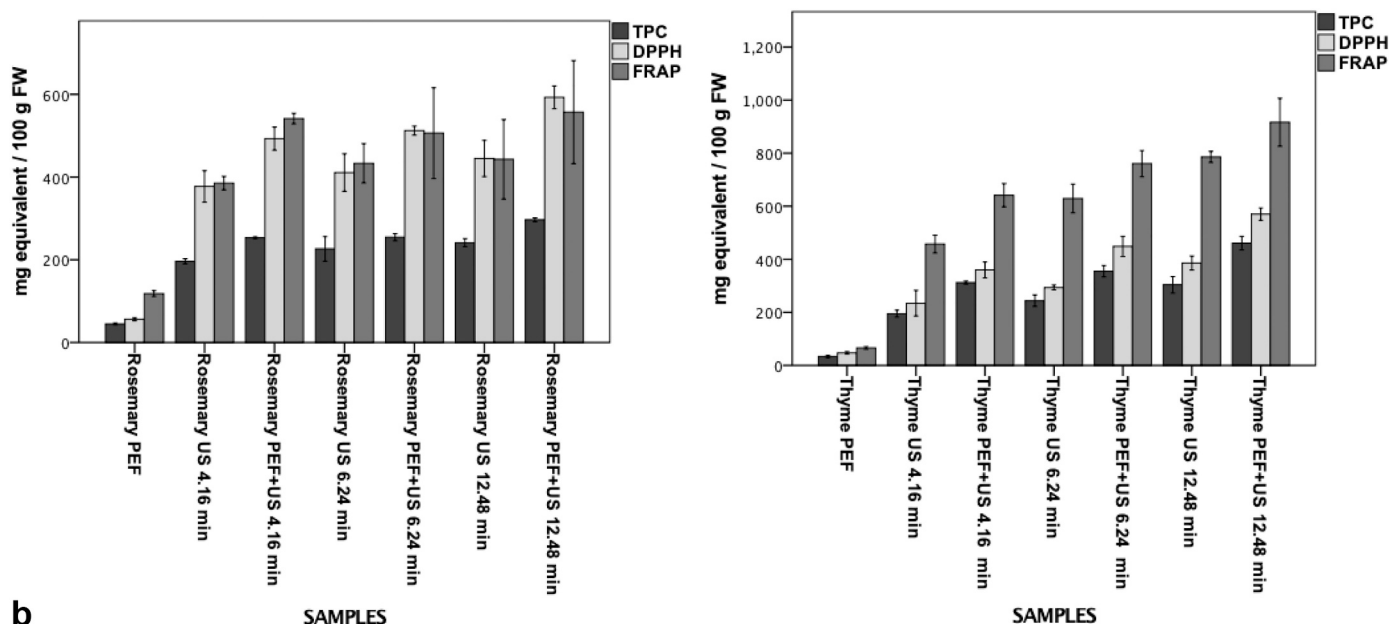


Fig. 3. Effect of PEF pre-treatment and time of extraction on the antioxidant activity and TPC of a rosemary and b thyme by-products.

studies is that PEF is a treatment that should be applied in raw food and plant materials (E. Vorobiev & Lebovka, 2006). Therefore, its utilization for instance after freeze-drying (Bozinou, Karageorgou, Batra, Dourtolglou, & Lalas, S., 2019), where the cells are already disrupted would not benefit more from additional cell destruction. The homogeneous starting material in these cases would also result in less variability and no profound effect.

Thyme samples exhibited a similar DPPH free radical scavenging trend to that of rosemary as presented in Fig. 3b and Table S3. The extracts were characterized by a higher activity after PEF pre-treatment with the highest found after 12.48 min of US extraction (570 mg TE 100 g⁻¹ FW). On the other hand, the lowest DPPH activities were recorded for the extracts after 4.16 and 6.24 min of US without prior to PEF that corresponded to 234 and 260 mg TE 100 g⁻¹ FW, respectively. In contrast to rosemary, PEF yielded significantly higher TPC values in thyme extracts compared to those obtained after only US for all extraction times. Similar to the behaviour observed for DPPH, 4.16 and 6.24 min of US without prior PEF resulted in the lowest phenolic contents (195 and 226 mg GAE 100 g⁻¹ FW, respectively), while after 12.48 min of PEF with subsequent US, a content of 460 mg GAE 100 g⁻¹ FW was observed. These results were in agreement to those reported by Liu, Esveld, Vincken, and Bruins (2019), when they studied the use of PEF as

an alternative drying pre-treatment to increase the recovery of phenolic compounds from fresh tea leaves. The authors suggested that 1.00 kV cm⁻¹ of electric field was capable of inducing cell rupture and formation of non-homogenous pores on tea leaves and were potentially responsible for the penetration of extraction solvent and the migration of phenolics (Liu et al., 2019). In an additional study, mint leaves were completely permeabilized after application of a higher electric field strength, which was equal to 3 kV cm⁻¹ (Fincan, 2015). However, as it has been previously indicated, and mainly in the case of culinary herbs, studies examining the application of PEF prior to US are limited for comparative purposes (Fincan, 2015). In addition, quantitative comparison with previous studies using fresh samples is not realistic as other authors have used different extraction conditions. For instance, Zheng and Wang (2001) showed that TPC of fresh rosemary and thyme after phosphate buffer homogenization were equal to 219 mg GAE 100 g⁻¹ FW and 178 mg GAE 100 g⁻¹ FW, respectively. However, even if the extraction conditions are not comparable to those obtained from this study, they are in the range of those reported here pointing to the validity of our data (Zheng & Wang, 2001). In respect to FRAP, higher but non-significant values were observed in the majority of thyme extracts, as for rosemary. Extracts measured after 4.16 min of only US were significantly lower compared to the rest with the exception to those after 6.24

Table 2
Identified phenolic compounds in PEF, US and PEF + US treated by-products of thyme and rosemary.

Identified compound	Thyme PEF	Thyme US	Thyme PEF + US	Rosemary PEF	Rosemary US	Rosemary PEF + US	Quantified as
1 protocatechuic acid ^a				c			n.q.
2 vanillic acid-O-hexoside ^b	c	c	c	c	c	c	n.q.
3 4-hydroxybenzoic acid ^a	c			c			n.q.
4 caffeic acid-O-hexoside ^b	d	d	d	c	c	c	Caffeic acid
5 coumaric acid-O-hexoside ^b	d	d	d	d	d	d	Carnosol
6 cryptochlorogenic acid ^a				d	d	d	Cryptochlorogenic acid
7 caffeic acid ^a	c	d	d	c	d	d	Caffeic acid
8 gallic acid ^b	d	d	d	d	d	d	Carnosol
9 medioresinol ^b	d	d	d	d	d	d	Luteolin-7-O-glucoside
10 4-O-p-coumaroylquinic acid ^b				c	c	c	n.q.
11 4-coumaric acid ^a	c			c			n.q.
12 luteolin-7-O-glucuronide ^a	d	d	d				Luteolin-7-O-glucuronide
13 luteolin-rutinoside ^b	c	d	d	c	c	c	Luteolin-7-O-glucoside
14 luteolin-7-O-glucoside ^a		d	d				Luteolin-7-O-glucoside
15 rosmarinic acid-O-hexoside ^b		d	d				Rosmarinic acid
16 narirutin ^b	c	c	c	c			n.q.
17 isorhamnetin-O-hexoside ^b				d	d	d	Rutin
18 rosmarinic acid ^a		d	d		d	d	Rosmarinic acid
19 hesperidin ^a				d	d	d	Hesperidin
20 hispidulin-rutinoside ^b				d	d	d	Luteolin-7-O-glucoside
21 trihydroxy-methoxyflavone ^b					d	d	Apigenin
22 phlorizin ^a				c			n.q.
23 medioresinol derivative ^b		c	c				n.q.
24 methyl rosmarinate ^b		c	c				n.q.
25 medioresinol glucuronide ^b		c	c	c	c	c	n.q.
26 luteolin ^a		d	d	d	d	d	Luteolin
27 naringenin ^a		c	c				n.q.
28 cirsimaritin ^b	c	d	d	c	d	d	Apigenin-7-O-glucoside
29 rosmanol ^a	c	c	c	d	d	d	Rosmanol
30 rosmanol isomer (epirosmanol) ^b	c	c	c	d	d	d	Rosmanol
31 carnosol methyl ether isomer ^b	d	d	d				Carnosol
32 carnosol isomer ^b		d	d				Carnosol
33 rosmadial isomer or rosmanol quinone ^b	c	c	c	d	d	d	Rosmanol
34 carnosol quinone ^b		d	d				Carnosol
35 carnosol ^a	c	d	d	d	d	d	Carnosol
36 rosmadial ^b				c	c	c	n.q.
37 rosmanol methyl ether ^b				c	c	c	n.q.
38 rosmaridiphenol ^b				d	d	d	Carnosol
39 carnosic acid ^a				c	d	d	Carnosic acid

n. q. not quantified.

^a Identification and/or quantification were performed with authentic standards.

^b Tentative identification and/or quantification.

^c The compound was detected but not quantified (low levels or traces).

^d The compound was detected and quantified.

min of US. The rest of the extracts did not exhibit statistically significant differences even though a clear tendency of higher levels was noticeable after longer extraction times and PEF applications. Finally, it should be noted that the higher level of cell destruction (as exhibited by a higher Z_{σ} value) in thyme after application of the same PEF conditions applied to rosemary could be the reason that the subsequent extraction steps had less pronounced effect in rosemary as compared to thyme.

3.3. Identification and quantification of major phenolic compounds

The UPLC-ESI-MS/MS mass spectra tentatively revealed the

existence of thirty-nine phenolic compounds in the different extracts of rosemary and thyme (Table 2). The main classes of phenolic compounds in the extracts were thirteen flavonoids (flavan-3-ols, flavones, flavanones, hydrochalcones and flavonols), twelve phenolic terpenes and eleven phenolic acids (hydroxybenzoic and hydroxycinnamic acids), whereas three lignans were also present. Fifteen compounds were identified with authenticated standards, while the additional twenty-four phenolics were tentatively identified as mentioned above (Materials and Methods section), through their comparison with literature acquired MS data. Thirty-one phenolics were detected in rosemary extracts and twenty-six in thyme with all the compounds having been

b	caffeic acid	rosmarinic acid	luteolin	carnosol	galloocatechin	cirsimaritin	coumaric acid-O-hexoside	medioresinol	luteolin-7-O-glucoside	luteolin-7-O-glucuronide	caffeic acid-O-hexoside	carnosol quinone	carnosol isomer	carnosol methyl ether isomer	luteolin-rutinoside	rosmarinic acid-O-hexoside
Thyme PEF	0.00	0.00	0.00	0.00	1.47	0.00	0.24	0.58	0.00	0.21	0.33	0.00	0.00	0.38	0.00	0.00
Thyme US 4.16 min	1.35	58.70	0.54	3.77	0.79	0.74	0.20	1.21	6.21	10.49	0.41	1.20	2.63	0.68	0.89	0.96
Thyme PEF+US 4.16 min	2.80	69.10	1.32	0.21	3.17	0.64	0.48	3.38	11.66	20.38	1.50	1.47	1.72	1.43	2.81	1.32
Thyme US 6.24 min	2.83	79.95	0.40	1.99	1.23	0.52	0.22	1.70	9.49	18.50	0.70	1.35	2.53	0.77	2.54	0.83
Thyme PEF+US 6.24 min	4.04	84.08	1.34	0.20	3.31	0.94	0.50	2.65	13.32	23.93	1.53	1.58	1.75	1.49	3.26	1.51
Thyme US 12.48 min	2.75	74.65	0.46	0.45	1.13	0.49	0.21	1.56	11.24	17.60	0.52	1.05	2.19	0.65	2.17	1.34
Thyme PEF+US 12.48 min	6.05	99.60	0.47	0.17	3.59	0.64	0.50	3.14	20.84	34.56	1.96	1.39	1.83	1.39	3.64	1.44

a	caffeic acid	rosmarinic acid	luteolin	carnosol	galloocatechin	cirsimaritin	coumaric acid-O-hexoside	medioresinol	cryptochlorogenic acid	rosmanol	hesperidin	carnosic acid	rosmaridiphenol	rosmadial isomer or rosmanol quinone	rosmanol isomer (epirosmanol)	ishorhamnetin-3-O-glucoside	hispidulin-rutinoside	trihydroxy-methoxy flavone
Rosemary PEF	0.00	0.00	0.18	0.00	2.95	0.00	1.07	0.86	0.21	0.84	3.71	0.00	0.09	3.14	1.03	0.17	0.61	0.00
Rosemary US 4.16 min	0.63	3.95	0.58	185.00	1.98	8.91	0.52	3.51	0.23	77.29	13.88	1.85	9.00	17.08	105.69	1.00	10.45	4.07
Rosemary US + PEF 4.16 min	0.46	12.11	0.86	72.47	5.28	9.09	1.43	5.38	0.85	116.29	17.38	0.95	8.83	35.95	173.90	1.45	9.61	3.98
Rosemary US 6.24 min	1.41	6.52	0.88	153.68	2.78	9.00	0.38	4.31	0.75	82.47	16.24	1.58	8.32	19.24	113.85	2.20	10.32	2.78
Rosemary US + PEF 6.24 min	0.76	16.94	0.95	72.10	5.80	9.47	1.44	5.64	1.23	125.28	18.77	0.97	9.02	42.02	183.12	1.93	11.47	8.10
Rosemary US 12.48 min	1.32	8.32	1.41	113.44	2.53	7.09	0.33	4.03	0.86	69.13	17.38	1.14	6.44	16.06	92.64	2.10	8.44	9.34
Rosemary US+PEF 12.48 min	1.06	24.94	1.58	77.16	5.89	9.74	1.54	5.48	1.55	126.25	24.35	0.94	9.16	46.31	192.44	2.50	12.56	7.81



Fig. 4. Heat maps of different polyphenols quantified a in rosemary and b thyme after application of different treatments and times in mg 100 g⁻¹ FW.

previously identified in the above stated literature sources. A number of phenolic acids including protocatechuic acid, 4-coumaric acid and 4-hydroxybenzoic acid were only identified in the extracts resulted from PEF and were not detectable after further UAE. This result demonstrated their presence in trace levels that were only detectable in a concentrated extract (34 g or 36 g of rosemary thyme respectively in 24 g of 0.1% NaCl in deionized water) (Table 2), but not after further dilution (1:20) solid-to-liquid-ratio during UAE. In parallel, additional compounds such as luteolin-7-O-glucoside and rosmarinic acid were not identified in the PEF extracts prior to the UAE. This implies that these compounds needed the subsequent use of US and the utilization of non-aqueous solvents and US to be released from the plant cells.

The quantification of phenolic compounds was carried out with authentic standards where commercially available. Relative or equivalent quantification with the use of reference compounds was conducted in the absence of commercial standards based on either structural similarities or when their limit of quantification could be reached. The concentration of the quantified phenolic compounds in rosemary and thyme extracts is illustrated with the use of heat maps illustrated in Fig. 4a and b, respectively. Other studies have suggested that carnosol, carnosic acid and rosmarinic acid constitute the most abundant antioxidant compounds in rosemary (Albu et al., 2004). However, the present study suggests that rosemary had a considerably lower content of both carnosic acid and rosmarinic acid in comparison to rosmanol, epirosmanol and carnosol (Fig. 4a, Table S4). The lower content of carnosic acid may be related to its degradation to carnosol as has been shown by Zhang et al. (2012), who assessed the degradation of ethanolic solutions of carnosol, carnosic acid and rosmarinic acid and their mixture with high-performance liquid chromatography (HPLC), after their exposure to light and at different temperatures (Zhang et al., 2012). However, compared to carnosic acid, rosmarinic acid was present in higher concentration in the different extracts and was significantly higher in the PEF-treated extracts, particularly after 12.48 min of US extraction. Rosmanol content seemed to be also significantly enhanced with prior application of PEF after 12.48 min of US. However, this difference was not significant based on the extraction time as it was shown from the PEF-treated samples after 4.16 and 6.24 min of subsequent US extraction. No significant difference was also observed for samples after 6.24 min of only US extraction. A similar effect was observed for epirosmanol, as PEF-treated samples after 12.48 min of US extraction had significantly higher values compared to the non-treated. As it was further shown, carnosol content was reduced after application of longer time of US. Though its content was lower mainly after application of PEF pre-treatment indicating the formation of other derivatives, such as epirosmanol and rosmanol.

The formation of the same derivatives has been also confirmed by Zhang et al. (2012). As it has been additionally suggested, US may lead to a number of chemical modifications as a result of the formation of free radicals within the cavitation bubbles (Paniwnyk, Beaufoy, Lorimer, & Mason, 2001). Therefore, the higher concentration of derivatives in the PEF-treated samples may be related to the more rapid release of intracellular carnosol and its prolonged exposure to US cavitation phenomena. Though, derivatives such as epirosmanol, rosmanol and rosmaridiphenol, also belong to the most potent antioxidant constituents of rosemary, (Niето, Ros, & Castillo, 2018) and an increase in their levels could explain the higher total antioxidant indices and phenolic content in these extracts.

With respect to thyme extracts, PEF pre-treatment (Fig. 4b, Table S5) resulted in extracts with different phenolic profiles, when compared to those after only US treatment. The most abundant phenolic constituents of thyme were luteolin-7-O-glucoside, luteolin-7-O-glucuronide and rosmarinic acid, and these are also the most abundant phenolic compounds reported in other studies (Pereira, Peres, Silva, Domingues, & Cardoso, 2013; Singh & Sharma, 2020; Tzima, Brunton, & Rai, 2020). Luteolin-7-O-glucoside exhibited significantly higher content only in the PEF-pretreated extracts after 12.48 min of US extraction. For luteolin-7-

O-glucuronide, a significant difference was observed after PEF-pre-treatment with subsequent US extraction for 4.16 min compared to US individually, while no significant differences were observed between the extracts after higher extraction times. Nevertheless, PEF pre-treated samples after 12.48 min had a significantly higher concentration compared to both PEF treated and untreated samples after 4.16 min of US extraction. Even if no significant differences were attained, a trend of higher extractability after PEF pre-treatment was apparent. Similarly, 12.48 min of US extraction with prior PEF application, led to a significantly higher content of rosmarinic acid compared to the extracts received after 4.16 min of US (with or without PEF pre-treatment). Though, its concentration was not significantly different between the extracts after 4.16 min of PEF + US or only US. In the case of rosemary extracts, significant differences and lower standard deviations were apparent for rosmarinic acid, indicating a significant effect of PEF in its recovery with US. As outlined in Section 3.2, high standard deviations were expected in this study, due to the use of fresh samples and the non-homogeneity of the starting material, which was deliberately created to mimic large scale industrial use of the pre-treatment and extraction techniques. However, these deviations may explain the lack of significance in many cases, especially after the assessment of individual compounds with a sensitive analytical technique such as UPLC-ESI-MS/MS. Nonetheless, clear trends were observed for several of the most abundant polyphenols, where minimal cell-permeabilization from PEF seemed to lead to higher recoveries of phenolic compounds and total antioxidant indices and total phenolic content. A higher number of replicates (>3) could result in more evident effects. Nonetheless, the use of fresh plant material and the limited time for its storage were some of the restrictive factors.

Overall, as the results indicated, PEF pre-treatment increased the solubility of phenolic compounds and the permeability of the plant cell walls. Therefore, the subsequent use of 55.19% aqueous EtOH during US application, successfully facilitated the transport of phenolics of different polarities and molecular weights through the cell membranes to the extraction medium, without exhibiting a specific pattern based on their structural characteristics. Solvent polarity constitutes a critical factor that determines the extraction efficiency (Xia et al., 2011). Moderate EtOH concentrations among 40% and 60% yielded the highest extraction efficiency of phenolic compounds from rosemary leaves, whereas 55.19% was the optimal concentration according to Hosseini et al. (2018) who determined the US conditions applied in this study. This could be potentially attributed to relative polarity of the bioactive compounds present, and the increased propagation of US waves in aqueous solvents (Hosseini et al., 2018). As it is known, the presence of certain H₂O concentration in the solvent can enhance the swelling of plant materials, and therefore, increase the contact of the plant matrix surface area and the solvent (Hosseini et al., 2018). Similar results were expected and observed for thyme after application of US with 55.19% EtOH, as it belongs to the same herbal family with rosemary (*Lamiaceae*) and has a similar phenolic profile.

It is also important to highlight a number of shortcomings that could be raised in relation to other studies on plant materials after the assessment and evaluation of their data and the detection of significance in their results. In most cases, researchers utilize ANOVA analysis, followed by post hoc tests such as Tukey's HSD. ANOVA falls into the category of parametric analysis and its prerequisites include the normality, independence, and homogenous variance of the examined datasets (Kim, 2017). As it has been indicated, the analysis of variances is critical for the correct assessment and definition of any potential statistical significance, whereas it is reliant on the satisfaction of the above mentioned prerequisites (Nunes, Alvarenga, de Souza Sant'Ana, Santos, & Granato, 2015). For instance, when the assumption of homogeneity of variances is violated, there is a high probability of Type I error (Moder, 2007) and detection of significances, whereas they are absent. Nonetheless, a limited number of publications comment on whether these prerequisites are satisfied. A further issue according to

(Nunes et al., 2015) is the “hypothesis of genuine replicates” as it most cases it is stated that “analyses were performed in triplicate” without defining whether they are independent (Nunes et al., 2015) (i.e. three independent extracts of a plant) or not (i.e. the analysis was conducted three times on the same extract). Therefore, as many researchers tend to quantify phenolics three times in the same extract, low standard deviations and high significance can be incorrectly attained.

4. Conclusions

A different selective release of the phenolic compounds was observed upon US, PEF and their combination, with different effects on rosemary and thyme fresh herbal by-products. At the same electrical field strength, a higher Z_{σ} index was achieved for thyme compared to rosemary. This may be related to the more pronounced effect of PEF pre-treatment in thyme extracts on the total phenolic content and antioxidant indices following the subsequent US aided extraction. In addition, PEF pre-treatment significantly ($p < 0.05$) increased the DPPH values of rosemary and thyme in most cases, and TPC of thyme. This fact indicates that the PEF treated samples had higher antioxidant and phenolic indices compared to the US treated independently, but also the highest indices at extraction time of 12.48 min. However, the FRAP assay indicated non-significant ($p > 0.05$) differences between the US and US after PEF for all the rosemary extracts. PEF pre-treatment increased the concentration of some of the major phenolic compounds of rosemary and thyme as revealed by the UPLC-ESI-MS/MS analysis, particularly after 12.48 min of US, where a significant ($p < 0.05$) increase was observed in a number of cases. However, PEF resulted in the reduction of carnosol concentration, a potential result of degradation and formation of its derivatives that possess less activity. Non-significant results could be attributed in several cases to the high variability in the extracts stemming from the use of fresh samples comprising of a mixture of stems and leaves. These findings suggest the choice of an extraction technology based on the required target compounds and the importance of its application during non-idealistic conditions to examine the variability of its potential industrial application. Therefore, our study is one of the few that examined fresh inhomogeneous plant material, which is closest to a real-life situation. Based on the obtained results, PEF pre-treatment can be used as a disintegration method prior to solid-liquid extraction processes, as beneficial effects were attained even with the use of low energy inputs. Even if PEF treatment yielded substantially lower specific energy inputs for both rosemary and thyme extracts in comparison to UAE, UAE step is still required for the extraction of the targeted compounds, while its energy input is lower from this of conventional extraction techniques. Hence, if further appropriately optimized, it could enhance the extraction of various phenolic compounds from fresh rosemary and thyme by-products and eliminate the economic barrier of drying by processors for their utilization.

Industrial relevance text

The utilization of herbal by-products can be a cost-efficient process for the recovery of functional and antioxidant compounds, particularly when large volumes are produced prior to retail. Therefore, this study examines the feasibility of novel extraction technologies, including pulsed electric field, ultrasound and their combination, as potential methods for the extraction of phenolic compounds from fresh rosemary and thyme by-products. The choice of these emerging technologies was based on the fact that a drying step prior to their use is unlikely to be used by herb processors due to its high energy inputs and its subsequent cost. The higher recovery of phenolic compounds that was achieved in several cases after the combined effect of pulsed electric field and ultrasound and the parallel use of low energy inputs, indicates the industrial relevance of these techniques after further optimization.

Author contributions

K.T. conducted the experiments and carried out the statistical analysis. D.K.R. and N.P.B. provided valuable editorial comments and supervised the experiments. D-F provided training in PEF treatment and disintegration index evaluation, and instructions for the application of UAE. K.T., D.K.R., N.P.B. and J.G.L. wrote, edited and reviewed the manuscript. All authors have read and approved the final manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

Author statement

Please find herewith the revised manuscript entitled “The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products” for consideration for publication in the *Innovative Food Science and Emerging Technologies*. I can confirm that this work is original, all authors have consented to its submission and have contributed to its composition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ifset.2021.102644>.

References

- Aadil, R. M., Zeng, X.-A., Han, Z., Sahar, A., Khalil, A. A., Rahman, U. U., ... Mehmood, T. (2018). Combined effects of pulsed electric field and ultrasound on bioactive compounds and microbial quality of grapefruit juice. *Journal of Food Processing and Preservation*, 42(2), Article e13507.
- Albu, S., Joyce, E., Paniwnyk, L., Lorimer, J. P., & Mason, T. J. (2004). Potential for the use of ultrasound in the extraction of antioxidants from *Rosmarinus officinalis* for the food and pharmaceutical industry. *Ultrasonics Sonochemistry*, 11(3), 261–265.
- Alvi, S. S., Ahmad, P., Ishrat, M., Iqbal, D., & Khan, M. S. (2019). Secondary metabolites from rosemary (*Rosmarinus officinalis* L.): Structure, biochemistry and therapeutic implications against neurodegenerative diseases. In M. K. Swamy, & M. S. Akhtar (Eds.), *Natural bio-active compounds: Volume 2: Chemistry, pharmacology and health care practices* (pp. 1–24). Singapore: Springer Singapore.
- Barba, F. J., Galanakis, C. M., Esteve, M. J., Frigola, A., & Vorobiev, E. (2015). Potential use of pulsed electric technologies and ultrasounds to improve the recovery of high-added value compounds from blackberries. *Journal of Food Engineering*, 167, 38–44.
- Baysal, T., & Demirdoven, A. (2011). Ultrasound in food technology. In D. Chen, S. K. Sharma, & A. Mudhoo (Eds.), *Handbook of applications of ultrasound: Sonochemistry for sustainability* (pp. 163–182). Boca Raton, Florida: CRC Press, Taylor & Francis Publishing Group.
- Bellumori, M., Innocenti, M., Binello, A., Boffa, L., Mulinacci, N., & Cravotto, G. (2016). Selective recovery of rosmarinic and carnosic acids from rosemary leaves under ultrasound- and microwave-assisted extraction procedures. *Comptes Rendus Chimie*, 19(6), 699–706.
- Bendif, H., Adouni, K., Miara, M. D., Baranauskienė, R., Kraujalis, P., Venskutonis, P. R., ... Maggi, F. (2018). Essential oils (EOs), pressurized liquid extracts (PLE) and carbon dioxide supercritical fluid extracts (SFE-CO₂) from Algerian *Thymus munbyanus* as valuable sources of antioxidants to be used on an industrial level. *Food Chemistry*, 260, 289–298.
- Bistgani, Z. E., Hashemi, M., DaCosta, M., Craker, L., Maggi, F., & Morshedloo, M. R. (2019). Effect of salinity stress on the physiological characteristics, phenolic

- compounds and antioxidant activity of *Thymus vulgaris* L. and *Thymus daenensis* Celak. *Industrial Crops and Products*, 135, 311–320.
- Bozinou, E., Karageorgou, I., Batra, G., Dourtoglou, V. G., & Lalas, S. I. (2019). Pulsed electric field extraction and antioxidant activity determination of *Moringa oleifera* dry leaves: A comparative study with other extraction techniques. *Beverages*, 5(1), 8.
- Brown, C. E. (1998). *Applied multivariate Statistics in geohydrology and related sciences* (1st ed.). Germany, Heidelberg: Springer.
- Clarke, G., Ting, K. N., Wiart, C., & Fry, J. (2013). High correlation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing activity potential and total phenolics content indicates redundancy in use of all three assays to screen for antioxidant activity of extracts of plants from the Malaysian rainforest. *Antioxidants (Basel, Switzerland)*, 2(1), 1–10.
- De Vito, F., Ferrari, G., Lebovka, I., Shynkaryk, N. V., & Vorobiev, E. (2008). Pulse duration and efficiency of soft cellular tissue disintegration by pulsed electric fields. *Food and Bioprocess Technology*, 1(4), 307–313.
- Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., & Mérillon, J. M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry*, 57(5), 1768–1774.
- El Darra, N., Grimi, N., Maroun, R. G., Louka, N., & Vorobiev, E. (2013). Pulsed electric field, ultrasound, and thermal pretreatments for better phenolic extraction during red fermentation. *European Food Research and Technology*, 236(1), 47–56.
- Fincan, M. (2015). Extractability of phenolics from spearmint treated with pulsed electric field. *Journal of Food Engineering*, 162, 31–37.
- Gachovska, T., Cassada, D., Subbiah, J., Hanna, M., Thipparedi, H., & Snow, D. (2010). Enhanced anthocyanin extraction from red cabbage using pulsed electric field processing. *Journal of Food Science*, 75(6), E323–E329.
- Gangopadhyay, N., Rai, D. K., Brunton, N. P., Gallagher, E., & Hossain, M. B. (2016). Antioxidant-guided isolation and mass spectrometric identification of the major polyphenols in barley (*Hordeum vulgare*) grain. *Food Chemistry*, 210, 212–220.
- Goupy, P., Hugues, M., Boivin, P., & Amiot, M. J. (1999). Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture*, 79(12), 1625–1634.
- Hosni, K., Hassen, I., Chaabane, H., Jemli, M., Dallali, S., Sebei, H., & Casabianca, H. (2013). Enzyme-assisted extraction of essential oils from thyme (*Thymus capitatus* L.) and rosemary (*Rosmarinus officinalis* L.): Impact on yield, chemical composition and antimicrobial activity. *Industrial Crops and Products*, 47, 291–299.
- Hosseini, H., Bolourian, S., Yaghoubi Hamgini, E., & Ghanuni Mahababadi, E. (2018). Optimization of heat- and ultrasound-assisted extraction of polyphenols from dried rosemary leaves using response surface methodology. *Journal of Food Processing and Preservation*, 42(11), Article e13778.
- Iswaldi, I., Arráez-Román, D., Rodríguez-Medina, I., Beltrán-Debón, R., Joven, J., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2011). Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT). *Analytical and Bioanalytical Chemistry*, 400(10), 3643–3654.
- Jacobo-Velázquez, D. A., Cuéllar-Villarreal, M. D. R., Welti-Chanes, J., Cisneros-Zevallos, L., Ramos-Parra, P. A., & Hernández-Brenes, C. (2017). Nonthermal processing technologies as elicitors to induce the biosynthesis and accumulation of nutraceuticals in plant foods. *Trends in Food Science & Technology*, 60, 80–87.
- Jovanović, A. A., Dorđević, V. B., Zduñić, G. M., Pljevljakusić, D. S., Savikin, K. P., Godevac, D. M., & Bugarski, B. M. (2017). Optimization of the extraction process of polyphenols from *Thymus serpyllum* L. herb using maceration, heat- and ultrasound-assisted techniques. *Separation and Purification Technology*, 179, 369–380.
- Katalinic, V., Milos, M., Kulisic, T., & Jukic, M. (2006). Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food Chemistry*, 94(4), 550–557.
- Kim, T. K. (2017). Understanding one-way ANOVA using conceptual figures. *Korean Journal of Anesthesiology*, 70(1), 22–26.
- Lebovka, N. I., Shynkaryk, N. V., & Vorobiev, E. (2007). Pulsed electric field enhanced drying of potato tissue. *Journal of Food Engineering*, 78(2), 606–613.
- Liu, Z., Esveld, E., Vincken, J.-P., & Bruins, M. E. (2019). Pulsed electric field as an alternative pre-treatment for drying to enhance polyphenol extraction from fresh tea leaves. *Food and Bioprocess Technology*, 12(1), 183–192.
- López-Gómez, G., Elez-Martínez, P., Martín-Belloso, O., & Soliva-Fortuny, R. (2020). Enhancing phenolic content in carrots by pulsed electric fields during post-treatment time: Effects on cell viability and quality attributes. *Innovative Food Science & Emerging Technologies*, 59, 102252.
- Luengo, E., Condón-Abanto, S., Alvarez, I., & Raso, J. (2014). Effect of pulsed electric field treatments on permeabilization and extraction of pigments from *Chlorella vulgaris*. *The Journal of Membrane Biology*, 247(12), 1269–1277.
- MacDonald-Wicks, L. K., Wood, L. G., & Garg, M. L. (2006). Methodology for the determination of biological antioxidant capacity in vitro: A review. *Journal of the Science of Food and Agriculture*, 86(13), 2046–2056.
- Manzoor, M. F., Zeng, X.-A., Rahaman, A., Siddeeg, A., Aadil, R. M., Ahmed, Z., ... Niu, D. (2019). Combined impact of pulsed electric field and ultrasound on bioactive compounds and FT-IR analysis of almond extract. *Journal of Food Science and Technology*, 56(5), 2355–2364.
- Medina-Meza, I. G., Bolioli, P., & Barbosa-Cánovas, G. V. (2016). Assessment of the effects of Ultrasonics and pulsed electric fields on nutritional and rheological properties of raspberry and blueberry purees. *Food and Bioprocess Technology*, 9(3), 520–531.
- Mena, P., Cirilini, M., Tassotti, M., Herrlinger, K. A., Dall'Asta, C., & Del Rio, D. (2016). Phytochemical profiling of flavonoids, phenolic acids, terpenoids, and volatile fraction of a rosemary (*Rosmarinus officinalis* L.) extract. *Molecules*, 21(11), 1576.
- Milevskaia, V. V., Temerdashev, Z. A., Butyl'skaya, T. S., & Kiseleva, N. V. (2017). Determination of phenolic compounds in medicinal plants from the *Lamiaceae* family. *Journal of Analytical Chemistry*, 72(3), 342–348.
- Milne, L., Stewart, I., & Bremner, D. H. (2013). Comparison of hydroxyl radical formation in aqueous solutions at different ultrasound frequencies and powers using the salicylic acid dosimeter. *Ultrasonics Sonochemistry*, 20(3), 984–989.
- Moder, K. (2007). How to keep the type I error rate in ANOVA if variances are heteroscedastic. *Austrian Journal of Statistics*, 36, 179–188.
- Mulinacci, N., Innocenti, M., Bellumori, M., Giaccherini, C., Martini, V., & Michelozzi, M. (2011). Storage method, drying processes and extraction procedures strongly affect the phenolic fraction of rosemary leaves: An HPLC/DAD/MS study. *Talanta*, 85(1), 167–176.
- Nieto, G., Ros, G., & Castillo, J. (2018). Antioxidant and antimicrobial properties of rosemary (*Rosmarinus officinalis*, L.): A review. *Medicines (Basel, Switzerland)*, 5(3), 98.
- Nunes, C. A., Alvarenga, V. O., de Souza Sant'Ana, A., Santos, J. S., & Granato, D. (2015). The use of statistical software in food science and technology: Advantages, limitations and misuses. *Food Research International*, 75, 270–280.
- Paniwnyk, L., Beaufoy, E., Lorimer, J. P., & Mason, T. J. (2001). The extraction of rutin from flower buds of *Sophora japonica*. *Ultrasonics Sonochemistry*, 8(3), 299–301.
- Parada, J., & Aguilera, J. M. (2007). Food microstructure affects the bioavailability of several nutrients. *Journal of Food Science*, 72(2), R21–R32.
- Pereira, O. R., Peres, A. M., Silva, A. M. S., Domingues, M. R. M., & Cardoso, S. M. (2013). Simultaneous characterization and quantification of phenolic compounds in *Thymus x citriodorus* using a validated HPLC-UV and ESI-MS combined method. *Food Research International*, 54(2), 1773–1780.
- Puértolas, E., Cregenzán, O., Luengo, E., Álvarez, I., & Raso, J. (2013). Pulsed-electric-field-assisted extraction of anthocyanins from purple-fleshed potato. *Food Chemistry*, 136(3), 1330–1336.
- Puértolas, E., Saldaña, G., & Raso, J. (2016). Pulsed electric field treatment for fruit and vegetable processing. In D. Miklavcic (Ed.), *Handbook of electroporation* (pp. 1–21). Cham: Springer International Publishing.
- Pyrzynska, K., & Pękal, A. (2013). Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples. *Analytical Methods*, 5(17), 4288–4295.
- Roohinejad, S., Everett, D. W., & Oey, I. (2014). Effect of pulsed electric field processing on carotenoid extractability of carrot purée. *International Journal of Food Science & Technology*, 49(9), 2120–2127.
- Segovia, F. J., Luengo, E., Corral-Pérez, J. J., Raso, J., & Almajano, M. P. (2015). Improvements in the aqueous extraction of polyphenols from borage (*Borago officinalis* L.) leaves by pulsed electric fields: Pulsed electric fields (PEF) applications. *Industrial Crops and Products*, 65, 390–396.
- Siddeeg, A., Faisal Manzoor, M., Haseeb Ahmad, M., Ahmad, N., Ahmed, Z., Kashif Iqbal Khan, M., ... Ammar, A.-F. (2019). Pulsed electric field-assisted ethanolic extraction of date palm fruits: Bioactive compounds, antioxidant activity and physicochemical properties. *Processes*, 7(9), 585.
- Singh, B., & Sharma, R. A. (2020). Thymus species. In *Secondary metabolites of medicinal plants, 4 volume set: Ethnopharmacological properties, biological activity and production strategies* (pp. 1164–1178). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.
- Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. R. (1999). Analysis of total phenols and other oxidation substrates and oxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*, 299, 152–178.
- Stratil, P., Klejduš, B., & Kubáň, V. (2006). Determination of total content of phenolic compounds and their antioxidant activity in vegetables evaluation of spectrophotometric methods. *Journal of Agricultural and Food Chemistry*, 54(3), 607–616.
- Tiwari, B. K. (2015). Ultrasound: A clean, green extraction technology. *TrAC Trends in Analytical Chemistry*, 71, 100–109.
- Tzima, K., Brunton, N. P., & Rai, D. K. (2020). Evaluation of the impact of chlorophyll removal techniques on polyphenols in rosemary and thyme by-products. *Journal of Food Biochemistry*, 44(3), Article e13148.
- Upadhyay, R., Nachiappan, G., & Mishra, H. N. (2015). Ultrasound-assisted extraction of flavonoids and phenolic compounds from *Ocimum tenuiflorum* leaves. *Food Science and Biotechnology*, 24(6), 1951–1958.
- Vallverdú-Queralt, A., Regueiro, J., Martínez-Huelamo, M., Rinaldi Alvarenga, J. F., Leal, L. N., & Lamuela-Raventos, R. M. (2014). A comprehensive study on the phenolic profile of widely used culinary herbs and spices: Rosemary, thyme, oregano, cinnamon, cumin and bay. *Food Chemistry*, 154, 299–307.
- Vinatoro, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry*, 8(3), 303–313.
- Vorobiev, E., & Lebovka, N. (2008). Pulsed-electric-fields-induced effects in plant tissues: fundamental aspects and perspectives of applications. In E. Vorobiev, & N. Lebovka (Eds.), *Electrotechnologies for extraction from food plants and biomaterials* (pp. 39–81). New York, US: Springer-Verlag.
- Vorobiev, E., & Lebovka, N. I. (2006). Extraction of intercellular components by pulsed electric fields. In J. Raso, & V. Heinz (Eds.), *Pulsed electric fields technology for the food industry: Fundamentals and applications* (pp. 153–193). Boston, MA: Springer US.
- Wiktor, A., Gondek, E., Jakubczyk, E., Dadan, M., Nowacka, M., Rybak, K., & Witrowa-Rajchert, D. (2018). Acoustic and mechanical properties of carrot tissue treated by pulsed electric field, ultrasound and combination of both. *Journal of Food Engineering*, 238, 12–21.
- Wiktor, A., Sledz, M., Nowacka, M., Rybak, K., Chudoba, T., Lojkowski, W., & Witrowa-Rajchert, D. (2015). The impact of pulsed electric field treatment on selected bioactive compound content and color of plant tissue. *Innovative Food Science & Emerging Technologies*, 30, 69–78.
- Wiktor, A., & Witrowa-Rajchert, D. (2016). Pulsed electric fields as pretreatment for subsequent food process operations. In D. Miklavcic (Ed.), *Handbook of electroporation* (pp. 1–16). Cham: Springer International Publishing.

- Wong, P. Y. Y., & Kitts, D. D. (2006). Studies on the dual antioxidant and antibacterial properties of parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) extracts. *Food Chemistry*, 97(3), 505–515.
- Xia, E.-Q., Ai, X.-X., Zang, S.-Y., Guan, T.-T., Xu, X.-R., & Li, H.-B. (2011). Ultrasound-assisted extraction of phillyrin from *Forsythia suspensa*. *Ultrasonics Sonochemistry*, 18(2), 549–552.
- Zderic, A., & Zondervan, E. (2016). Polyphenol extraction from fresh tea leaves by pulsed electric field: A study of mechanisms. *Chemical Engineering Research and Design*, 109, 586–592.
- Zderic, A., & Zondervan, E. (2017). Product-driven process synthesis: Extraction of polyphenols from tea. *Journal of Food Engineering*, 196, 113–122.
- Zhang, Y., Smuts, J. P., Doddiba, E., Rangarajan, R., Lang, J. C., & Armstrong, D. W. (2012). Degradation study of Carnosic acid, carnosol, rosmarinic acid, and rosemary extract (*Rosmarinus officinalis* L.) assessed using HPLC. *Journal of Agricultural and Food Chemistry*, 60(36), 9305–9314.
- Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, 49(11), 5165–5170.