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Pulsed Electric Field-Based Extraction of Total Polyphenols from *Sideritis raiseri* Using Hydroethanolic Mixtures

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Abstract: Polyphenols are an important class of compounds, due to their excellent antioxidant properties. Lately, much effort has been placed into developing new extraction techniques and optimizing them, so that polyphenols can be retrieved more efficiently from the plant materials. One of the most recent advances in extraction techniques is pulsed electric field extraction (PEF). This new technique is environmentally friendly and has the potential to maximize the recovery of compounds from plant tissues. Although the efficiency of PEF depends, among others, on the nature of the solvent used, up to date, there are no reports on the optimization of the PEF extraction of polyphenols, using hydroethanolic solutions of varying content in ethanol. In this study, three hydroethanolic solutions, water, and ethanol were used for the PEF-based extraction of total polyphenols from *Sideritis raiseri*. Results were conclusive that the 1:1 mixture of ethanol and water can increase by up to 146% the yield of polyphenols in the extract, highlighting the need to study more extensively, in the future, mixtures of solvents and not just plain water.

Keywords: pulsed electric field extraction; polyphenols; *Sideritis raiseri*; Folin–Ciocalteu; HPLC-DAD

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1. Introduction

Oxidative damage is a broad term, that is used to define the state where there is a perturbation in the balance between the number of reactive oxygen species (ROS) and the number of antioxidant compounds, within an organism, in favor of ROS, which in turn results in damage of various cellular components [1]. Oxidative damage has been found to be linked to numerous diseases, abnormal health conditions, and is even linked to cancer [2]. Therefore, much effort has been placed to counteract the detrimental effects of oxidative damage within living systems. However, it is well known that prevention is better than treatment. Therefore, preventing oxidative damage is of much higher importance. To this end, multiple studies have been carried out, evaluating the performance of various compounds, in terms of antioxidant activity [3–6]. A class of chemical compounds that holds great promise since it has well-grounded antioxidant activities are the phenolic compounds and polyphenols.

Polyphenols are a class of organic compounds that are abundantly being found in plants since they are used by the plants to protect them from ultraviolet radiation [7]. Polyphenols can be found in all fruits and vegetables that are widely consumed in human diets, in varying concentrations. Among others, tea-like beverages are among the 100 richest dietary sources of polyphenols [8]. There are many species of plants used for tea production, with the most famous being the *Camellia sinensis*, from which green and black tea is produced. In the Mediterranean area, however, since ancient times, an endemic plant (i.e., *Sideritis*) was used extensively in folk medicine [9]. *Sideritis* (mountain tea) is mainly used for tea production and consumed as such. Due to its medicinal usage, much

effort has been placed in detecting and isolating active phytoconstituents. However, the reports about the extraction of polyphenols from *Sideritis*, are scanty and sparse [10–15]. In these reports, classical techniques have been employed, including Soxhlet extraction [11], ultrasonic-assisted extraction [13], and homogenization-assisted extraction [10] or simple liquid extraction using various solvents [15]. Recently, our laboratory showcased the applicability of pulsed electric field (PEF) as a standalone extraction procedure [14] and highlighted the instrumental conditions that favor the extraction of polyphenols from plant material.

This extraction method has multiple benefits, including among others: minimum environmental impact due to its low energy usage, capability to alter the composition of the obtained extract, depending on the experimental parameters (e.g., electric field strength, pulse duration, pulse period, pulse frequency, etc.), limited damage to heat-sensitive substances, since no heat is generated during PEF extraction) and improved extraction yields, compared to other techniques [16–18]. The novelty of PEF lies within the electroporation that takes place, during which the cellular membranes of the plant tissue, are rendered more permeable, and thus, the phytoconstituents can more readily diffuse. Due to all the above-mentioned advantages, there is increasing interest in the preparation of extracts using PEF, that can be used in the food and pharmaceutical industries [16]. Up to date, PEF there are multiple reports regarding the use of PEF for the extraction of compounds from plant material, such as the extraction of polyphenols from olive leaves [19], tea leaves [20], citrus fruits [21], potato peels [22] and Merlot grapes [23].

Despite the fact that the nature of the solvent can affect the efficacy of PEF extraction [16], little emphasis has been placed on this, and in most studies, water is used as the sole solvent [14,24–27]. Since in previous studies, employing other extraction techniques showcased that a mixture of water and ethanol achieves better extraction of polyphenols [28], our aim was to examine the effect of water:ethanol mixture composition on the extractability of total polyphenols from *Sideritis*, using the PEF extraction method. To this end, the *Sideritis raiseri* was employed, and using three mixtures of water and ethanol, along with pure water and pure ethanol in combination with two different pulse duration times the polyphenol extracts were obtained. The extracts were assessed in terms of total polyphenol content (TPC) (using the Folin–Ciocalteu assay) and a high-performance liquid chromatography (HPLC) assay so that results about their content in total polyphenols could be obtained.

2. Materials and Methods

2.1. Chemicals

Acetonitrile, water, and ethanol were of HPLC grade. Solvents and formic acid were obtained from Carlo Erba (Val de Reuil, France). Anhydrous sodium carbonate (>99%) and gallic acid monohydrate were purchased from Penta (Prague, Czech Republic). Folin–Ciocalteu reagent was obtained from Panreac (Barcelona, Spain).

2.2. Plant Material

The aerial parts of *Sideritis raiseri* were a kind offer of BioPetersHerbs (Makrichori, GR-43100, Karditsa, Greece). The plants were collected from the Makrichori area (Karditsa, Greece), in June 2021. After the plants were collected, they were placed in airtight bags and into a fridge, until transported to the laboratory. Then, the plant material was washed with ice-cold water and dried using a paper towel. An appropriate amount of the material was then subjected to PEF extraction and the rest was stored in airtight containers, at 4 °C.

2.3. Instrumentation

The PEF system used in this study consisted of a high voltage power generator (Leybold, LD Didactic GmbH, Huerth, Germany) (maximum voltage 25 kV), a digital oscilloscope (Rigol DS1052E, Rigol Technologies, Inc, Beaverton, OR, USA), a function/arbitrary

waveform generator (UPG100, ELV Elektronik AG, Leer, Germany), and two custom-made stainless-steel chambers (Val-Electronic, Athens, Greece) [14].

A Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany) was used for the absorbance measurements.

The HPLC system was a Shimadzu CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany), coupled to a diode array detector (Shimadzu SPD-M20A). A Phenomenex Luna C18 column (5 μm, 4.6 × 250 mm) (Phenomenex Inc., Torrance, CA, USA) was used as a stationary phase and placed in an oven so that the temperature of the stationary phase was kept constant at 40 °C during all runs. The mobile phase consisted of (A) water containing 0.5% v/v formic acid and (B) a mixture of acetonitrile:water (60:40) containing 0.5% v/v formic acid. The elution program was as follows: 5% B to 40% B in 40 min, then to 50% in 10 min, and finally to 70% in 10 min and kept constant for 10 more minutes, with a flow rate of 1 mL min^{-1}. The total program run time was 70 min. The injection volume was 20 μL and injections were made using a rheodyne injector.

2.4. Dry Weight Determination

In all PEF-based extractions, it is of paramount importance that the extractable tissue contains humidity, in order for electroporation to take place. Therefore, dry tissues cannot be used for PEF extractions. On top of that, in the case that dry tissue needs to be used, it is a common practice to re-hydrate the plant tissue [29]. However, in order to have a basis to express the results, the amount of humidity in the plant needs to be determined. In order to determine the water content of the plant material, a portion of the plant was placed in an oven and heated at 105 °C, until constant weight.

2.5. PEF Extraction

For the extraction of total polyphenols, 4.0 g of fresh, cleaned plant material (not dry) were ground into smaller pieces and mixed with 80 mL of the extraction solvent (at a ratio of 20:1 mL g^{-1}). The extraction solvents were: 100% water, 25% ethanol in water, 50% ethanol in water, 75% ethanol in water, and 100% ethanol. After thorough mixing, the mixture was placed in the chamber of PEF and the extraction was carried out for 20 min. For the extraction of total polyphenols, two pulse durations were selected: 10 μs and 100 μs. The period was 1 ms (frequency: 1000 Hz) and a total of 100 pulse cycles were completed [30]. The electric field density was set to 1.0 kV cm^{-1}. The temperature was monitored at the beginning and the end of PEF, and no significant difference was recorded (less than one degree Celsius). After PEF was completed, the mixture was placed in a Falcon tube and centrifuged at 4500× g for 10 min. Then the supernatant (comprised of the extraction solvent and the polyphenols) was immediately subjected to further analyses, as stated in Sections 2.6 and 2.7. Control samples for each extraction solvent were also prepared, which were extracted by placing the plant–solvent mixture in the PEF chamber for 20 min but without applying pulses.

2.6. Folin–Ciocalteu Assay

A previously reported method was used to determine the total polyphenol content of the extracts [30,31]. In brief, the plant extracts (obtained after centrifugation) were firstly diluted with a formic acid solution (0.5% v/v) at a ratio of 1:50 (plant extract:formic acid solution). Then, 100 μL of the diluted sample was mixed with 100 μL of Folin–Ciocalteu reagent and vortexed. After 2 min, 800 μL of a sodium carbonate solution (5% w/v) was added. After incubating for 20 min at 40 °C the absorbance of the solution was measured at 740 nm. In order to determine the total polyphenol concentration (TPC), an appropriate calibration curve was prepared using gallic acid and the results were expressed as mg of gallic acid equivalents (GAE) per gram of dry weight.

2.7. HPLC-Based Determination of Total Polyphenol Content

A Shimadzu liquid chromatograph (CBM-20A) and a Shimadzu diode array detector (SPD-M20A) were used. For the detection of the compounds, a UV–vis spectrum was recorded from 190 nm to 800 nm. Identification of individual polyphenols (chlorogenic acid, verbascoside, 5-caffeoylquinic acid, and apigenin 7-*O*-glucoside) was carried out by comparing the retention time and the absorbance spectra with that of known standards. Integration of the chromatographic areas was carried out using the Shimadzu LC solution software. Results were expressed as the total chromatographic area for each extract.

2.8. Statistical Analysis

Extracts were prepared in triplicates and for each extract, three replicate analyses were carried out. Results are expressed as means of all measurements (nine measurements per condition). Statistically significant differences were evaluated by Kruskal–Wallis for $p < 0.05$, using SPSS (SPSS Inc., Chicago, IL, USA) software, after testing for normality of data with the Shapiro–Wilk test.

3. Results and Discussion

In our previous study, we showcased that the different parameters related to PEF can enhance the extraction of polyphenols. However, to a varying degree, compared to other plant species [14]. Therefore, we opted for different extraction solvents to maximize the extraction yield. Prior to the analysis of the TPC of the extracts, the percentage humidity of the plant material was assessed, so that the results can be expressed in terms of dry weight, and can be comparable, regardless of the water content of the plant. The average humidity of the plant was found to be 9 ± 2%.

As can be seen in Figure 1, there are notable differences between the different solvents used for the extraction. More specifically, using plain water, the TPC of the obtained extract was found to be 13.9 ± 0.2 mg GAE g^{-1} dw of *Sideritis raiseri*. When a mixture of 25% (*v*/*v*) ethanol:water mixture was used, a notable increase was recorded, yielding 24.6 ± 0.3 mg GAE g^{-1} dw (77% increase). A further 39% increase in the TPC was also recorded when the content of ethanol in the final mixture increased to 50%. However, neither further increase in the TPC was recorded, as the content of ethanol increased to 75%, nor in the case that pure ethanol was employed. Furthermore, according to the results, it is evident that the total polyphenol content of the extracts is not dependent on the pulse duration since all extracts obtained by using pulses with 10 μs and 100 μs were found to have nearly the same content of polyphenol compounds (except the case of 25% ethanol content, where 10 μs pulse yielded higher content of TPC). In all the above cases (except the case of 0% and 100% ethanol content for 10 μs pulse duration), the differences between the different extraction solvents employed were found to be statistically significant for $p < 0.05$. However, no statistically significant differences were recorded between most of the extracts obtained with the same solvent, but with different pulse duration. In addition to the above, in all cases, the control samples, extracted without the PEF technique, contained statistically significant ($p < 0.05$) fewer total polyphenols (5–19%), compared to the PEF extracted samples (except the case of 25% ethanol content and 100 μs pulse). This finding validates the superiority of the PEF being used as an extraction technique.

As regards the extraction solvent in PEF, many factors can affect the outcome, such as the polarity of the solvent and the solubility of the compounds in the solvent, as well as the electrical conductivity [16]. The electrical conductivity of pure ethanol, employed herein is <0.1 (μS cm^{-1}), and that of water is 2.3 (μS cm^{-1}) [19]. Therefore, as the percentage of ethanol in the water:ethanol mixture increases, the electrical conductivity of the mixture decreases. As stated previously, the higher the electrical conductivity of the solvent, the better the cell membrane electroporation that occurs during PEF extraction [16]. As a result, better extraction of the compounds from the plant cells occurs. This is contradicting the fact that when the mixtures of ethanol and water were used, a higher content of the extracts

in polyphenol compounds was recorded. This hints toward another explanation for the recorded results.

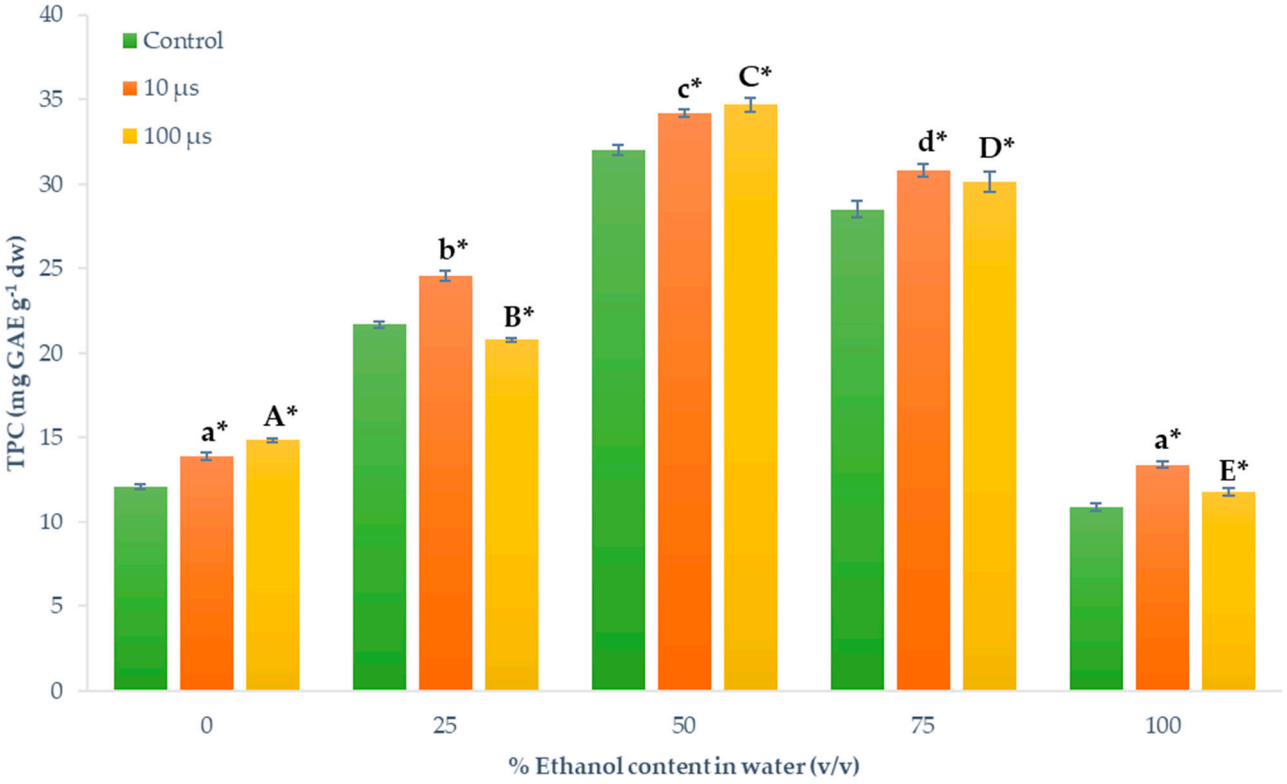

Figure 1. Total polyphenols, TPC (mg GAE g^{-1} dw), of the *Sideritis raiseri* extracts, obtained with mixtures of water:ethanol of different compositions, using PEF extraction. Statistically significant differences for $p < 0.05$ are denoted with lower letters (e.g., a–d) for 10 μs pulse duration and with capital letters (e.g., A–E) for 100 μs pulse duration samples; asterisks (*) denote statistically significant differences for $p < 0.05$ between samples and the respective control.

A putative explanation would be the polarity of the solvent. It is known that the polarity index of water is 10 and the polarity index of ethanol is 5.2 [32]. Therefore, as the percentage of ethanol in the mixture increases, the polarity of the mixture decreases. Using the equation reported by Hemwimon et al. [33] (i.e., $Pm = R_1P_1 + R_2P_2$, where R_1 and R_2 are the volume fractions of solvent 1 and 2, respectively, and P_1 and P_2 are the polarity indices of the two solvents) the polarity index of the mixtures are the following: 25% ethanol in water: 8.8, 50% ethanol in water: 7.6, 75% ethanol in water: 6.4. A solvent with a higher polarity index can extract more polar phenolic compounds, whereas solvents with a lower polarity index, such as the 50% ethanol in water mixture are able to extract phenolics with a broader range of polarity. The fact that the extracts obtained with 0% ethanol and 100% ethanol contained similar TPC can be justified by the fact that as the percentage of ethanol increases, the swelling of the plant is weaker, leading to decreased extraction efficiency. On the other hand, using plain water results in high polarity of the solvent, decreasing the extraction of less polar compounds [34]. Our results are in accordance with previous studies, showcasing that mixtures of ethanol and water yield extracts that contain more polyphenols [28,33]. Furthermore, our findings highlight the need for alternative solvents to be used, instead of plain water in PEF extractions.

As regards the separation of the polyphenols with the HPLC and their consecutive analysis, results expressed as the total chromatographic area can be seen in Figure 2. It can be seen that the extract obtained by using the 50% ethanol in the water mixture yielded the extract with the highest content in total polyphenols. Higher content of ethanol in the extraction solvent, resulted in extracts with decreased chromatographic areas, as in the case

of solvents with lower ethanol content than 50%. In all cases, the individual polyphenols examined followed the same trend, as the total chromatographic areas. The results are in accordance with the results of the Folin–Ciocalteu assay and further highlight the importance of examining the use of solvent mixtures, instead of plain water, in PEF extractions.

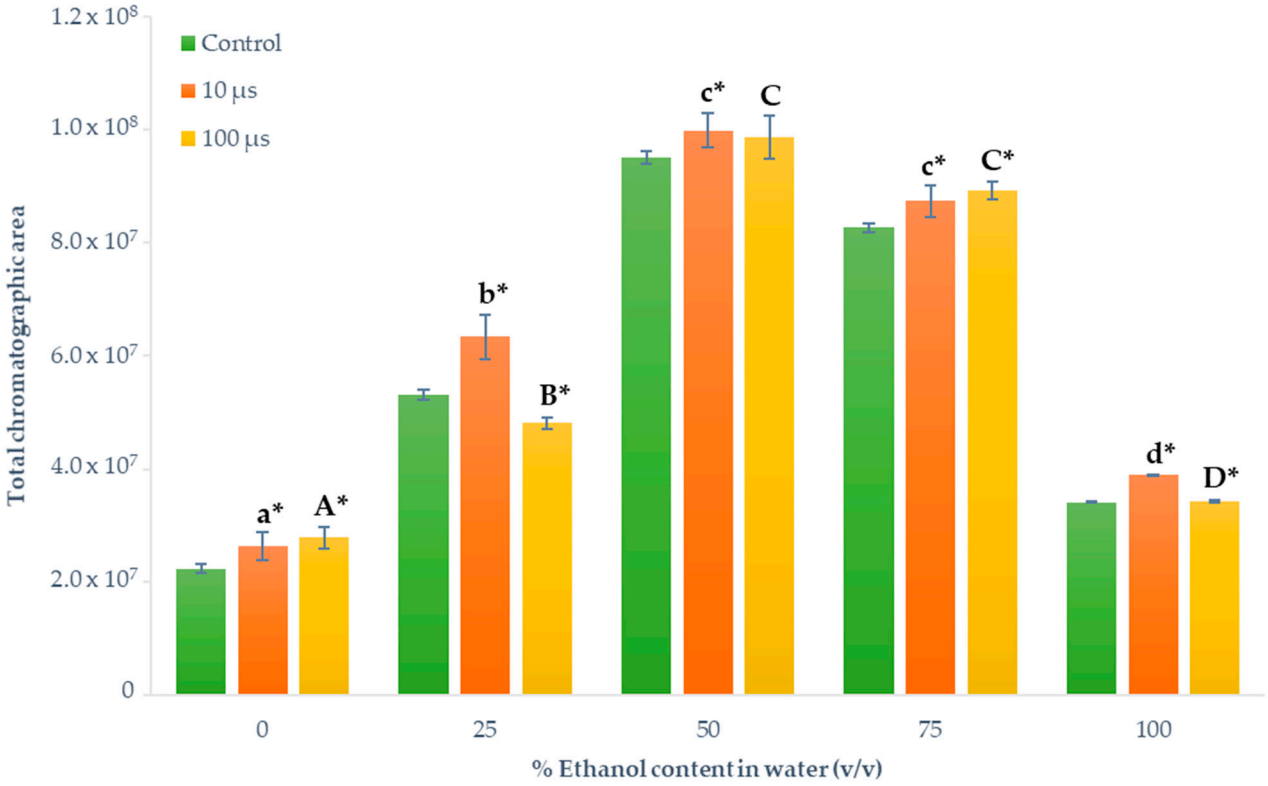

Figure 2. Total chromatographic area of the *Sideritis raiseri* extracts (20 mL solvent per g of plant), obtained with mixtures of water:ethanol of different compositions, using PEF extraction. Statistically significant differences for $p < 0.05$ are denoted with lower letters (e.g., a–d) for 10 µs pulse duration and with capital letters (e.g., A–D) for 100 µs pulse duration samples; asterisks (*) denote statistically significant differences for $p < 0.05$ between samples and the respective control.

4. Conclusions

Maximizing the yield of bioactive compounds from plant materials is of utmost importance for many reasons. Although the use of PEF extraction is highly promising, compared to other techniques there are still many parameters that can be optimized, so as to obtain extracts with better properties. As regards the extraction of total polyphenols from *Sideritis raiseri*, our results were conclusive that a 1:1 mixture of water and ethanol can increase the content of polyphenols in the extract by 146%, compared to the use of plain water. Although the use of organic solvent may be discouraging in terms of environmental friendliness, this study highlights the fact that PEF extraction can be further enhanced by optimizing the extraction solvent and obtaining extracts, with higher antioxidant properties.

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