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Combination of Pulsed Electric Field and Ultrasound in the Extraction of Polyphenols and Volatile Compounds from Grape Stems

George Ntourtoglou [1,2], Fotini Drosou [1], Theodoros Chatzimitakos [2], Vassilis Athanasiadis [2], Eleni Bozinou [2], Vassilis G. Dourtoglou [1], Abeer Elhakem [3], Rokayya Sami [4], Amal Adnan Ashour [5], Alaa Shafie [6] and Stavros I. Lalas [2,*]

[1] Department of Wine, Vine, and Beverage Sciences, School of Food Science, University of West Attica, 12243 Athens, Greece; gntourtoglou@uniwa.gr (G.N.); fdrosou@uniwa.gr (F.D.); vdourt@uniwa.gr (V.G.D.)
[2] Department of Food Science and Nutrition, University of Thessaly, 43100 Karditsa, Greece; tchatzimitakos@uth.gr (T.C.); vaathanasiadis@uth.gr (V.A.); empozinou@uth.gr (E.B.)
[3] Department of Biology, College of Science and Humanities in Al-Kharj, Prince Sattam Bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia; a.elhakem@psau.edu.sa
[4] Department of Food Science and Nutrition, College of Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; rokayya.d@tu.edu.sa
[5] Department of Oral & Maxillofacial Surgery and Diagnostic Sciences, Faculty of Dentistry, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; a.a.ashour@tu.edu.sa
[6] Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; a.shafie@tu.edu.sa
* Correspondence: slalas@uth.gr; Tel.: +30-24410-64783; Fax: +30-24410-64781

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Abstract: Increasing the yield of extraction of bioactive compounds from plants is of high importance. Grape stems are widely discarded during the wine-making process, despite their high content in many valuable compounds. The aim of this work was to examine whether the use of pulsed electric field (PEF) treatment of the stems could increase the yield of polyphenol and volatile compounds in the extracts. For this reason, a relatively low-energy consuming PEF process was employed (low-electric field strength, 1 kV/cm) for a short time (30 min) at the grape stems. In addition, the effect of different solvents during this pretreatment step was examined. With the use of Folin–Ciocalteu assay, the extracts were compared with the respective control samples (not pretreated with PEF). Moreover, extracts were prepared to assess whether changes occur to the volatile profile of the extracts. The results were conclusive that not only PEF can increase the yield of polyphenols (up to 35% increase recorded), but also that the solvent used during PEF pretreatment can affect the process. Furthermore, a 234% increase in the total content of volatile compounds was recorded, when PEF was used as a pretreatment step. Therefore, the combination of PEF and ultrasound-assisted extraction is highly promising to obtain grape stem extracts with a higher content of bioactive compounds.

Keywords: by-product; extraction; phenolics; pulsed electric field; ultrasonication; gas chromatography–mass spectrometry

1. Introduction

Today, more than ever, there is a growing interest in sustainability, including, but not limited to crop by-products. Consequently, adding value to biowaste is more important than ever [1]. Considering and treating biowaste as a source of bioactive compounds can result in significant benefits in terms of sustainability, as well as major benefits to the food and beverage industries [2]. Grape stems are widely known materials that are left over from grapes and wine-making. Every year, millions of tons of waste are produced from the cultivation of grapes with only a small percentage of them (<4%) remaining for the feeding of animals. Grape stems represent about 5% of the waste from the process of wine-making, and in general, the production of grapes [3]. Regarding the grape stems, researchers worldwide have successfully identified their composition [4,5]. They mainly consist of water [6], cellulose and hemicellulose, lignin, proteins, acids, sugars, as well as polyphenolic compounds, particularly those derived from red grapes. The possibility of creating value from the by-products of the abovementioned process has been studied by several researchers [7–10]. For instance, Quero et al. [11] studied the antiproliferative effect of grape stem extracts against three cancer cell lines. Similarly, Leal et al. [7] studied the antioxidant, antimicrobial, anti-inflammatory, and anti-aging properties of the grape stem extracts. Of all their components, phenolic compounds are of great importance owing to their antioxidant, antibacterial, and antifungal properties [12–15]. To this end, multiple extraction techniques have been employed to prepare grape stem extracts, including solid–liquid extraction [16], ultrasonication (US) [17], and pressurized liquid extraction [9]. However, to the best of our knowledge, the pulsed electric field has not been employed to date. The pulsed electric field (PEF) is a green extraction technique that has been used in recent years to optimize the extraction process of precious substances [18–20]. PEF is an extraction technique, based on electro-permeabilization, aiming at the creation of pores in the cellular membranes, and thus, assist in the extraction of compounds. Owing to its inherent advantages, PEF has been successfully employed for the improved extraction of polyphenols from citrus fruits [21], potato peels [22], *Sideritis raiseri* [23], Merlot grapes [24], etc. Moreover, it has been shown that the use of PEF in combination with ultrasound can increase the extraction of phenolics from plant by-products [25]. The PEF is typically applied prior to the ultrasound-assisted extraction as a pretreatment step. However, this step does not exclude the release of ingredients from the raw materials during PEF treatment. In particular, short-duration pulses between 100 ns–1 ms are used with a voltage between 1 and 3 kV.

The present study aimed at investigating whether the use of PEF prior to the ultrasound-based extraction of polyphenols from grape stems can enhance their extraction, and to what extent. In addition, volatile compounds contained in the extracts were examined, since they are valuable molecules widely used in the food industry. To this end, grape stem extracts were prepared, using the ultrasound treatment. Moreover, PEF pretreated samples (with various solvents) were subjected to the ultrasound treatment and compared.

2. Materials and Methods

2.1. Chemicals

Folin–Ciocalteu reagent and all solvents used were at least of analytical grade and purchased from Chem Lab (Zedelgem, Belgium).

2.2. Plant Material and Sample Processing

Grape stems from the Merlot variety were obtained from Kavala, Greece region. The stems were collected in air-tight bags and placed in a freezer during transportation. Prior to further processing, stems were washed with deionized water and dried using paper towels. Then, 30 g of stems were chopped into smaller pieces (between 45 and 50 mm). The stems were equally shared within 6 beakers (5.0 g in each beaker). Half of the beakers were used to prepare the control samples and the others for the PEF treated samples. Extraction was carried out in two steps. In the first step, grape stems were immersed in a solvent and subjected to PEF treatment. In the second step, the PEF treated and non-treated samples were subjected to ultrasonic extraction. More specifically, three sets of experiments were carried out: (I) In one beaker, the stems were left intact, without the addition of any medium. Simultaneously, the second portion of stems was inserted into the PEF treatment chamber. After 30 min, the stems were transferred into two Duran bottles (250 mL) and 50 mL of extraction solvent (methanol:deionized water, 70:30 v/v) was added to fully cover the stems. (II) For the second experiment, another 5 g of stems were inserted in a 250 mL Duran bottle with 50 mL of deionized water. At the same time, 50 mL of deionized water with 5 g of stems were added to the PEF treatment chamber. After 30 min (with or without the PEF treatment), the solvent was retracted and subjected to further analysis. The stems in both cases were transferred to new containers and the extraction solvent (methanol:deionized water, 70:30 *v*/*v*) was added. (III) For the third experiment, 5 g of stems were inserted in a 250 mL Duran bottle and followed by the addition of a methanol:water mixture (50:50 *v*/*v*). The same mixture of solvents along with 5 g of stems was added to the PEF treatment chamber. Following the treatment, the solvent was retracted (and subjected to further analysis) and the stems were transferred to new beakers, where the extraction solvent (methanol:deionized water, 70:30 *v*/*v*) was added. In all cases, after the addition of the final extraction solvent, the mixtures (solvent and stems) were placed in an ultrasonic bath for 15 min. Each experiment was repeated three times.

2.3. Instrumentation

The equipment used for the PEF treatment is provided in detail in our previous studies [19,20,23]. In brief, the system was comprised of a high voltage power generator (Leybold, LD Didactic GmbH, Huerth, Germany), a digital oscilloscope, a function/arbitrary waveform generator, and a treatment chamber made of two stainless steel plates (10 cm length and 10 cm height) and 1 cm Teflon between them for isolation purposes. The pulse duration used for the treatment of the samples was 1 ms and the frequency of the pulses was 1 Hz. The wave type is a typical square wave with a maximum delay of 20 ns. Absorbance measurements were carried out at a Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany). Ultrasound treatment of the samples was carried out at a TRANSONIC 570/H (ELMA) unit, with a volume of 4.25 L, frequency of 35 KHz, and HF peak of 320 W.

GC–MS analyses were carried out using an Agilent 6890 series GC System (Agilent Technologies, Santa Clara, CA, USA), coupled to a 5975C MSD mass detector. A fused silica capillary column (30 m × 0.32 mm i.d. × 0.25 µm film thickness (HP-5MS, Agilent Technologies) was used for the separation of compounds, and helium was used as carrier gas at a flow rate of 1 mL/min. The sample (1 µL) was injected using a split ratio of 100:1. The injector temperature was set at 250 °C and the temperature of the transfer line was set at 280 °C. The detector was operated at the ionization mode (EI), using a voltage of 70 eV, and spectra were recorded in the mass range of 40–550 amu. Oven temperature was set at 70 °C for 0.5 min, increased to 100 °C with a rate of 8 °C per min and remained for 0.5 min, and finally, increased to 250 °C with a rate of 5 °C per min and remained for 15 min. The total run time was 49.75 min. Data were recorded with the Turbomass 5.0 ChemStation software. Identification of the compounds was carried out using the NIST 11 library.

2.4. Measurement of Total Phenolic Content

The measurement of total phenolic content (TPC) is based on the oxidation of phenols in an alkaline environment (Folin–Ciocalteu method) [26,27]. In a vial containing 7.9 mL of deionized water and 500 µL of Folin–Ciocalteu reagent, 100 µL of the samples (as mentioned in Section 2.2) were added. After mixing at a vortex for 1 min, 1.5 mL of saturated Na_2CO_3 solution was added. A blank sample was prepared by substituting the sample with deionized water. After mixing again in a vortex, samples were incubated at 40 °C for 30 min. Finally, absorbance spectra were recorded in the range of 500–800 nm and comparisons between samples were carried out by measuring the absorbance at 765 nm. Results were expressed as absorbance units (AU) and not as equivalents of other compounds, with calibration curves.

2.5. Measurement of Volatile Compounds

Extracts of the control samples and the PEF treated samples (using methanol as solvent), after ultrasound extraction, were dried using sodium sulfate. After filtering, using syringe filters, 10 µL of internal standard solution (3-octanol in pentane) was added, and 1 µL of the sample was inserted into the GC–MS system.

2.6. Statistical Analysis

All of the experiments were performed in triplicates. Results are expressed as means of all measurements (three replicate extractions x three analyses = nine total measurements). Statistically significant differences were evaluated using ANOVA, followed by Duncan's multiple range test for $p < 0.05$. Statistical analysis was carried out using SPSS (SPSS Inc., Chicago, IL, USA) software.

3. Results and Discussion

3.1. Phenolic Content of the Extracts

To date, a significant amount of effort has been placed to examine the phenolic content of extracts obtained solely by ultrasound treatment of plant mixtures and lesser effort to study the effect of PEF treatment. In addition, studies focusing on the combination of the two aforementioned techniques are insufficient and in short supply [25,28]. The results obtained from PEF pretreated and non-pretreated samples subjected to ultrasound extraction are summarized in Table 1. In accordance with the results, the use of PEF without the aid of ultrasonication does not lead to notable changes in the polyphenolic content of the extracts (the recorded increase in the TPC was <4% and was not found to be statistically significant). This minuscule increase in the TPC can possibly be attributed to the fact that even though the cell membranes are partially damaged, the phenolic compounds cannot, yet, be released [25]. On the contrary, when PEF was used as a pretreatment step, prior to the ultrasonication extraction step, a notable increase in the polyphenolics contained in the extract was recorded. More specifically, when 1:1 v/v methanol:water was used as a solvent, during the PEF pretreatment step, a 17% increase in the polyphenolic content of the extract (after US extraction) was recorded, compared with the non-pretreated sample. Similarly, when water was used as a solvent during the PEF pretreatment step, the respective extract contained 35% more polyphenols, compared with the non-pretreated sample with PEF extract.

Table 1. Absorption of samples, resulting from various treatments, using the Folin–Ciocalteu method. Statistically significant differences ($p < 0.05$) are denoted with superscript letters; AU: Absorbance units.

Solvent Used before Treatment	PEF Treatment	US Extraction	Extract from the Control Beaker (AU)	Extract from the PEF Beaker (AU)	% Increase in TPC
only stems	×	✓	0.081 ± 0.001^{a}	0.084 ± 0.002^{a}	4
50% v/v methanol:water	✓	×	0.053 ± 0.001^{a}	0.055 ± 0.001^{a}	4
	✓	✓	0.086 ± 0.006^{a}	0.101 ± 0.005^{b}	17
water	✓	×	0.051 ± 0.001^{a}	0.053 ± 0.001^{a}	4
	✓	✓	0.085 ± 0.005^{a}	0.115 ± 0.005^{b}	35

In a previous study, a similar approach was employed to examine whether PEF pretreatment can increase the TPC content of rosemary and thyme extracts, obtained by ultrasound-assisted extraction [25]. In accordance with the authors, the use of PEF as a pretreatment step was found to be beneficial in both cases. However, the PEF pretreatment was found to increase the TPC of thyme extracts, compared with rosemary extracts. In another study, the PEF pretreatment increased by nearly 15% of the TPC content of almond extract (obtained by US-assisted extraction), compared with PEF non-pretreated extract [28]. Therefore, in accordance with our results, it can be concluded that not only PEF can be used as a pre-treatment step to maximize the extraction yield of polyphenols, but the solvent used during the PEF treatment step, is also of high importance to obtain the optimum extract.

3.2. Effect on Volatile Compounds

It is known that there are cases of wine-making in which stems are used to bestow the wine with green and vegetal aroma [6]. Therefore, grape stems can be used as a source to extract aroma compounds for further use in the food industry. In this context, we examined whether treating stems with PEF, prior to the US-assisted extraction of the volatile compounds, can also increase the yield of aroma compounds. Representative chromatograms of the extracts are provided in Figure 1 and the results in Table 2. In the samples extracted without PEF pretreatment, a total of six volatile compounds were identified, whose total content was 0.73 mg/Kg. Regarding the extracts obtained using PEF treatment, prior to the US-assisted extraction, a total of eight volatile compounds were identified, whose total content was 2.44 mg/Kg. The new compounds identified were 1,14-tetradecanediol and 1-methoxy-4-methyl-benzene. Therefore, PEF pretreatment increased the total volatile content of the extract by 234%. There are even more pronounced increases for some individual compounds, such as 4-dodecanol, whose content was increased by 2100%, and hexanedioic acid, bis(2-ethylhexyl) ester, whose content increased by 716%. In all cases (except for 14- and 16-heptadecenal), the content of each compound was found to be statistically significantly higher in PEF pretreated samples. Therefore, our results highlight the superiority of PEF for use as a pretreatment step.

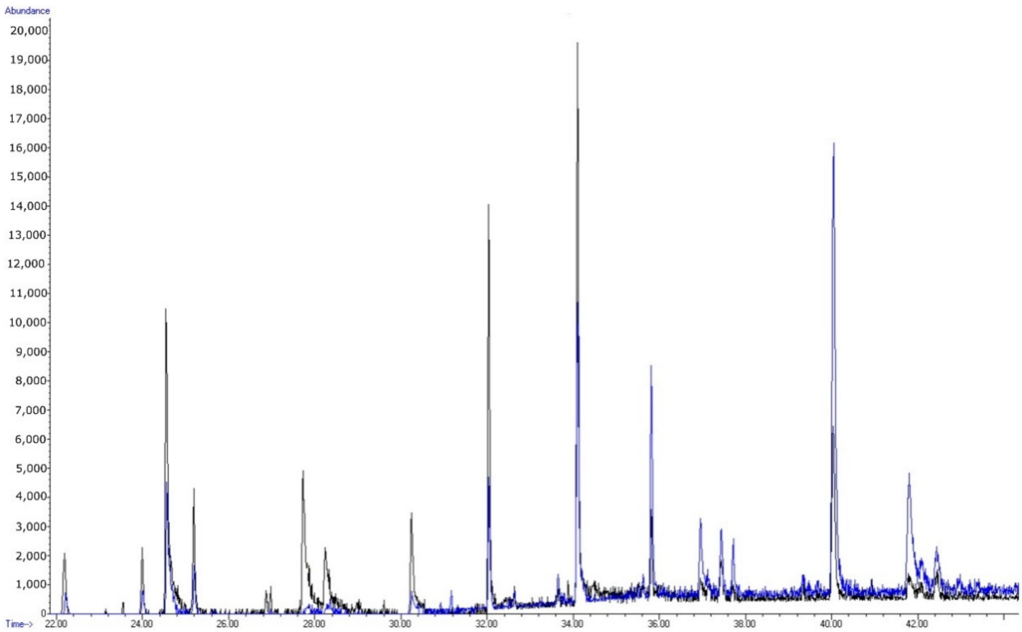

Figure 1. Representative chromatograms of grape stem extracts obtained by ultrasound-assisted extraction (blue chromatogram) and obtained by ultrasound-assisted extraction, accompanied by a PEF pretreatment step (black chromatogram).

Table 2. Volatile compounds detected by gas chromatography–mass spectrometry in PEF treated and non-treated samples. Statistically significant differences ($p < 0.05$) are denoted with superscript letters.

Compounds	Control (mg/Kg)	PEF (mg/Kg)	% Increase
Phenylethyl Alcohol	0.04 ± 0.01^{a}	0.10 ± 0.02^{b}	150
Benzene, 1-methoxy-4-methyl-	ND	0.07 ± 0.02	-
n-Hexadecanoic acid	0.10 ± 0.03^{a}	0.59 ± 0.09^{b}	490
1,14-Tetradecanediol	ND	0.39 ± 0.14	-
4-Dodecanol	0.01 ± 0.01^{a}	0.22 ± 0.01^{b}	2100
Hexanedioic acid, bis(2-ethylhexyl) ester	0.06 ± 0.01^{a}	0.49 ± 0.05^{b}	716
14-Heptadecenal	0.13 ± 0.02^{a}	0.15 ± 0.02^{a}	15
16-Heptadecenal	0.39 ± 0.09^{a}	0.43 ± 0.05^{a}	10
Total volatile content	*0.73*	*2.44*	*234*

The composition of volatile components of the grape stems is an understudied area, resulting in a lack of data regarding this topic. Hashizume et al. [29] examined the volatile components of grape stems of the Cabernet Sauvignon and Chardonnay varieties and identified seven compounds. Similarly, Ruiz-Moreno et al. [30] identified seven compounds in the Syrah variety grape stems. Since grape stems can contribute unique aromas to the wines, further research should focus on the determination of volatile profiles of grape stems to obtain wines with the desired properties.

4. Conclusions

Grape stems are a natural by-product of the wine-making industry, which contains valuable compounds. These compounds can be retrieved using various extraction techniques, with the most common as ultrasound-assisted extraction. In accordance with our results, the use of PEF as a pretreatment step can significantly enhance the extraction of polyphenolic compounds from grape stems. Moreover, it was apparent that the use of different solvents during PEF treatment of the stems can further increase the yield of polyphenols. Similarly, PEF not only was found to increase the yield of volatile compounds in the extracts, but also assisted in the extraction of two additional volatile compounds, compared with bare ultrasound-assisted extraction-derived extracts. As a concluding remark, the use of PEF as a pretreatment step in the extraction of various volatile substances as well as polyphenolic compounds, in a very short period (30 min) is a promising approach that can be utilized for the enhancement of the extraction process. Furthermore, by studying other parameters of the extraction, such as extraction solvents, time, and temperature, further enhancement of the extraction yield can be achieved.

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