Mass transfer intensification in bioactive compounds recovery by alternative extraction methods: effects of solvent

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Abstract

Recently, the exploration of new alternatives to common organic solvents in combination with non-conventional energy sources is a subject of intense research and development as tools for green extraction of plant-derived natural products. This paper deals with the use of low frequency ultrasound (100 W, 32 kHz) and pulsed electrical fields (0.86 kV cm^{-1}, 13 Hz) to raise the efficiency of the extraction of selected plant species rich in valuable phenolic compounds. Four types of natural deep eutectic solvents were applied for the ultrasound-assisted extraction of silymarin from milk thistle among which a choline-chloride-based mixture with glycerol was proved to be the most promising one. Additionally, the usage of high ultrasound power significantly increased the concentration of sylimarin as compared to traditional ethanol extraction, generating rapidly for 10 min considerable temperature rise as a side effect under non-cooling conditions. The application of PEF as a pre-treatment for 0.8 s to the extraction allowed reducing the ethanol percentage in the ethanol/water mixtures giving comparable phenolic yields, without any, or very little, increase in temperature. As such, optimized power intensity in conjunction with appropriate solvent system provides a potential to improve the release of thermally unstable bioactive compounds under mild processing conditions.

Keywords: Deep eutectic solvents, pulsed electric field, energy consumption, polyphenols, milk thistle, grape seeds.

1. Introduction

Processing natural product extracts is become increasingly important in many research and development areas owing to their functional and health-beneficial properties. Considering the highly diversified nature of active ingredients and their properties, appropriate separation methods are required to extract specific compounds or compound groups, and then to remove the solvent and separate of high added value compounds, rendering them useful either as pure compounds or as standardized extracts. In the field of natural compounds extraction, attention has been recently devoted not only to the compounds that are present but also to their localization inside the microstructural arrangement of cells, extracellular spaces, capillaries and pores. These structural characteristics are recognized as a factor in optimization processes, as they control the diffusion of solvent and solutes as well as influencing the concentration fields within the solid matrix.

From all the valuable biomass extractives, phenolic phytochemicals represent a large and diverse group of secondary metabolites, widely produced by plants. A number of studies have indicated that they are differentially distributed in the plant tissues according to the solubility which is caused by their polarity (Medina-Torres *et al.,* 2017). Characteristically, hydrophilic substances are stored in cell vacuoles, while others such as the majority of lignin polymers, complex flavonoids, and water insoluble polyphenols are deposited in the cell walls through different interactions, as physical and chemical adsorption, strong covalent bonding, or entrapment. The liberation of the intracellular content and cell wall-bound compounds could be a rate limiting step, causing slow process kinetics, low recovery efficiency or high operation costs. This is the reason why various ways of extracting the active compounds are available and several advanced processes using non-conventional energy sources, such as microwave, ultrasound, as well as electric fields have already been applied in process intensification, mainly on a laboratory scale (Azwanida, 2015; Misra *et al.*, 2017). In addition to the increased yield and rate of mass transfer, the proposed benefits include also the possibility of use of alternative solvents, that are both more eco-friendly and more efficient (Selvamuthukumaran *et al.*, 2017).

In this regard, recent advances may be represented by the deep eutectic solvents (DES) that are a new generation of solvents following ionic liquids in the area of sustainable solvent systems (Duan *et al.*, 2016). The DESs result from the mixture of two or more components with the ability to donate and accept protons and electrons to form hydrogen bonds, thus increasing their dissolution capacity. Compared to water and common organic solvents, they possess unique characteristics, such as

negligible volatility, high ionic conductivity, chemical stability, and thermal behavior very distinct from the one of their components. Natural DES - NADES - have also been formed using easily available natural raw materials, such as sugars, carboxylic acids and amino-acids, urea, choline chloride, glycerol, typically coming from renewal sources (Paiva *et al.,* 2014). Because the chemical inertness, low toxicity, good biocompatibility and low cost, applications of NADESs are growing fast with the aim to replace the conventional solvents in research, industry and new process developments (Bakirtzi *et al.,* 2016; Bubalo *et al.,* 2016).

In this study, two different approaches to facilitate the mass transfer during extraction have been developed and applied. In the first case study, the advantages of NADES as tunable solvent systems and the assistance of ultrasound as effective energy source (NADES-UAE) were used to extract silymarin from milk thistle seeds. The second approach considers the combination of pulsed electric fields (PEF) and solid-liquid extraction of polyphenols from grape seeds by using different hydro-alcoholic solutions of water and water as treatment media for electrical processing and as a extraction solvent. Among the two methods, the UAE is still more widely used, especially because of the simplified manipulation and scale-up procedure. Currently, most such applications deal with ultrasonic waves of low frequencies (20-40 kHz) with relatively high intensities (10-1000 W cm^{-2}) in processing of various natural products (Medina-Torres *et al.,* 2017), including milk thistle (Gağdaş *et al.,* 2011). The mechanisms of action and acceleration that take place is the propagation of pressure waves resulting in the phenomenon of acoustic cavitation (Misra *et al.,* 2017). Multiple impacts as mixing effect, thermal effect and possible rupture of cell wall due to formation of microcavities are supposed to occur as cavitation bubbles collapse in the liquid near the solid surface, and reducing mass-transfer resistances. In the area of non-thermal processing technologies, the use of PEFs is regarded as one of the most promising, mainly because its potential to change the structural characteristics of plant-based product at low temperatures and very short treatment duration. The application of an external electric field of high intensity was supposed to affect mainly the cell membranes, inducing local changes in their structures and promoting the formation of pores, named electroporation (Puértolas *et al.,* 2012). As a result, cell membranes lose their semi-permeability, either temporarily or permanently that facilitates the release of intracellular components at a more rapid rate. Most reported applications concern the aqueous extraction from soft plant materials with high moisture content (>70%). Short duration pulses (micro to milliseconds) of moderate or high electric field strengths (0.5-10 kV cm^{-1}) and total specific energy below 20 kJ kg^{-1} generally achieved effective electroporation (Puértolas *et al.,* 2015). Compared to other organic matter, dry seeds and seed meals more difficult to process with regard to hard and more compact solid matrix and very rigid cell walls which makes cell disruption more energy demanding.

In this work the possibility to apply the UAE and PEF to improve the extraction rate of the compounds targeted by the process was investigated. Particularly, the influence of solvent composition affecting the extent of process efficiency was evaluated searching the proper non-toxic and safe solvents. The experimental methodology followed was to test alternative extraction protocols and to make comparison with commonly used extraction technique and solvents, such as ethanol and ethanol/water mixtures that are recognized as adequate for extraction of bioactive compounds, especially phenolic ones.

2. Materials and methods

2.1. Materials

For extracts preparation, two popular plant materials, namely the medicinal herb milk thistle (*Silybum marianum L.*), and the seeds of red grape (*Vitis Vnifera L.*) were chosen due to their high-value phytochemicals and/or importance of application. Milk thistle, and in particular the silymarin complex (1-3%) possesses several therapeutic properties including antihepatitis activity while grape seeds contain a diverse assortment of protective phenolic compounds in relevant quantities (about 6-8%) which are responsible for its high antioxidant activity. Plant materials were obtained from local growers or producers and stored at room temperature under dark conditions until they were processed. Meal of milk thistle seeds was used after fatty oil has been squeezed whole grape seeds of mean size 3.6 mm was used without prior defatting and mechanical pretreatment. Pure standards of Gallic acid 99% (CAS 149-91-7), DPPH radical (CAS 1898-66-4) as well as other reagents and solvents used such as choline chloride, lactic acid, glycerol, and ethanol were of analytical grade.

2.2. Deep Eutectic Solvents Preparation

Previously reported preparation process (Duan *et al.,* 2016), was applied to obtain four kinds of binary and ternary deep eutectic solvents to test their extraction potency toward sylimarin. NADESs were prepared by combining the type of hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) components in the proper molar ratios (Table 1).

Table 1. Compositions of the synthesized NADESs

	HBD	HBA1	HBA2	HBA/HBD molar ratio
DES1-LA	Choline chloride	Lactic acid		1:2
DES1-Gl		Glycerol		1:2
DES1-Gl-U		Glycerol	Urea	1:2:2
DES2 Gl-U	Lactic acid	Glycerol	Urea	2:1:2

The mixture was heated at 80-90°C for 25-35 min, under continuous stirring on a magnetic stirrer, at 400 rpm, until perfectly transparent liquids were formed. The liquids were stored in sealed glass containers in a dark chamber,

at room temperature and were visually inspected for eventual development of crystals over a period of 10 days.

2.3. Ultrasound assisted extraction

The experimental tests were carried out using a 100 W and 32 kHz ultrasonic processor (model UW 100 Bandelin Sonopuls, Germany), which was submersed in the liquid medium. The sample was mixed with the different solvents at sample-solvent ratio of 1:50. The mixtures were processed at 10% of the nominal power for 10 min. The power densities of sonication (W_{US}) can be estimated as follows:

$$W_{US} = \frac{P_g \cdot t_{US}}{m} \qquad (1)$$

where P_g is the ultrasonic processor power, t_{US} is the sonication time, and m the amount of treated sample. During sonication, the temperature of the solvent was measured to see the additional thermal effect. The recovered crude extracts were characterized by a direct measure of the UV-Vis absorption spectra in the range of 200-800 nm.

2.4. Pulsed electric field

A lab-scale pulsed electric field *(*PEF) assisted grape seeds extraction was developed and evaluated. The experimental procedure consisted in three steps: preheating to 40°C and wetting the sample matrix, pre-treatment using PEF, and a diffusive step, using water and ethanol, either as pure solvents or in combination, in various concentrations (0-95%, v/v). PEF system consists of a power source; a high voltage pulse generator to transform the DC power into short pulses; fast switches; and a static treatment chamber with parallel-plate stainless still electrodes. Before the PEF treatment itself, the seeds were placed between the two electrodes, mixed with the treatment solution, and subjected to a sequential square-wave electric pulses. Figure 1 shows a schematic diagram of the PEF system with a typical treatment pulse recorded by the oscilloscope.
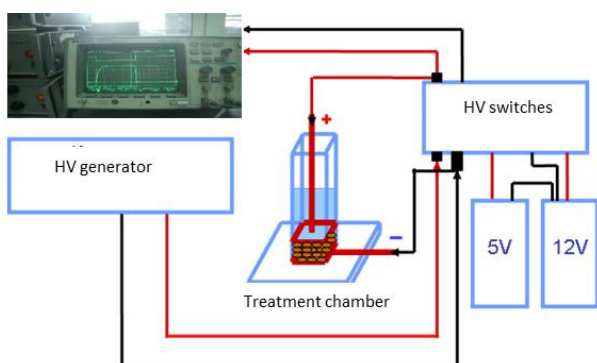

Figure 1. Schematic diagram of the PEF system

The operating parameters for PEF, namely the nominal electric field strength calculated as the potential difference between the electrodes, divided by the electrode distance, pulses characteristics and total t_{PEF} used are presented in Table 2. The electrical conductivity of the solvents and grape seeds-solvent suspensions

subjected to PEF treatment was also measured in a specially designed cell (Iatcheva *et al.,* 2013) to characterize the amount of energy used.

Table 2. Experimental conditions used to study the effect of different solvents on PEF performance

Electrical field parameters				
Field strength	Number of pulses	Pulse duration	Pulse interval	Treatment time, t_{PEF}
0.86 kV cm^{-1}	900	900 μs	75 ms	810 ms
Tap water	**Distilled water**	**40 % ethanol**	**75% ethanol**	**95% ethanol**
Electrical conductivity of solvents (S m^{-1} x 10^{-4})				
158.7±14.4	27.6±0.7	5.8±0.8	2.3±0.2	1.1±0.4
Initial electrical conductivity of the suspension (S m^{-1} x 10^{-4})				
115.4±11.2	76.4±9.5	75.4±6.1	68.9±8.4	68.8±6.4
Specific energy (W_{PEF}), kJ kg^{-1}				
59.2	48.2	30.8	20.6	13.1

The specific energy (W_{PEF}) deliverable by the PEF system was calculated as:

$$W_{PEF} = \frac{\sigma_m E_0^2}{m} t_{PEF} \qquad (2)$$

where E_0 is the strength of the applied external field and σ_m the initial electrical conductivity of the suspension, measured after soaking grape seeds in selected solvents. The temperature of the treatment medium was also measured with a thermocouple before and after the PEF application. By fixing extraction time (up to 120 min) and extraction temperature at 40°C, batch solid-liquid extraction with stirring was performed by adjusting the solid to liquid ratio of 1:5. Determination of total phenolic content (TPC) in the extracts was performed following the standard Folin-Ciocalteu method (Singleton, 1999) by measuring absorbance at 765 nm, and the results were expressed as mg gallic acid equivalent (GAE) per g dry weight of solid (mg GAE g^{-1}).

3. Results and discussion

3.1. Ultrasonic-assisted extraction

To find a suitable solvent to produce milk thistle extracts, binary and ternary eutectic mixtures with different HBD and HBA were examined (Table 1). Actually, common solvents having moderate polarity like ethanol, methanol, and acetone, at different volume fractions in water, have been used for the extraction of milk thistle active compounds, ranging from highly polar taxifolin to less polar silymarin complex that is unique isomeric mixture of flavonolignans - the two diastereomers, silybin A and B, being the major and more active polyphenolic flavonoids (Saleh, 2016). On the other hand, the times of extraction reported are on average quite long; given that the extraction is restricted also by diffusive effect; in that ultrasounds treatment was introduced to enhance the extraction.

After conducting the experiments under ultrasound treatment for only 10 min using NADESs, as well ethanol as solvents and analyzing the resulting solutions, the

following absorption spectra have been obtained as shown in Figure 2. They are compared to a reference extract representing semi-purified commercial extract from defatted seed meal of milk thistle (standard solution) to evidence their similarities and dissimilarities. In the case of standard solution, the UV scan showed the absorption maxima at wavelength of 288 nm, which is the characteristic absorbance of the predominant silymarin fraction, the commercial preparation being standardized at 70-80% silymarin. The same characteristic wavelength for silimarin was also found in other works (Chaudhari *et al.,* 2016).
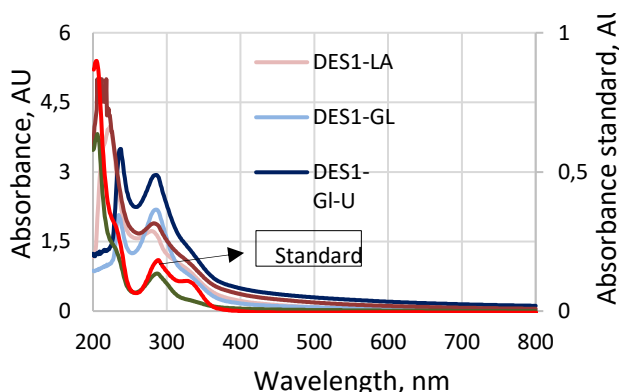

Figure 2. UV-VIS spectra of milk thistle extracts obtained by different solvent systems with respect to the commercial extract used as standard

The absorbance values expressed in term of absorbance units (AU) at 288 nm give information about the concentration of silymarin in different extracts, and extraction enhancement was seen through the rise in absorbance relative to the standard solution. For the ethanol extract, the absorbance intensity is only 0.8, for DES1-GL, extracts the absorbance is very similar and ranges from 1.8 to 2.1, and for DES1-Gl-U it reaches the value of 3.3. Thus, by changing the NADES composition, its extractive power partially changed and decreased in the order: DES1-Gl > DES1-LA > DES2-Gl-U > DES1- DES1-Gl-U. The higher absorbance values in all the sonicated extracts obtained with NADESs are indicative for the higher solubilization and extraction ability when compared to ethanol.

Even if the shape of the spectra is very similar in the whole scanned UV-Vis region, in the case of crude extracts of NADESs there is, however, the appearance of additional peaks in the wavelength region shorter than 288 nm, where no absorption peak is found for ethanol extract and standard solution. The peak absorbance centered around 237 nm for DES1-GL and DES1-GL extracts, tend to be shifted toward the shorter wavelengths and the absorption peaks tend to be large for other two NADES systems. The most significant changes was found the DES2-Gl-U, where the sample has too much absorbance and saturates the detector. The absorption peaks in this region would indicate the presence of other compounds with small conjugated system, such as simpler phenolic acids, flavonoids and their derivatives. This result in both

different total yields and variation in the purity of the extracts. The presence of various compounds in NADES extracts is probably because of their more polar nature due to strong hydrogen bonding. Their polarity ranges between that of water and alcohols making them suitable to extract a wide variety of polar to medium polar phenolic compounds. Furthermore, most of NADESs are denser than water and ethanol, and are highly viscous at room temperature which is not good for the penetration of the solvent into the plant tissue. According to Abbott *et al.* (2011) the viscosity of the NADES systems is expected to decrease by the addition of ChCl to glycerol by interrupting the intermolecular hydrogen bonding of the glycerol molecules. This is presumably one of the reason why mixtures composed of choline chloride and glycerol more efficient and more selective against the targeted sylimarin fraction than those containing lactic acid as the HBD.

The superimposed UV-Vis spectra of the extraction without and with ultrasound indicated that the use of these alternative solvents and ultrasound together showed the positive interaction between both, as illustrated in the case of ChCl-glycerol-urea solution (DES1-Gl-U) in the Figure 3. The two peaks appear at roughly the same position and their absorbances are increased in an appreciable manner employing sonication while the ratio of the absorbance values (A_{288}/A_{237}) remain almost the same. This suggests that UAE can help to extract higher content of the same classes of compounds maintaining the selectivity toward flavanolignans.
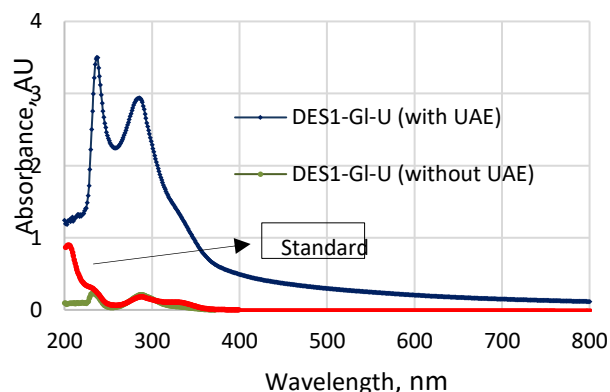

Figure 3. Influence of sonication on the milk thistle extracts in the case of ChCl-glycerol-urea solution (DES1-Gl-U)

These experimental trials were performed without external cooling and the high ultrasound power employed increased the temperature to 40°C in the case of ethanol and up to 80°C in all cases of NADESs. Consequently, the significant and positive effect of UAE could be explained by the synergism between mechanic and thermal effect, resulting in increased solutes solubility, solvent penetration rate and mass transfer. Moreover, the spectrophotometric analysis of the NADESs alone confirmed their thermal stability (data not shown) at the temperatures reached through processing. According to these preliminary essays, UAE can be successfully employed with the selected NADESs, but have some

drawbacks, such as a decrease in selectivity toward the targeted compounds and the rapid raise in the processing temperature that need to be monitored and controlled to ensure the quality requirements of extracts.

3.2. Application of pulsed electric field treatment

In this case study, PEF was applyed on whole intact grape seeds as a pretreatment prior to conventional extraction with the purpose of reducing extraction times and obtaining higher extractability of different phenolic compounds ranging from simple phenolic acids to complex flavonoids. The recovery of total phenolic content (TPC) in extracts at equal periods of extraction time was used as a criteria for quantifying the efficiency of the PEF treatment intensity (E_0, W_{PEF}) in the presence of different binary mixtures of ethanol and water and to identify a more suitable treatment medium raising the efficiency of the entire process.
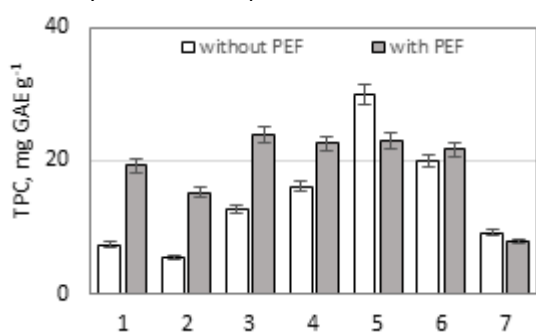

Figure 4. Total phenolic content (TPC) of extracts obtained without and with PEF-treatment of grape seeds in different solvents (1. tap water; 2. distilled water; 3. 20% ethanol; 4. 40% ethanol; 5. 75% ethanol; 6. 80% ethanol; 7. 95% ethanol)

Figure 4 shows the TPC values obtained after 120 min by direct extraction (without PEF) and after PEF pretreatment (with PEF) of grape seeds at a fixed field strength E_0 of 1 kV cm^{-1} for 810 ms and energy input (W_{PEF}) varying from between 13 to 59 kJ kg^{-1}. Since the W_{PEF} is a direct function of the suspension conductivity σ_m, in which grape seeds were immersed, the increase of PEF input energy was achieved by decreasing the concentration of ethanol (or increasing volume fraction of water), according to the Table 2.

In the extraction trials performed with untreated seeds (control extraction), water and ethanol extracted low quantities individually, whereas their mixtures made it possible to achieve notably higher recoveries, particularly when used at 75% ethanol (27.8±1.8 mg g-1). This variation in extractability could be partially explained by the distinction in the relative polarity of heteregeneous fraction of phenols in grape seeds and solvents involved (Galanakis, 2013)**.** Structurally, phenols include one or more hydroxyl groups (polar part) attached directly to an aromatic ring (non-polar part). Protic polar solvents as ethanol that have a lower polarity index (PI = 5.2) favor the solubility and diffusion of the phenolic compounds by reducing the dielectric constant and viscosity of the solvent. On the other hand, water (PI = 9) has been referred to as the universal solvent dissolving better

polymerized procyanidins and flavonoid glycosides. For dry grape seeds (< 8 % initial moisture content), water acts also as a swelling agent of the plant matrix, thereby causing an increase in the pores of the cellular walls, while ethanol could act by dissolving hydrophobic components in the wall or structural rearrangement in the phospholipid layer of the cell membrane (Gurtovenko *et al.,* 2009). For these reasons, concentration of ethanol higher than 50% have commonly been used for the extraction of polyphenols from different plants.

Differently to the direct extraction, when PEF was used as a sample pretreatment step, the content of phenolics recovered after 120 min in different aqueous solutions of ethanol (except ethanol alone) was about the same, in the range of 15.23±0.8 to 24.8±3.2 mg g^{-1}. The relatively low field strength (E_0 = 1 kV/cm) delivered between the two electrodes was not sufficient strong to induce further improvements of TPC in respect to the content quantified by the direct extraction but gave better extraction of more polar phenolic classes by using less concentrated mixtures of ethanol with water. Specifically, the release of poorly water-soluble compounds after applying PEF has more than doubled and became greater than those extracted by ethanol. This implicates that higher amount of water is needed during PEF treatment, favoring the wetting of matrix molecules by solvent and possible destabilization of the cellular membrane. It is presumable that the conductivity of the media surrounding the biological cell influences both cell disruption efficiency and energy consumption (Ivorra *et al.,* 2010). According to Table 2 and Figure 4 data, the improvement of the extraction started at about 30 kJ kg^{-1} (40% ethanol), and the highest PEF-effect was achievable when 60 kJ kg^{-1} was delivered during tap water extraction. Ethanol itself is highly resistive (non-electrolyte), it does have a significantly lower conductivity (1.1 x 10^{-4} S m^{-1}) than distilled water (27.6 x 10^{-4} S m^{-1}) and tap water (158.7 x 10^{-4} S m^{-1}). Moreover, there was a definite non-linear decreasing of conductivity with increasing ethanol concentration, and the electric conductivity of the solid-liquid suspensions decreased even in the presence of small percentage of ethanol. The results obtained support the argument that materials having very low electrical conductivity (< 1 x 10^{-4} S m^{-1}) are difficult to process by PEF, since it impairs the generation of sufficient strong local electric fields, and higher field may be required to achieve the same degree of electroporation. However, increasing the electric field increases energy consumption in the square-law dependency on electric field strength (eq.2), and this places a limitation on application of PEF for increasing the extraction yields in the case of more difficult separations.

Another consequence of PEF treatment was the acceleration of the extraction rate, allowing to obtain similar yields by using the shorter extraction time. The experimental test presented in Figure 5, compares the recovery at different time intervals (30, 60, 90 and 120 min) during extraction from PEF-treated seeds in presence of 20 % ethanol and direct extraction with 75%

ethanol that maximized the TPC. The polyphenols content increased significantly in the initial 30 min, giving about 2 times more rapid extraction rate compared to control. Overall, about 90 min should be enough to approach the concentration equilibrium, instead of 120 min during direct extraction.
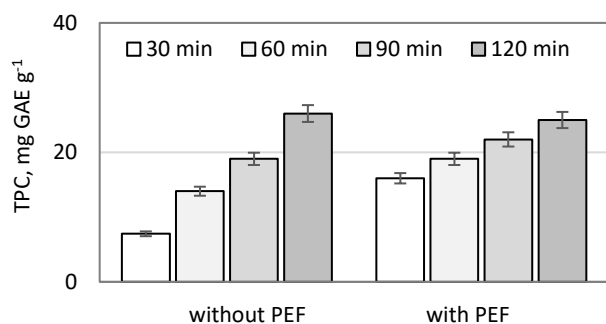

Figure 5. Content of total polyphenols in grape seed extracts depending on solvent and extraction time
(1: untreated seeds, 75% ethanol; 2: PEF-treated seeds, 20% ethanol)

In comparison to the UAE method, where a part of ultrasonic energy was used as a source of heating of the solvent-matrix solution, in the case of PEF experiments the measured temperature raise in the treatment medium after the PEF application was as low as 5-9°C, even if the energy input W_{PEF} was high because of long pulse-duration of 900 μs for up to 900 pulses. The very short treatment time (< 1 s) limits the Joule effect and does not produce any considerable macroscopic thermal response, and accordingly there is not alteration of the thermo-labile compounds. Thus, based on the presented results it can be concluded that the pretreatment by PEF at a moderate temperature (30-40 °C) can be carried out at much lower concentration of ethanol, even water and can help to improve process performance by reducing the extraction time to give similar recovery. To ensure the desired quality of extracts, the ratio of ethanol/water can be further adjusted during extraction for profiling a particular phenolic class.

4. Conclusions

The comparative results revealed a specific importance of the type of extraction solvent for the efficiency of the ultrasound or PEF treatment of vegetable materials. Besides the solubility of the molecules of interest, also several major characteristics of the treatment media, such as relatively low vapor pressure, high viscosity or suitable electrical conductivity relevant to the technological applications should be considered because of the interacting multiphysics phenomena. Results could be associated with the synergy of physical and thermal effects at the microscopic and structural levels of the vegetable matrix, and consuming only a fraction of the thermal energy normally needed for conventional extraction methods. Although, some treatment conditions have been successful to some extent, the results consist of a first step to processes characterization.

Further experiments are needed to maximize the obtained effects and to confirm the effectiveness of different approaches to meet green extraction principles with optimal consumption of raw materials, solvents and energy.

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