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Antioxidant Extraction and Bioactivity Preservation from Winery by-Products by Natural Deep Eutectic Solvents (NaDES)

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In recent times, the feasibility of sourcing the bioactivity and health-promoting activity of anthocyanins, flavanols, stilbenes and phenolic acids from viticulture and winery by-products was explored. In particular, pomace, wine lees, and pruning stalks attracted a great deal of interest among scientists, technologists, and businesses. Traditional solid-liquid extraction methods employ hazardous solvents, such as hydrocarbons, alcohols and chloro-alkanes. As an attempt to convey compliance to the pillars of Green Chemistry, the possibility to choose innovative eco-friendly solvents was evaluated. Furthermore, this approach could preserve the activity of the bioactive compounds for possible applications in food, pharmaceutical, cosmetic and phytosanitary domains.
In this work, natural deep eutectic solvents (NaDES) were used to investigate Solid-Liquid Extraction of phenolic species from Merlot marc. Two different operating temperatures were tested and the influence of biomass grinding was investigated. Experimental results show that both kinetic and final anthocyanins extraction efficiency are positively affected by biomass grinding and by temperature increase.

1. Introduction

Grapes are one of the world's most cultivated fruit crops, with a production of approximately 77.8 million metric tons in 2018 (OIV). The leading grape producer nations are China, Italy and the United States, followed by France and Spain. Of the over 70 million tons of grapes produced yearly, about 50% is destined to winemaking, while the rest comprises table grape productions. The primary by-product of wine production is called grape pomace or grape marc, which includes the stalks, skin, pulp, and seeds that are the leftovers after pressing the grapes during the winemaking process or after wine racking. The disposal of these waste materials in large amounts poses ecological and economic difficulties. Often, wineries use these by-products as fertilizers or animal feeds, and sometimes sell them to biogas plants for the production of renewable energy. However, from an economic point of view, wine production wastes have a much higher potential, given the amount of valuable chemical compounds that can be recovered from them. Therefore, the extraction of said compounds is of utmost importance to value the by-products of winemaking. Grape skins represent about 10% of grape pomace mass, but the most is separated with the seeds after wine or juice productions. This winemaking by-product has been used as compost or animal feed in the past, however the large amounts of phenolic compounds in grape skins makes them a valuable source rich in bioactive phytochemicals. A neat upgrade pathway has been investigated for more than a century with the production of "enocyanin", a deeply colored extract of red grape pomace, which has been produced commercially in Italy since 1879 (Mazza, 2018) and is referred to as E163 in the European list of the allowed food colouring agents.

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Enocyanin production, which was initially carried out by extraction in ethanol and subsequent drying, is currently obtained by a three-step process: (1) maceration of the pomace in a mild solution of sulfur dioxide; (2) separation of the water phase from the spent pomace; and (3) vacuum concentration of the water extract, with or without recovery of ethanol. Although the phenolic content and compositions differs with grapevine variety and cultivation conditions, grape skins contain the highest amount of anthocyanins compared to the other grape parts, whereas flavan-3-ols are present in similar quantities in skin and seeds. The most valuable anthocyanins found in grape skins are malvidin-3-O-glucoside and peonidin-3-O-glucoside. Phenolic compounds are found in the hypodermis, the inner layer of the grape skins. These high-value molecules have antioxidative properties and their separation could be considered a possible first step of an integrated winery by-product biorefinery. Phenolic compounds are effective free radical scavengers and studies show they have the potential to prevent cancer, cardiovascular diseases, cellular oxidative stress, diabetes, and proliferation of pathogens. Antioxidant compounds can be extracted from viticulture leftovers through conventional and nonconventional methods. Conventional methods include solid-liquid extraction, heating, grinding and enzymatic treatments. Nonconventional methods seek to increase the extraction efficiency by applying particular chemical-physical properties to improve upon conventional ones. According to the principle of green chemistry, extraction techniques for such by-products for their evaluation and utilisation should be based on green and suitable technologies that involve the usage of new environmentally friendly and tunable solvents that can meet both the technological and economic demands (Bosiljkovet al. 2017). Over the last few years, Natural Deep Eutectic Solvents (NaDES) have emerged as a key candidate alternative to conventional solvents. They represent a new generation of liquid salts, based on mixtures of relatively cheap and readily available components, such as non-toxic quaternary ammonium salts (e.g., choline chloride) with naturally derived uncharged hydrogen-bond donors, which provide a ‘green profile’ and have good prospects for wider use in the field of green technologies (Abbott et al., 2004) although the open issues concerning the actual mode the extract can be used (as such, or treated to separate the solute from the solvent (Panić et al., 2019). A number of studies have reported NaDES applications for the extraction of phenolic compounds, among which grape by-products (Radošević et al., 2016). The aim of the present study was to evaluate the possibility to carry out the extraction of bioactive compounds (anthocyanins) from spent grape skins employing acid NaDES. Characterization of solvents and raw biomass was performed. Furthermore, extraction kinetics studies were performed and the influence of temperature and biomass mechanical pre-treatment was elucidated.

2. Materials and Methods

2.1 Chemicals and materials

Choline Chloride, Acid L(+)-tartaric, ethanol and trolox were purchased from Sigma (St. Luis, MO, USA). Folin-Ciocalteu reagent (FC) were obtained from Merk (Darmstadt, Germany). Demineralized water was employed throughout all the experiments. The chemicals employed did not undergo any further purifications. Grape pomace (Merlot cultivar) was supplied by the Oenological Institute of Conegliano (Treviso, Veneto) after a macerative vinification. Pomace was pressed, spread out in a thin layer, and stabilised by sulphuric acid (1% v/v) and potassium metabisulphite and refrigerated (4 °C) until use. Enocyanin E163 (50% w/w) was provided by Ruffini distillery (Tavarnelle Val di Pesa, Italy). Its anthocyanins profile was characterised by HPLC and found consistent with typical ranges.

2.2 NaDES preparation

Two choline chloride (ChCl) based NaDES were prepared. The acid character was imparted by tartaric acid (TA) dosed at the 1:2 molar ratio ratio with choline chloride. The solvents were prepared from the powdered raw materials by mixing them in a capped beaker at 90°C until a homogeneous pale amber colour liquid was obtained. Subsequently the water content of the solvent was adjusted to the adopted value for extraction or viscosity characterization.

2.3 NaDES characterisation

The water content of the biomass was measured by gravimetric method putting a small amount of grape skin in an oven at 105°C to costant weight. The dimensional characterization of marc was made by image analysis using the open source ImageJ software (imagej.nih.gov/ij/) on pictures acquired by a Basler A641fc camera interfaced to a personal computer. The biomass was wet-ground in the solvent adopted for the extraction step, for selected times, in a food grinder with 200 mL capacity (VeoHome) suited for both dry and wet grinding.

2.4 Grape skin characterisation

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2.5 Design of extraction experiments

All the extractions with NaDES were carried out using a solid-liquid ratio equal to 2.5% (w/w) in 250 mL Erlenmeyer flasks kept under agitation (250 rpm) in a thermostated orbital shaker (B. Braun CERTOMAT H). Extraction performance is expected to be the result of the ability of solvent to cause the detachment of polyphenols remaining in the pomace (maceration under winemaking is known to remove most part of vacuolar anthocyanins) and ease their migration through the film of solvent surrounding the particle surface to the solvent bulk. Being an acidic pH known to be favourable for the extraction of residual anthocyanins, and being NaDES pH almost insensitive to water content (data reported in the following), extraction was not parameterised with regard to pH. However, pomace anthocyanins are known to exhibit different hydrophilic/hydrophobic character, and NaDES polarity was adjusted because polarity effects can be expected to affect the detachment of individual polyphenolic species from the matrix. Following detachment, diffusion first through the solid matrix, and then through the solvent film surrounding the fragment is necessary for anthocyanins to enrich the extracting solvent bulk. In order to assess the mass transfer resistance in the pomace matrix, unground and (moderately) ground pomace was used during the experiments. Three parameters were therefore selected to investigate the extraction kinetics with NaDES: the NaDES water content (22% and 44% by weight, denoting the relevant solvents as NaDES28 and NaDES44), the extraction temperature (25 and 38 °C) and the specific surface of pomace, as obtained without and after applying a short (30" or 60") wet grinding procedure in the presence of the NaDES. The thermodynamic (maximum extraction) and kinetic (matrix-side and bulk liquid-side mass transfer resistance) controlling factors were investigated by carrying out the extractions and assessing the effect of contact time (0.5, 1, 2, 4 and 24 hours, or when stability was achieved). The extracts were centrifuged for 10 min at 10000 rpm and the liquid was separated from the solid residue. The NaDES-mediated efficiency extraction was compared to the efficiency reached with an acidified hydroalcoholic solvent. An aqueous solution of ethanol (H2O:EtOH equal to 30:70 v/v) was acidified with HCl (5% v/v) and mixed with the biomass (Panić et al., 2019). Three subsequent extraction steps, each lasting 30 min, were carried out at 60°C by replacing the solvent of the preceding extraction with an equal amount of fresh solvent to exhaust the pomace matrix.

2.6 Colorimetric analysis of the extracts

After liquid-solid separation samples were analysed using a spectrophotometer at a wavelength of 528 nm to measure the concentration of anthocyanins in the liquid. The quantification was done by means of a calibration line obtained with solutions of pure enocyanin in the pure solvent (NaDES or water-ethanol) according to the Lambert-Beer law. Afterwards, the anthocyanins concentration in the extracts was evaluated by spectrophotometrically measuring the absorbance at 528 non and expressing their anthocyanins content as grams of enocyanin per litre of solvent. Anthocyanins content was expressed as a concentration of the total anthocyanin found in enocyanin. Control extracts obtained using the acidified hydroalcoholic solvent were analysed following the Folin-Ciocalteau Assay (Folin et al., 1927) to quantify the content of total polyphenols. The Folin-Ciocalteau was not applied to NaDES extracts due to the interference of the Folin reagent with choline chloride.

3 Results and discussion

3.1 Grape skin characterisation

The very first step of this work involved characterising the water content of the grape pomace, which was found to be equal to 64.4%. Skin particle size distribution was also characterised before and after grinding. Size distribution data were used to calculate skin specific surface, whose value is reported in Table 1.

Table 1: Grape skin particle size characterisation results

Parameter	Unground skin	Ground skin (30 s)	Ground skin (60 s)
Surface area/weight ($cm^2\ g^{-1}$)	11.2	13.8	15.7
Average diameter of the equivalent sphere (mm)	9.3	5.7	3.5

3.2 NaDES characterisation

In the second phase of the work, the pH, density and viscosity of the two tested NaDES formulations was assessed at 20 °C. The results, reported in Table 2, show that pH exhibits a minor dependence upon water content, while the influence of water content on density is in line with expectations.

Table 2: NaDES properties at room temperature

Water content	Viscosity (mPa s)	Density (kg m^{-3})	pH
28%	13.5	1215	0.70
44%	4.2	1156	0.85

The rheology of the adopted NaDES was investigated systematically. Choline chloride:tartaric NaDES viscosity was never found to be a function of shear rate, thereby showing that these NaDES are Newtonian fluids. The dependence of viscosity upon temperature and water content was then characterised and the results were correlated by using Arrhenius equations, reported in equations 1 to 3.

$$\eta = \eta_0 * e^{E_a/RT} \ (mPa * s) \quad (1)$$

$$\eta_0 = 1.08 * x_{H2O}{}^{5.03} \ (mPa * s) \quad (2)$$

$$E_a = -19526 * \ln(x_{H2O}) - 2503 \ (J * mol^{-1}) \quad (3)$$

The results of the measurements are reported in the figure below (Figure 1). NaDES with a lower content of water (5%) shows a higher viscosity. A marked reduction in viscosity is observed up to a dilution of 15-20% (w/w) of the mixture ChCl:TA. Above this water content, viscosity curves exhibit a trend that asymptotically tends to that of water.

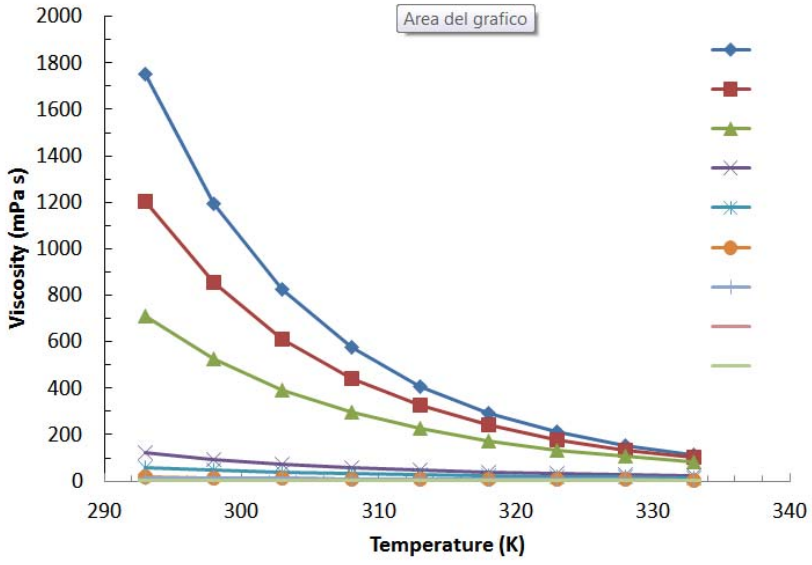

Figure 1: Choline chloride:tartaric acid:water NaDES viscosity as a function of temperature at different water contents

3.3 Anthocyanins extraction

Extraction runs results, reported in Table 3, show that NaDES44 attained the highest overall extraction of anthocyanins (3.3 g/l) among the tested solvents at stability, obtained after 24 hours of extraction performed at

room temperature on unground pomace, at stability. When the same extraction conditions were adopted on ground pomace, a 60% higher extraction was measured after stability was attained. Considering that anthocyanins are completely miscible with NaDES44,, it can be argued that part of the unground skin matrix was not subjected to extraction, probably due to hindrances in its structure that prevented the solvent from accessing bound anthocyanins to free and deliver them to the solvent's bulk. When the matrix was physically fragmented, the extent of accessed volume increased warranting 48% higher extraction performance. This hypothesis can be reasonably confirmed by the consideration that ground skin shows a specific surface area increase equal to 40%, a figure which is likely underestimated because initial fragments were not spherical (crushed berries are lenticular in shape) while grinding reduces aspect ratio (i.e., the ratio of the largest to the smallest dimension of the particles). The fact that the grinding pretreatment does not affect significantly the optimal extraction time can be taken as an argument to reinforce this interpretation. A further question is in order to investigate the reason of NaDES44 higher performance with respect to NaDES28. One fact is that NaDES44 is less polar that NaDES28 (choline chloride-based NaDES are among the most polar solvents in nature, as reported by Dai, 2013). Ethanol is also less polar than water, and performs worse than NaDES44. One could say that NaDES44 has exactly the right polarity which quantitatively maximises extraction, thus reinforcing the "designer's solvent" reputation that NaDES have been awarded for. The reasons for the lower performance of the ethanolic solvent could also include the less acidic pH value compared to the NaDES' one, even though it was the recommended value in the literature (Patil et al., 2009).

Table 3: Equilibrium data. Optimal extraction time refers to the time taken to reach 90% of the maximal extraction.

	Anthocyanins (g/l)	Optimal extraction time (h)
NaDES44, T=25°C, unground skin	1.85	8
NaDES44, T=25°C, ground skin 60''	3.33	6
NaDES28, T=25°C. unground skin	0.81	2
NaDES28,T=30°C, unground skin	0.84	<1
NaDES28, T=38°C, unground skin	1.03	<1
H2O:EtOH:HCl, T=60°C, unground skin	0.84	
H2O:EtOH, T=60°C, unground skin	0.29	

As far as extraction temperature effects are concerned, increasing process temperature from 25 °C to 38 °C resulted in increasing maximal extraction by 29% (reaching 1.85 g/L) on NaDES28, which is the more viscous tested solvent in the group, lowering it to 9 cP, a value which falls in the middle between those of NaDES28 and NaDES44 at room temperature. It should be noted that, while the extraction rate during the first period (a few hours) appears to be affected even by a modest temperature increase (from 25 °C to 30 °C) without changing the ultimate yield, if extraction temperature is increased further (38 °C) an increase is observed both in extraction rate and ultimate extraction yield. In general, the obtained extraction curves, which are collectively expressed in Table 3 by the optimal extraction time, become steeper as the working temperature increases and the water content in the solvent decreases. While the increased diffusion rates at the higher temperatures could be attributed to the higher solute diffusivity in the impregnated matrix and to the increased external film coefficient (increased diffusivity and reduced viscosity), the ratio of mass transfer velocity (k·L) to diffusion velocity (D), named after Sherwood (Sh), is a function of the ratio of the momentum to the mass diffusion rate, v/D, or μ/ρ D, in the form Sh = f(Sc)1/3. A large Sh in solid-liquid extraction is usually taken as suggestive of a controlling step for mass transfer located in the solid matrix. In order to better characterise the specific features of NaDES extraction, extracts were characterised in terms of their anthocyanins distribution by HPLC and the results obtained are reported graphically in Figure 2.

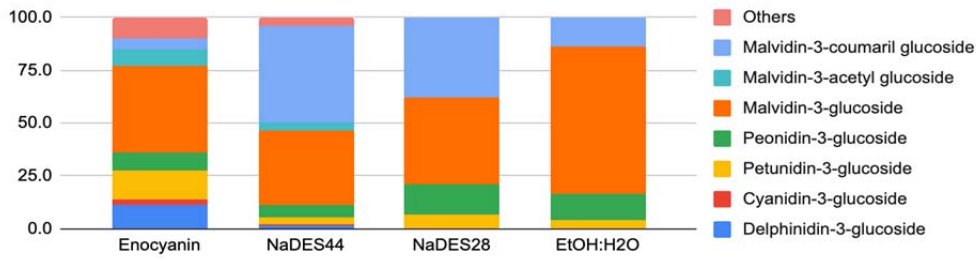

Figure 2: Anthocyanins profile in pure Enocyanin, NaDES and ethanolic extracts.

It can be observed that, when compared to enocyanin the NaDES extracts are slightly poorer in malvidin, delphinidin and petunidin glucosides and significantly richer in malvidin-3-coumaril glucoside. The ethanolic extract, conversely, is richer in malvidin glucoside than enocyanin, which recalls the fundamental difference between the macerative extraction occurring in vinification.

4. Conclusions

An innovative natural deep eutectic solvent based on choline chloride, tartaric acid and water was tested in the extraction of polyphenols from grape skin obtained after red winemaking. The NaDES solvent has shown extraction performance 112% higher than the control (acidified ethanol) in comparable extracting conditions (unground pomace). The NaDES formulation containing 44% of water (w/w) exhibited the most favourable extraction features among the tested solvent formulations. The reported results show that NaDES are promising food-safe solvents to improve a conventional method for the recovery of anthocyanins from spent grape marc leaving full freedom to apply the extract itself in compatible biologically-relevant applications in the food, agricultural, dermatological, and pharmaceutical domains.

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