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Extraction of Anthocyanins from Grape (*Vitis vinifera*) Skins Employing Natural Deep Eutectic Solvents (NaDES)

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In the last years, the possibility to exploit the high added value bioactive compounds obtained by the solid residues of the winemaking process in cosmetic, food supply and pharmaceutical field was explored. To enhance the biocompatibility of the final products and reduce the environmental impact of traditional extraction routes, researchers attempted at developing sustainable and green extraction processes. In the present work, the feasibility of employing NaDES (Natural Deep Eutectic Solvents) as a cheap and non-toxic alternative to traditional solvents for the extraction of bioactive compounds from winery wastes of Merlot marc was studied. Furthermore, a two-level factorial design was used to study the single and combined effect of process variables, namely, solid to liquid ratio dry grinding time and extraction time, on the extraction yield. Experimental show that, in the range studied, solid to liquid ratio has a predominant effect and both grinding and extraction times have a slightly appreciable effect on the extraction yield.

1. Introduction

Grapes (*Vitis vinifera*) are among the most cultivated and consumed fruits in the world, especially in the wine industry. Winemaking leads to the production of a very large amount of waste, in particular pulp, skins and shoots. The conservation and disposal of residues represents a significant cost for wine industries. Winemaking wastes are generally disposed of or, alternatively, used for composting. In order to limit the environmental impact of the wastes and the disposal costs, wastes were employed for production of heat, exploiting their high lignocellulosic fraction, or destined to the field of bio-fertilization and animal feed (Rani et al., 2020). In the last years, several studies reported that the solid residues of the winemaking process are particularly rich in high commercial value bioactive compounds, such as phenolic compounds (stilbenes, phenolic acids, flavonoids), pigments, tannins and alcohols (Teixeira et al., 2013; Chen et al., 2018; Šuković et al., 2020). Anthocyanins are the most common and valuable pigments in red grapes and are extensively studied in the field of natural dyes, in order to replace synthetic dyes that are often toxic to humans. Among these, enocyanin is coloured extract of an intense red obtained from grape pomace, which is produced as described by Mazza and Miniati (1993). The most common extraction solvents are hydroalcoholic, such as mixtures with different titers of ethanol or methanol (Librán et al., 2013; Makris et al., 2007). These methods, however, require large amounts of solvent and long extraction times (Wang and Weller, 2006). To reduce extraction times, the use of microwave and / or ultrasound systems was combined with traditional methods to facilitate the release of compounds in the plant matrix (Beres et al., 2017). Other unconventional approaches involve the use of sub- and supercritical CO_2, ASE (accelerated solvent extraction), pressurized hot water, overheated hydroalcoholic mixtures (Schieber, 2017). Although many of these techniques allow obtaining high extraction yields, in recent years, sustainable and greener extraction techniques were studied. Researchers attempted at developing extraction processes that would allow an appropriate exploitation of raw materials and optimal consumption of solvents, minimizing energy consumption.

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The use of NaDESs (Natural Deep Eutectic Solvents) as an alternative to traditional solvents for the extraction of bioactive compounds from agro-industrial wastes was considered in agreement with many of the principles of Green Chemistry (Cicci et al., 2019; Smith et al., 2014). NaDESs have unique physicochemical properties, are easily adaptable to process requirements and are generally composed of low cost, readily available and non-toxic compounds. Among these, there are quaternary ammonium salts (such as choline chloride) with neutral donors of naturally derived hydrogen bonds, such as vitamins, amines, carboxylic acids, alcohols and sugars (Dai et al., 2013). In different studies, NaDES composed of choline chloride, proline, betaine as hydrogen bond acceptors and organic acids (malic, oxalic, citric acids), sugars (glucose, fructose, xylose) and polyols (glycerol) as hydrogen bond donors, in opportune molar ratios, were employed for the extraction of bioactive compounds from wine industry wastes (Bosiljkov et al. 2017; Radošević et al., 2016; Panić et al. 2019). In our previous work (Sapone et al., 2020), choline chloride and L (+) tartaric acid based NaDES was employed for the extraction of phenolic species from Merlot marc. In the present contribution, as a further step forward, a preliminary study of the effect, single and combined, of operating parameters such as solid to liquid ratio, dry grinding time and extraction time on the anthocyanins extraction yield was carried out. To assess the single and combined effect of process parameters, a two-level factorial design was used.

2. Materials and methods

2.1 Chemicals

Choline chloride, L (+) tartaric acid, DPPH (2,2-Diphenyl-1-picrylhydrazyl), methanol (purity ≥ 99.5 %) and hydrochloric acid (37 w/v %) were purchased from Merck (Darmstadt, Germany). E163 enocyanin (50 % w/w) was made available as a courtesy by Ruffini Aromi. Demineralized water was used for the experiments. All reagents were used without further purification.

2.2 Biomass preparation

Merlot cultivar grape pomace 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 employing 1 % v/v sulphuric acid and potassium metabisulphite, portioned, vacuum-packed and frozen until use. Before the extraction, the skins were manually separated from the pomace and ground in a coffee grinder.

2.3 NaDES preparation

NaDES was obtained by mixing choline chloride and tartaric acid in 3:2 molar ratio inside a jacketed glass vessel at 90 ± 0.1 ° C, until a clear and homogeneous mixture was obtained. The mixture was subsequently cooled down to room temperature and 40 % (w/w %) water was added to obtain a lower solvent viscosity. The NaDES obtained was stored at room temperature in a closed bottle.

2.4 Extraction procedure

Preliminarily to the actual extraction, a two-stage total extraction was carried out to assess the maximum anthocyanin content, according to the procedure described by Bonfigli et al. (2017), with slight modifications. Briefly, a small amount of grape skins was ground for 30 s and treated with 70 % methanol-water at pH 0.7 (obtained by adding opportune volume of hydrochloric acid), with a solid to liquid ratio of 0.03 g/mL at 45 °C, under magnetic stirring, for 24 hours. The extraction stirring rate was 250 rpm. Subsequently, the solid was separated from the liquid by filtration and the solid was treated with the same volume of fresh solvent in the same conditions of the first stage for 1 hour. The NaDES extraction of anthocyanins was carried out in 100 mL glass flasks in the solid to liquid ratios 0.13 - 0.44 g/mL, dry grinding times 6 - 24 s, for extraction times of 120 - 390 min, in a horizontal stirrer (170 rpm) at 25°C. The solid-liquid separation was achieved by centrifugation (1165 g) for 10 minutes. The extracts obtained were stored at 4 °C for further analyses.

2.5 Analytical methods

The moisture content of the biomass was measured gravimetrically by oven drying the biomass at 105°C to constant weight. The total anthocyanin content was determined spectrophotometrically (Shanghai Mapada Spectrophotometer UV- 1800 PC) by reading the absorbance at 528 nm against a calibration curve obtained with standard solutions of enocyanin. The extraction yield was obtained dividing the anthocyanin content of each sample by the equivalent enocyanin content obtained with the two-stage total extraction, expressed as weight of enocyanin equivalents on dry basis (DB). The antioxidant activity was evaluated by the DPPH assay (Kedare and Singh, 2011) and the results were expressed as trolox equivalents antioxidant capacity (TEAC) on dry basis, using a calibration curve obtained with standard solutions of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

2.6 Factorial design

For the preliminary investigation of the effects of solid to liquid ratio (X_1), dry grinding time (X_2) and extraction time (X_3) on the extraction yield, a two-level factorial design was employed. The factorial design was composed of 8 points and 6 replicated points to ensure accuracy in the estimation of the experimental error. The selection of factor levels, which are reported in Table 1 in both uncoded and coded values, was carried out according to preliminary experiments. Coded values were obtained as reported in Eq(1), where x_i represents the coded value of i^{th} variable, X_i is the uncoded value of the i^{th} factor, $X_{i,0}$ is the value of the i^{th} factor at the central level and ΔX_i is the step size. The experimental design layout is shown in Table 2. The design of experiments and statistical analysis was performed by using Design Expert 7.0 software® (Version 7.1.6, Stat-Ease Inc., Minneapolis, MN, USA).

$$x_i = \frac{X_i - X_{i,0}}{\Delta X_i} \quad (1)$$

Table 1: Actual and coded levels of the experimental design factors

Factor	Unity	Levels		
		-1	0	1
Solid to liquid ratio (X_1)	g/mL	0.13	0.29	0.44
Grinding time (X_2)	s	6	15	24
Extraction time (X_3)	min	121	255	388

Table 2: Experimental design layout, observed response (Yield) and antioxidant activity of the extracts

Run	X_1	X_2	X_3	Yield (%)	TEAC (mg TE/g DB)
1	0.44	24	388	25.67	3.57
2	0.29	15	255	32.16	3.92
3	0.44	6	388	25.15	3.31
4	0.13	6	121	42.81	4.20
5	0.29	15	255	35.14	4.09
6	0.13	24	121	45.54	5.36
7	0.13	24	388	53.56	6.08
8	0.29	15	255	34.96	4.7
9	0.44	24	121	25.34	3.05
10	0.29	15	255	34.10	4.76
11	0.13	6	388	48.33	5.33
12	0.44	6	121	21.07	2.39
13	0.29	15	255	34.17	5.06
14	0.29	15	255	35.80	4.41

3. Results and discussion

3.1 Extract characterization

The moisture content measured for the grape skins was 64.4 %. Spectrophotometric analyses for each extract show an intensity peak the wavelength band comprised between 500 and 550 nm, suggesting the presence of a anthocyanins' residue after maceration. The UV-Vis spectrum of the central point is reported in Figure 1 and shows an intensity peak at 520 nm. The antioxidant activities of the extracts are reported in Table 2. The results show that the antioxidant activity increases according to the extraction yield.

3.2 Extraction modelling

The results in terms of extraction yield and antioxidant activity are reported in Table 2.

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (2)$$

The influence of the three factors, namely, solid to liquid ratio (g/mL), grinding time (s) and extraction time (min) was evaluated by means of the factorial design previously described. The model was represented by a second-order polynomial equation, Eq(2), where β_i are the coefficients associated to the three main effects, β_{ij} are those associated to the cross interactions, x_i are the coded independent variables and y is the process response variable.

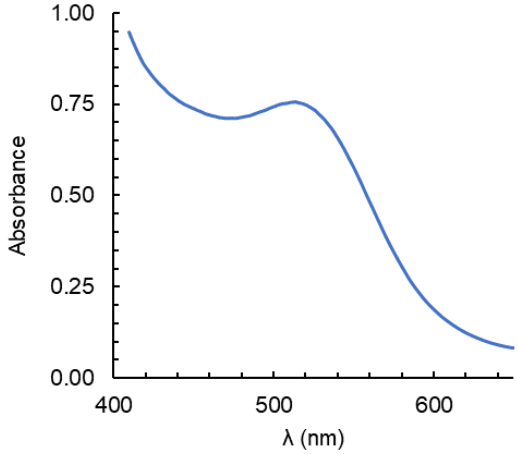

Figure 1: UV-Vis spectrum of the extract obtained employing choline-chloride

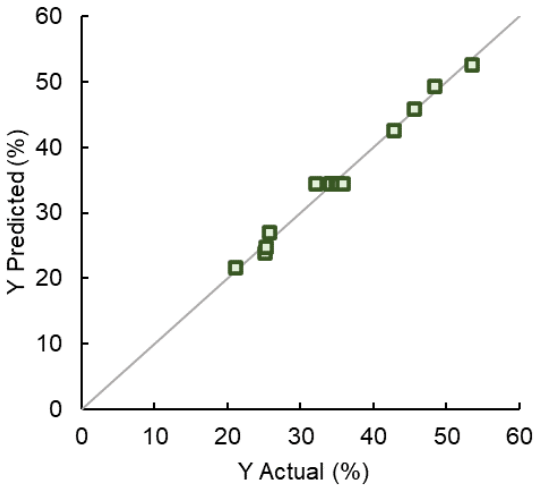

Figure 2: Parity plot

In Figure 2, the parity plot is reported. It can be observed that the mathematical model provides a good approximation of the experimental data, being the points uniformly distributed around the bisector. Furthermore, values of coefficient of determination, R^2 (0.9838), adjusted-R^2 (0.9766), prediction-R^2 (0.9508) witness a good fit of data. The low value of the coefficient of variation (4.13 %) ensured a high reproducibility of the experiments. The lack of fit was not significant and there were no violations of basic analysis of variance. In Table 3, the model β_i coefficients, standard errors, F-values and p-values are reported. The three factors were all statistically significant and influenced the extraction yield. The predominant effect on the response variable was exerted by the solid to liquid ratio, as it can be observed by the high negative value of β_1 coefficient. In fact, as the solid to liquid ratio increases, the extraction yield decreases. This factor is also involved in a statistically significant interaction with extraction time, as it can also be observed in Figure 3.

Table 3: Regression coefficients (β_i) of the polynomial model (Eq.2) with their standard errors (SE), F-values (F) and p-values (p).

Coeff	Term	Value	Standard Error	F-value	p-value
β_0	Intercept	+35.27	0,47		
β_1	X_1	-11.36	0,47	432.47	<0.0001
β_2	X_2	+1.59	0,47	8.13	0.0191
β_3	X_3	+2.24	0,47	16.11	0.0030
β_{13}	X_1X_3	-1.14	0,47	4.17	0.716

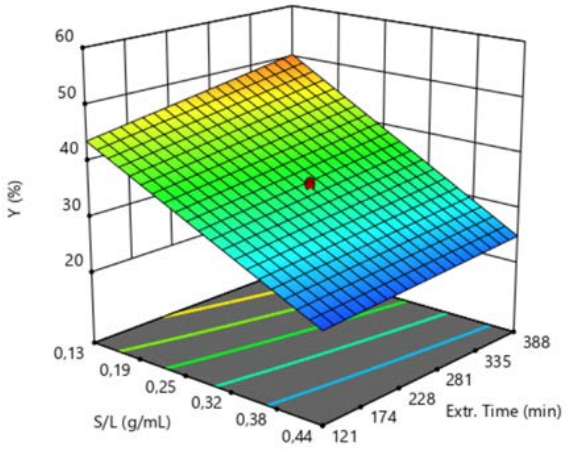

Figure 3: 3d plot showing the effect of solid to liquid ratio (g/mL) and extraction time (min) on the extraction yield.

Several studies employing factorial designs indicated extraction time as a significant factor in a solid-liquid extraction of bioactive compounds from plant biomass (Lavecchia et al., 2015; Kaur et al., 2008). However, in the present study, the effect of extraction time exerts a slightly positive effect on the extraction yield, according to the positive, but low value of coefficient β_2. This behaviour could be attributed to the time range used in the present study, where shorter extraction times were excluded. According to the value of β_3 coefficient, dry grinding time exerts a rather negligible effect on the extraction yield. This behaviour could be due to the conditions of spent grape skins, that already underwent a maceration and the previously described downstream operations. Therefore, further mechanical operations might have not substantially affected the biomass. The results in terms of extraction yield obtained are apparently comparable to those obtained with hydroalcoholic solvents (75 % EtOH-H_2O v/v) by Librán et al. (2013). However, they are far lower than those obtained in the study of Bosiljkov et al. (2017), with a choline chloride – malic acid based NaDES. This behaviour could be attributed to a higher solubility of anthocyanins and to the different density of the solvent due to the use of malic acid, instead of tartaric acid. Furthermore, the authors employed wine lees and an ultra-sound equipment to enhance the extraction process.

4. Conclusions

Wastes from the wine industry are still very rich in bioactive compounds and can represent a promising raw material for cosmetic, nutraceutical and food products. The use of NaDES could represent an effective alternative to traditional solvents for the extraction process, as they can be formulated employing components of natural origin, compatible with the final use of the bioactive compounds. In the present work, a NaDES based on choline chloride and citric acid was used to recover anthocyanins from red grape pomace, obtaining yields up to 54%. A two-level factorial design was employed to evaluate the effects of three factors, namely, solid to liquid ratio, biomass dry grinding time and extraction time. A second order polynomial equation provided a good fit of the experimental data. Liquid to solid ratio appeared to be the factor exerting the highest effect on the extraction yield.

The results in terms of anthocyanin content and antioxidant activity of the extracts, encourage further studies to expand extraction time range and explore the combination of the use of NaDES with pretreatments, such as ultrasound, microwaves and enzymatic processes, in order to fully explore the potential of this approach.

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