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Ultrasound-assisted extraction of anthocyanins and total phenolic compounds in *Vitis vinifera* L. 'Tempranillo' winemaking lees

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Summary

Winemaking lees (WL) are the less exploited by-products from the winemaking industry. This by-product is generated after the fermentation of grape must and its composition is variable, although it is mainly made up of lysed yeasts, tartaric acid, colloids, polyphenols and ashes. Of all the compounds that can be found in lees, polyphenols exhibit particularly powerful bioactive properties and their extraction for their reuse in different matrices could be a matter of particular interest.

Ultrasound-assisted extraction (UAE) has been used to extract both total anthocyanins (TA) and total phenolic compounds (TPC) from lees from *Vitis vinifera* L. 'Tempranillo' vinification. The optimization of six extraction variables (% methanol, pH, temperature, amplitude, cycle and ratio solvent:mass) was performed using a Box-Behnken design (BBD) where TA and TPC were considered independent variables. The UAE method that has been developed in this research could be a new rapid and efficient method to extract TPC and, coupled to chromatographic methodologies, identify up to fourteen different anthocyanins from WL. The objective of this study is to determine WL polyphenolic content and its potential for further uses in other procedures aiming to create value from vinification waste.

Key words: anthocyanins; winemaking lees; phenolic compounds; ultrasound-assisted extraction.

Introduction

The wine making industry is a very important sector with a worldwide production around 279 million hectolitres (OIV 2018). This activity generates a huge amount of wastes and by-products. In Spain alone, the wine making industry generates 2-3 million tons of wastes and by-products, where 14 % correspond to winemaking lees (WL) (Ruggieri *et al.* 2009). WL are defined by the European Economic Community regulation no. 337/79 as the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following filtration or centrifugation. These WL, are formed by microorganisms (mainly yeasts), tartaric acid, colloids and inorganic matter (Pérez-Serradilla *et al.* 2008). Traditionally, WL have been used as animal food stock, as a nutrient for yeast development during wine alcoholic fermentation or as a substrate for tartaric acid recovery in wineries (Versari *et al.* 2001, Dimou *et al.* 2015). However, in most cases WL have been disposed of at landfill contributing, in this way, to an environmental problem because of their low pH, high chemical oxygen demand and biological oxygen demand (Lafka *et al.* 2007, Bustamante *et al.* 2008).

There are not too many studies in the published literature focused on the extraction of TA and TPC from an analytical design point of view. On the one hand, WL UAE have been studied during the vinification process. More concretely, García-Martín *et al.* (2013) and Fresno *et al.* (2018) studied the WL UAE, with the aim of extracting proteins and carbohydrates in order to improve final wine sensory properties. On the other hand, WL UAE has been studied for industrial purposes, focused mainly on the WL reuse. Tao *et al.* (2014) studied the influence of four variables in the WL extraction procedure and the stability of the extracts obtained during storage, and Romero-Díez *et al.* (2019) pointed at WL as an important source of polyphenols, mainly anthocyanins. Among others, delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, delphinidin 3-*O*-(6"-*p*-acetylglucoside), cyanidin 3-*O*-6(6"-*p*-acetylglucoside), have been identified in red WL. WL TPC like the above mentioned anthocyanins exhibits the potential to be employed in the food, cosmetic or pharmaceutical industries because of its antioxidant, antimicrobial, anti-inflammatory and cardioprotective properties (Landeka *et al.* 2017). In this way, WL exploitation through its polyphenolic compounds extraction may contribute to a greater environmental balance and, at the same time, to obtain extracts with superior bioactive potential.

UAE has been gaining relevance over the last few years as a method to extract polyphenols from different matrices, since it is easy to implement, it produces large extraction yields and exhibits high reproducibility and low solvent con-

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sumption as well as a reduced carbon print and investment (Tao *et al*. 2013). In addition, it has been proved that UAE has some advantages as extraction method, such as a lower solvent requirement than other commonly used extraction methods (Picó, 2013, Gonzalez de Peredo *et al*. 2019). Heat and mass transfer induced by ultrasonic waves is attributed to the effects that accompany ultrasonic cavitation, such as mechanical agitation, local extremely high temperature and pressure, etc. (Horžić *et al*. 2012, Tao *et al*. 2013). However, the efficiency level of UAE may be affected by different factors, such as the type of solvent and its volume, pH, temperature as well as wave cycle and amplitude (Vázquez-Espinosa *et al*. 2018). In fact, hydroalcoholic mixtures with a similar polarity to the polyphenolic compounds should be used to obtain a higher extraction yield according to the literature (Cai *et al*. 2016, Pasquel-Reátegui *et al*. 2014).

In view of these facts, it is necessary to optimize the different UAE variables to obtain higher extraction yields, which may depend on the nature of the samples and on the extractable compounds in them (Kazemi *et al*. 2019). Hence, since it has already been proved as a useful three level procedure, six factors Box-Behnken Design (BBD) in combination with response surface methodology (RSM) has also been used for this research study. This experimental design allows a reduction in the number of experiments when compared to other statistical designs (Watson *et al*. 2015). The results obtained from this kind of experiments are then treated by RSM to generate an adjusted mathematical equation that, when solved, provides the optimum values for the studied variables (Samuel *et al*. 2015, Altemimi *et al*. 2016).

This research intends to contribute to the exploitation and valorisation of red WL by optimizing simultaneously, for the first time, six variables during the UAE procedure for the extraction of TA and TPC from red wine lees and the identification of fourteen different anthocyanins.

Material and Methods

Raw material: Fresh WL from 'Tempranillo' vinification were provided by a private winery in Jerez de la Frontera (Cadiz, Spain). WL were recovered from the bottom of fermentation tanks. Once collected, WL were centrifuged in an OrtoAlresa TORNAX centrifuge (Madrid, Spain) for 20 min at 2000 g. Afterwards, WL were freeze-dried for 72 h in a SP Scientific Virtis Benchtop K™ freeze dryer (New York, United States), grounded in a Thermomix TM31 mill (Wuppertal, Germany) until its homogenization and stored in absence of light with a dessicator until its use. These lyophilized WL were used for further extractions and method characterization.

Reagents: the solvents for extraction used was a mixture of methanol (Fisher Scientific, Loughborough, United Kingdom) and Milli-Q water obtained from a Millipore water purification system (Bedford, United States). pH was adjusted by means of hydrochloric acid and sodium hydroxide (Panreac, Barcelona, Spain). For the Folin-Ciocalteu process, anhydrous sodium carbonate (Panreac, Barcelona, Spain) and Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) were employed. Cyanidin chloride and gallic acid (Sigma-Aldrich Chemical, Saint Louis, United States) were employed as the anthocyanin and phenolic standards respectively. For the UHPLC analyses, methanol (Fisher Scientific, Loughborough, United Kingdom) and formic acid (Scharlau, Barcelona, Spain) were used. These UHPLC solvents were degassed and filtered through a 0.22 μm nylon filter (FILTER-LAB, Barcelona, Spain) before their use.

Extraction procedure: A Hielscher Ultrasound Technology UP200S model probe (Berlin, Germany) coupled to an amplitude and cycle adjustable processor was used to extract TA and TPC from WL. A P-Selecta Frigiterm-10 model thermostatic batch (Barcelona, Spain) was employed for temperature adjustment. The experiments temperature, wave cycle and amplitude were set up based on previous results obtained from some of the members in our research group (González de Peredo *et al*. 2019). The UAE parameters for the study ranged as follows: solvent methanol percentage (25-75 % for phenolic compounds and 0-50 % for anthocyanins), temperature (10-60 °C), amplitude (30-70 %), cycle (0.2-0.7 s), pH (2-7) and solvent:mass ratio (10:0.5-20:0.5 mL:g). For each extraction procedure, about 0.5 g of lyophilised WL was weighed and the corresponding volume of solvent, depending on each experiment, was added. The extraction time was 10 min, followed by a variable cooling period after the extraction. Then, the extracts were centrifuged at 5900 g for 5 min and the supernatant was stored in 25 mL volumetric flasks. The precipitates from the extraction were dissolved in 5 mL of the same solvent and centrifuged under the abovementioned conditions. The supernatant was placed in the same volumetric flask and was filled up with the same solvent. Once the extracts were ready, they were stored at -20 °C in absence of light prior to their analysis.

Total phenolic compounds in WL: TPC content in WL was determined according to Singleton *et al*. (1999) modified method. For the absorbance measurements a Thermo Scientific Genesis UV-Vis model spectrophotometer (Whaltman, United States) was used. All the extracts were analysed in duplicate following the protocol explained by Espada-Bellido *et al*. (2017). The calibration curve for total phenol content quantification was plotted using a standard gallic acid pattern under the same conditions as the extracts. The regression equation obtained for a range between 6.25 to 400 $mg \cdot L^{-1}$ of gallic acid was $y = 0.0059x + 0.108$ with a correlation coefficient $R^2 = 0.9998$. The results are expressed in gallic acid milligrams per gram of lyophilized WL.

Anthocyanins identification by UHPLC-QToF-MS: Anthocyanin content in UAE extracts was identified by means of an ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole-time-of-flight mass spectrometry (QToF-MS) (XevoG2 QToF, Waters Corporation, Milford, United States). The extracts were filtered through 0.22 μm pore size syringe filters (Membrane Solutions, Dallas, United States) prior to analysis. The injection volume was 3 μL. Chromatographic separation was performed according to the method proposed by Pereira *et al*. 2019 on a reverse phase C18 analytical column of 2.1 mm x 100 mm and 1.7 μm of particle size (Acquity

UPLC CSH C18, Waters Corporation, Milford, United States). The following anthocyanins were identified in the samples (Fig. 1): delphinidin 3-*O*-glucoside (*m/z* = 465), cyanidin 3-*O*-glucoside (*m/z* = 449), petunidin 3-*O*-glucoside (*m/z* = 479), peonidin 3-*O*-glucoside (*m/z* = 463), malvidin 3-*O*-glucoside (*m/z* = 493), delphinidin 3-*O*-(6''-acetyl)-glucoside (m/z = 507), cyanidin 3-*O*-(6''-acetyl)-glucoside (*m/z* = 491), petunidin 3-*O*-(6''-acetyl)-glucoside (*m/z* = 521), peonidin 3-*O*-(6''-acetyl)-glucoside (*m/z* = 505), malvidin 3-*O*-(6''-acetyl)-glucoside (*m/z* = 535), cyanidin 3-*O*-(6''-coumaroyl)-glucoside (*m/z* = 595), petunidin 3-*O*-(6''-p-coumaroyl)-glucoside (*m/z* = 625), malvidin 3-*O*-(6''-p-coumaroyl)-glucoside (*m/z* = 639) and peonidin 3-*O*-(*6''-p*-coumaryl)-glucoside (*m/z* = 609).

Anthocyanins determination by UHPLC-UV-Vis: Once the anthocyaninswere identified, their separation in UAE extracts was carried out on a VWR Hitachi Elite UHPLC LaChrom system model (Tokyo, Japan). This system consists on an L-2200U autosampler, an L2300 column oven, two L-2160 pumps and an L-2420U UV-Vis detector. The column used was a PhenomenexKitenex Fused Core C18, 2.1 mm x 100 mm with a 2.6 µm particle size (Torrance, United States). The method employed was the one published by Pereira *et al.* (2019). A cyanidin chloride (commercial anthocyanidin standard) calibration curve in a range from 0.36 to 34 mg·L^{-1} of cyanidin chloride (y = 232219.56x – 3574.06) with a correlation coefficient (R^2 = 0.9997) was calculated. The 14 anthocyanins concentrations were calculated using the abovementioned curve. The analyses were carried out in duplicate and the results obtained are expressed as anthocyanins milligrams per gram of lyophilized WL.

UAE method design and analysis: A Box-Behnken Design (BBD) is a design that consists on a spherical response surface methodology (RSM) that consists on a central point plus several middle points on the edges of a cube inside a sphere (Ding *et al.* 2016). BBD requires a shorter number of experiments compared to other statistical experiment designs. In the present research, a six factor, three-level BBD was employed to establish the optimal UAE conditions for the highest possible extraction yield of TA and TPC from WL. All the extractions were optimized for six different variables: solvent methanol percentage (X1), temperature (X2), ultrasonic wave amplitude (X3), ultrasonic wave cycle (X4), solvent pH (X5) and solvent:mass ratio (X6). The whole design consisted on 54 extractions with six repetitions at the central point carried out in a random order. The six variables were studied at three different levels; -1 (low), 0 (middle) and +1 (high). TA (Y_{TA}, mg·g^{-1} WL) and TPC (Y_{TP}, mg·g^{-1} WL) results and the whole experimental layout are shown in Tab. 1. To correlate the relationships between the variables, the results obtained after the analysis were entered into the following second order polynomial equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_{11}X_1^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 - \beta_{14}X_1X_4 + \beta_{15}X_1X_5 + \beta_{16}X_1X_6 + \beta_{22}X_2^2 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{25}X_2X_5 + \beta_{26}X_2X_6 + \beta_{33}X_3^2 + \beta_{34}X_3X_4 + \beta_{35}X_3X_5 + \beta_{36}X_3X_6 + \beta_{44}X_4^2 + \beta_{45}X_4X_5 + \beta_{46}X_4X_6 + \beta_{55}X_5^2 + \beta_{56}X_5X_6 + \beta_{66}X_6^2$$

where Y is the aforementioned response of the extractions, β_0 is the model constant, β_i are the linear coefficients, β_{ij} are the interaction coefficients, β_{ii} are the quadratic coefficients and X_i and X_j are the independent variables.

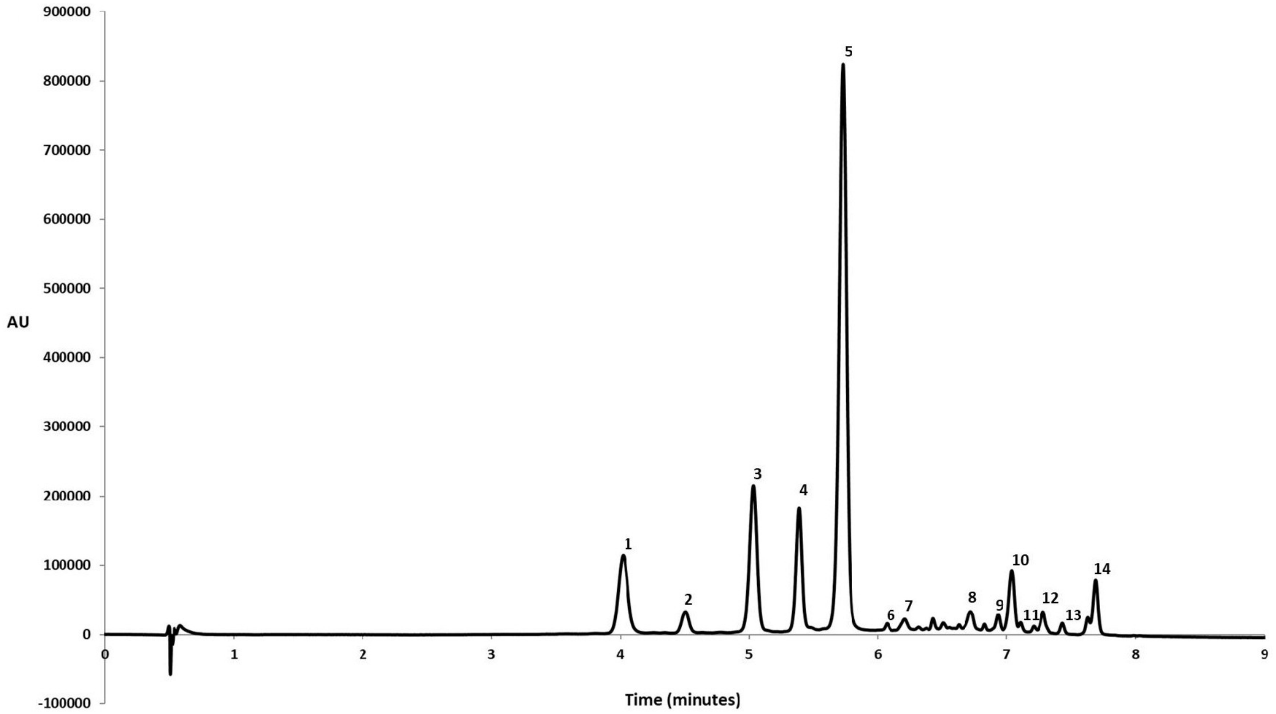

Fig. 1: Chromatogram of anthocyanins analysed by UHPLC-UV-Vis in the UAE extracts from WL. Peak assignment: (**1**) delphinidin 3-O-glucoside, (**2**): cyanidin 3-O-glucoside, (**3**): petunidin 3-O-glucoside, (**4**): peonidin 3-O-glucoside, (**5**): malvidin 3-O-glucoside, (**6**): delphinidin 3-O-(6''-acetyl)-glucoside, (**7**): cyanidin 3-O-(6''-acetyl)-glucoside, (**8**): petunidin 3-O-(6''-acetyl)-glucoside, (**9**): peonidin 3-O-(6''-acetyl)-glucoside, (**10**): malvidin 3-O-(6''-acetyl)-glucoside, (**11**): cyanidin 3-O-(6''-coumaroyl)-glucoside, (**12**): petunidin 3-O-(6''-p-coumaroyl)-glucoside, (**13**): malvidin 3-O-(6''-p-coumaroyl)-glucoside, (**14**): peonidin 3-O-(6''-p-coumaryl)-glucoside.

Table 1

BBD matrix with coded variables and measured and predicted responses for TA and TPC

Run	Factors						Responses			
	Solvent	Temp.	Amplitude	Cycle	pH	Ratio	Y_{TA} (mg·g^{-1} WL)		Y_{TP} (mg·g^{-1} WL)	
	X1	X2	X3	X4	X5	X6	Measured	Predicted	Measured	Predicted
1	0	0	-1	0	-1	-1	6.564	6.886	37.890	33.901
2	0	0	1	0	-1	-1	6.617	6.498	44.817	48.026
3	0	0	-1	0	1	-1	4.682	3.792	21.308	28.483
4	0	0	1	0	1	-1	4.651	3.363	66.945	61.038
5	0	0	-1	0	-1	1	4.259	5.331	42.777	48.748
6	0	0	1	0	-1	1	4.729	5.834	48.785	41.544
7	0	0	-1	0	1	1	4.576	4.479	36.949	33.805
8	0	0	1	0	1	1	5.049	4.941	41.108	45.031
9	0	-1	0	-1	-1	0	3.251	4.139	87.116	74.381
10	0	1	0	-1	-1	0	5.569	5.316	45.288	50.547
11	0	-1	0	1	-1	0	18.553	13.701	40.307	60.076
12	0	1	0	1	-1	0	5.273	6.760	68.316	55.077
13	0	-1	0	-1	1	0	3.007	2.622	41.918	51.427
14	0	1	0	-1	1	0	6.217	9.958	73.495	57.454
15	0	-1	0	1	1	0	3.708	5.070	60.227	51.238
16	0	1	0	1	1	0	6.295	4.296	59.638	76.101
17	-1	0	-1	-1	0	0	1.913	1.492	26.848	19.814
18	1	0	-1	-1	0	0	9.197	8.203	33.172	39.839
19	-1	0	1	-1	0	0	2.081	1.799	21.332	11.571
20	1	0	1	-1	0	0	6.077	5.632	65.410	72.904
21	-1	0	-1	1	0	0	2.362	2.617	13.281	16.557
22	1	0	-1	1	0	0	8.171	8.641	47.918	46.908
23	-1	0	1	1	0	0	4.457	5.261	4.742	8.844
24	1	0	1	1	0	0	7.800	8.406	84.239	80.502
25	0	-1	-1	0	0	-1	5.368	5.386	46.305	34.801
26	0	1	-1	0	0	-1	5.274	5.234	33.512	49.727
27	0	-1	1	0	0	-1	4.333	4.930	55.723	71.611
28	0	1	1	0	0	-1	5.340	4.867	62.991	59.597
29	0	-1	-1	0	0	1	3.952	4.640	42.500	45.828
30	0	1	-1	0	0	1	5.500	5.112	74.825	58.870
31	0	-1	1	0	0	1	5.257	5.081	77.458	61.308
32	0	1	1	0	0	1	5.871	5.636	35.842	47.411
33	-1	-1	0	0	-1	0	4.745	5.975	28.014	33.058
34	1	-1	0	0	-1	0	8.508	9.501	80.854	74.046
35	-1	1	0	0	-1	0	2.994	1.530	17.589	18.465
36	1	1	0	0	-1	0	8.575	8.172	65.866	59.775
37	-1	-1	0	0	1	0	1.755	1.047	12.634	12.454
38	1	-1	0	0	1	0	3.909	4.262	69.974	62.827
39	-1	1	0	0	1	0	2.663	2.788	24.616	27.723
40	1	1	0	0	1	0	9.195	9.093	87.223	78.448
41	-1	0	0	-1	0	-1	1.558	1.217	31.294	38.616
42	1	0	0	-1	0	-1	6.779	8.125	80.218	72.366
43	-1	0	0	1	0	-1	1.912	3.426	44.692	18.805
44	1	0	0	1	0	-1	8.304	8.648	54.162	62.882
45	-1	0	0	-1	0	1	2.314	1.781	12.240	14.290
46	1	0	0	-1	0	1	7.743	6.416	46.782	61.897
47	-1	0	0	1	0	1	4.317	4.158	31.036	28.116
48	1	0	0	1	0	1	7.956	8.107	82.601	86.048
49	0	0	0	0	0	0	5.208	5.260	66.806	53.215
50	0	0	0	0	0	0	5.326	5.260	54.686	53.215
51	0	0	0	0	0	0	5.287	5.260	38.166	53.215
52	0	0	0	0	0	0	5.216	5.260	66.624	53.215
53	0	0	0	0	0	0	5.210	5.260	43.377	53.215
54	0	0	0	0	0	0	5.313	5.260	49.631	53.215

Statistical analysis: The results obtained from the BBD with RSM were represented by means of Pareto charts and 3D-surface plots generated by Statgraphics Centurion XVII software.

Results and Discussion

UAE method development: To optimize the six variables that can influence the TA and TPC yields using the UAE method, a BBD was applied. The studied variables ranged as follows: X_1: 0-25-50 % and 25-50-75 % methanol:water (v/v) for TA and TPC respectively, X_2: 10-35-60 °C, X_3: 30-50-70 %, X_4: 0.2-0.45-0.7 s, X_5: 2-4.5-7 and X_6: 10:0.5-15:0.5-20:0.5 mL:g. Afterwards, an analysis of variance (ANOVA) was performed to evaluate the effect of the different variables studied and their possible interactions. The coefficients for the quadratic polynomial equation and their significance (*p*-values) are also shown in Tabs 2 and 3. With regards to anthocyanins the *p*-values X_4, X_5, X_2X_4, X_2X_5 andX_4X_5 were significant factors that influenced the response, with *p*-values lower than 0.05 and a confidence level of 95 %. As it can be seen in Tab. 2, X_1 (solvent methanol percentage) was the most significant variable with a *p*-value < 0.01. The analysis of the model clearly showed that the solvent methanol percentage, pH and cycle were the most significant factors. The solvent methanol percentage and the ultrasonic wave cycle had both a positive effect ($b_1 = 2.464$ and $b_4 = 1.085$ respectively), which means that an increase in temperature and cycle favoured anthocyanins extraction yields and their recovery. Conversely, pH had a negative effect ($b_5 = -0.997$) which implies, therefore, that high pH values diminished extraction yields and anthocyanins recovery. Regarding solvent methanol percentage, it is an important variable since a similar polarity between the solvent and the target compound is necessary to maximise extraction yields (Machado *et al.* 2015). Results agree with those obtained by Tao *et al.* (2014) where the solvent composition was the most influential variable during the UAE procedure. With respect to the cycle, some authors reported that ultrasonic waves cycle is an influent variable, since the use of ultrasonic pulses improves the extraction of polyphenolics among others compounds (You *et al.* 2014, Hashemi *et al.* 2015, Kazemi *et al.* 2019). The positive effect of this variable may be due to the cavitation effect caused by the ultrasonic waves (Machado *et al.* 2017). Lastly, the negative effect showed by pH and its combination with other variables such as wave cycle (X_2X_4) might be due to the transformation and degradation caused on polyphenols (Machado *et al.* 2015).

Table 2

Analysis of variance for the model adjusted to the extraction of TA

	Total anthocyanins						
	Source	Coefficient	Sum of squares	Degrees of squares	Mean square	*F*-Value	*p*-Value
b_0	Model	5.260					
Solvent	X_1	2.464	145.746	1	145.746	57.59	0.000
Temp.	X_2	0.100	0.244	1	0.244	0.10	0.758
Amplitude	X_3	0.018	0.008	1	0.008	0.00	0.955
Cycle	X_4	0.975	22.818	1	22.818	9.02	0.006
pH	X_5	-0.997	23.860	1	23.860	9.43	0.005
Ratio	X_6	0.005	0.000	1	0.000	0.00	0.986
Solvent · Solvent	X_1^2	-0.529	2.888	1	2.888	1.14	0.295
Solvent · Temp	X_1X_2	0.774	4.798	1	4.798	1.90	0.180
Solvent · Amplitude	X_1X_3	-0.719	4.138	1	4.138	1.64	0.212
Solvent · Cycle	X_1X_4	-0.171	0.471	1	0.471	0.19	0.669
Solvent · pH	X_1X_5	-0.082	0.054	1	0.054	0.02	0.884
Solvent · Ratio	X_1X_6	-0.318	0.809	1	0.809	0.32	0.576
Temp. · Temp	X_2^2	0.266	0.732	1	0.732	0.29	0.595
Temp. · Amplitude	X_2X_3	0.020	0.003	1	0.003	0.00	0.970
Temp. · Cycle	X_2X_4	-2.020	32.890	1	32.890	13.00	0.001
Temp. · pH	X_2X_5	1.539	37.936	1	37.936	14.99	0.000
Temp. · Ratio	X_2X_6	0.156	0.195	1	0.195	0.08	0.783
Amplitude · Amplitude	X_3^2	-0.134	0.184	1	0.184	0.07	0.789
Amplitude · Cycle	X_3X_4	0.584	2.733	1	2.733	1.08	0.308
Amplitude · pH	X_3X_5	-0.010	0.000	1	0.000	0.00	0.985
Amplitude · Ratio	X_3X_6	0.222	0.794	1	0.794	0.31	0.580
Cycle · Cycle	X_4^2	0.661	4.497	1	4.497	1.78	0.194
Cycle · pH	X_4X_5	-1.770	25.309	1	25.309	10.00	0.004
Cycle · Ratio	X_4X_6	0.042	0.014	1	0.014	0.01	0.940
pH · pH	X_5^2	0.295	0.900	1	0.900	0.36	0.555
pH · Ratio	X_5X_6	0.560	2.514	1	2.514	0.99	0.328
Ratio · Ratio	X_6^2	-0.280	0.812	1	0.812	0.32	0.576

Table 3

Analysis of variance for the model adjusted to the extraction of TPC

	Total Phenolic compounds						
	Source	Coefficient	Sum of squares	Degrees of squares	Mean square	*F*-Value	*p*-Value
b_0	Model	53.215					
Solvent	X_1	22.920	12608.800	1	12608.800	58.88	0.000
Temp.	X_2	0.257	1.587	1	1.587	0.01	0.932
Amplitude	X_3	6.337	964.006	1	964.006	4.50	0.043
Cycle	X_4	1.085	28.268	1	28.268	0.13	0.719
pH	X_5	-0.482	5.591	1	5.591	0.03	0.872
Ratio	X_6	-0.289	2.015	1	2.015	0.01	0.923
Solvent · Solvent	X_1^2	-10.532	1141.000	1	1141.000	5.33	0.029
Solvent · Temp	X_1X_2	0.088	0.062	1	0.062	0.00	0.986
Solvent · Amplitude	X_1X_3	10.326	853.136	1	853.136	3.98	0.560
Solvent · Cycle	X_1X_4	2.581	106.615	1	106.615	0.50	0.486
Solvent · pH	X_1X_5	2.353	44.318	1	44.318	0.21	0.652
Solvent · Ratio	X_1X_6	3.464	96.004	1	96.004	0.45	0.509
Temp. · Temp	X_2^2	7.117	521.116	1	521.117	2.43	0.130
Temp. · Amplitude	X_2X_3	-6.374	362.879	1	362.879	1.69	0.204
Temp. · Cycle	X_2X_4	4.708	177.393	1	177.393	0.83	0.371
Temp. · pH	X_2X_5	7.465	891.747	1	891.747	4.16	0.050
Temp. · Ratio	X_2X_6	-0.470	1.773	1	1.773	0.01	0.928
Amplitude · Amplitude	X_3^2	-8.724	728.869	1	728.869	3.66	0.060
Amplitude · Cycle	X_3X_4	0.132	0.140	1	0.140	0.00	0.979
Amplitude · pH	X_3X_5	4.607	169.838	1	169.838	0.79	0.381
Amplitude · Ratio	X_3X_6	-5.332	454.945	1	454.945	2.12	0.156
Cycle · Cycle	X_4^2	3.159	102.669	1	102.669	0.48	0.494
Cycle · pH	X_4X_5	3.529	99.634	1	99.634	0.47	0.501
Cycle · Ratio	X_4X_6	8.409	565.690	1	565.690	2.64	0.116
pH · pH	X_5^2	-3.954	160.825	1	160.825	0.75	0.394
pH · Ratio	X_5X_6	-2.381	45.367	1	45.367	0.21	0.649
Ratio · Ratio	X_6^2	2.035	42.632	1	42.632	0.20	0.652

With regards to TPC, the most significant variables were solvent methanol percentage (X_1 and X_1^2) and amplitude (X_3). Both variables had a positive influence (b_1 = 22.920 and b_3 = 6.337), which indicates that as methanol concentration and wave amplitude increase, phenolic compounds yields from WL also grow. In addition, a quadratic interaction with the solvent methanol percentage has been observed ($p < 0.05$). According to Machado *et al.* (2015), hydroalcoholic mixtures improve polyphenolic compounds extractions, in a better way than pure solvents do. This may be because the use of methanol increases the solubility of the polyphenolic matter and, at the same time, a small percentage of water contributes to the solute desorption from the sample (Mustafa and Turner 2011).

For a visual representation of the variables and its combination effects, standardized Pareto charts are displayed in Fig. 2a and b in a decreasing order of significance and with a line that crosses each chart to determine which factors or combinatory factors are statistically significant (*p*-value < 0.05). As shown in the standardized Pareto charts, for the TA extractions, the vertical line was surpassed only by solvent methanol percentage, pH, cycle and the following combined effect: temperature-pH, temperature-cycle and cycle-pH. On the other hand, regarding TPC extractions, solvent methanol percentage itself, its quadratic effect, and ultrasonic amplitude were the only ones to go beyond the vertical line. The results that can be observed in both tables (Tabs 2 and 3) agree with those published by Cavalcanti *et al.* (2011) and Gonzalez de Peredo *et al.* (2019), where solvent methanol percentage and pH were influential variables on the extraction and stability of the anthocyanins extracted from biological products like myrtle berries.

Based on the analyses results, 82.81 % and 78.31 % of the total variability of TA and TPC respectively can be explained. Thus, the values obtained in both cases have shown statistically significant agreement between the estimated and measured responses. The anthocyanins and polyphenols extraction yields have been related to the experimental conditions by a second-order polynomial equation. These polynomial equations have been obtained from the variables coefficients and their interactions (Tabs 2 and 3). In this way, the prediction of Y_{TA} and Y_{TP} response values have allowed to obtain the following two second-order equations:

$Y_{TA} = 5.26 + 2.464\cdot X_1 + 0.100\cdot X_2 + 0.018\cdot X_3 + 0.975\cdot X_4 - 0.997\cdot X_5 + 0.005\cdot X_6 - 0.529\cdot X_1^2 + 0.774\cdot X_1X_2 - 0.719\cdot X_1X_3$

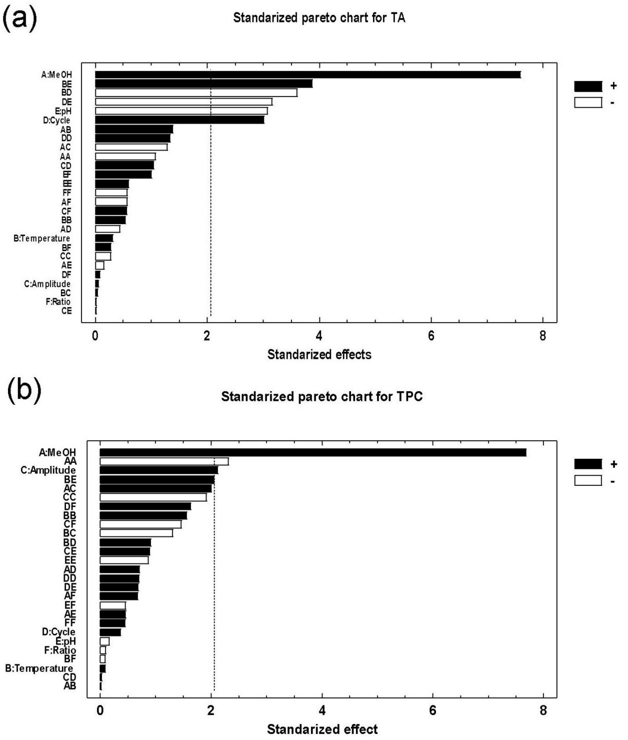

Fig. 2: Standarized Pareto charts for: (**a**) Total Anthocyanins (TA) and (**b**) Total Phenolic Compounds (TPC).

$$- 0.171 \cdot X_1X_4 - 0.082 \cdot X_1X_5 - 0.318 \cdot X_1X_6 + 0.266 \cdot X_2^2 + 0.020 \cdot X_2X_3 - 2.027 \cdot X_2X_4 + 1.539 \cdot X_2X_5 + 0.156 \cdot X_2X_6 - 0.134 \cdot X_3^2 + 0.584 \cdot X_3X_4 - 0.010 \cdot X_3X_5 + 0.222 \cdot X_3X_6 + 0.661 \cdot X_4^2 - 1.77 \cdot X_4X_5 + 0.042 \cdot X_4X_6 + 0.295 \cdot X_5^2 + 0.560 \cdot X_5X_6 - 0.280 \cdot X_6^2$$

$$Y_{TP} = 53.215 + 22.920 \cdot X_1 + 0.257 \cdot X_2 + 6.337 \cdot X_3 + 1.085 \cdot X_4 - 0.482 \cdot X_5 - 0.289 \cdot X_6 - 10.532 \cdot X_1^2 + 0.088 \cdot X_1X_2 + 10.326 \cdot X_1X_3 + 2.581 \cdot X_1X_4 + 2.353 \cdot X_1X_5 + 3.464 \cdot X_1X_6 + 7.117 \cdot X_2^2 - 6.734 \cdot X_2X_3 + 4.708 \cdot X_2X_4 + 7.465 \cdot X_2X_5 - 0.470 \cdot X_2X_6 - 8.724 \cdot X_3^2 + 0.132 \cdot X_3X_4 + 4.607 \cdot X_3X_5 - 5.332 \cdot X_3X_6 + 3.159 \cdot X_4^2 + 3.529 \cdot X_4X_5 + 8.409 \cdot X_4X_6 - 3.954 \cdot X_5^2 - 2.381 \cdot X_5X_6 + 2.035 \cdot X_6^2$$

Given these full second-order equations, the two models let us largely comprehend the variability and which non-significant terms ($p < 0.05$) could be removed from the equations. This would result in a simplified second-order equation as follows:

$$Y_{TA} = 5.26 + 2.464 \cdot X_1 + 0.975 \cdot X_4 - 0.997 \cdot X_5 - .027 \cdot X_2X_4 + 1.539 \cdot X_2X_5 - 1.77 \cdot X_4X_5$$

$$Y_{TP} = 53.215 + 22.920 \cdot X_1 + 6.337 \cdot X_3 - 10.532 \cdot X_1^2$$

All the previous trends have been recorded in 3D-Response surface plots (Fig. 3a and b) using the fitted model to represent the influences of the selected factors (solvent methanol percentage and temperature) for each response.

Model optimization: According to the experimental design for TA and TPC extraction above explained, and once the optimization study had been completed, it was concluded that the optimal UAE conditions for TA and TPC were the ones displayed in Tab. 4.

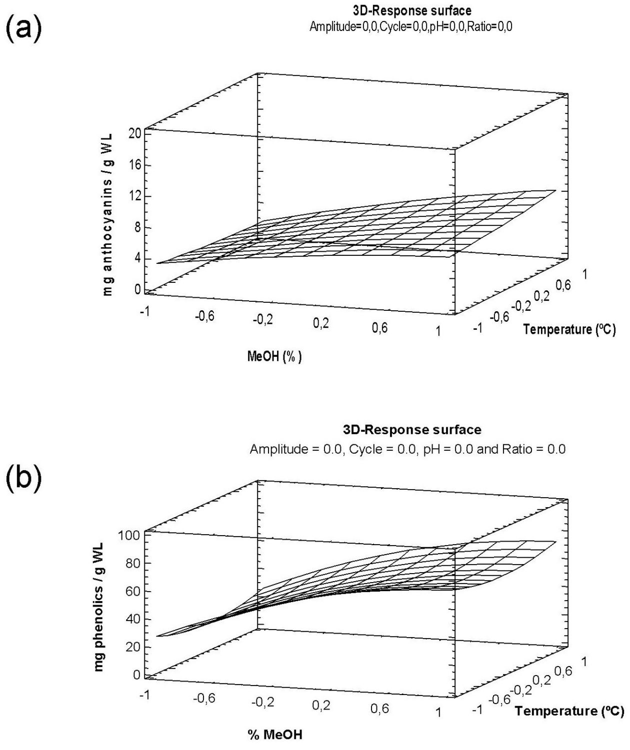

Fig. 3: Response surface plots of the BBD using the polynomial equations. Temperature and solvent composition on the Total Anthocyanins (TA, **a**) and Total Phenolic Compounds (TPC, **b**).

Regarding the optimal conditions for anthocyanins extraction, it has been observed how low temperatures ensure good anthocyanin preservation, since these compounds are thermally sensitive (González de Peredo *et al.* 2019). In addition, low temperatures would prevent methanol evaporation and would maintain the desired solvent:mass ratio values. pH values are near its neutral point, and even though acid pH generates stable anthocyanin conformations, some authors have reported how pH between 3 and 7 improves extraction yields (Machado *et al.* 2017). With respect to the cycle, almost the highest value has been reached in both cases. This may be because high cycle values increase the ultrasonic wave compression and refraction and, as a consequence, a higher compound extraction is obtained (Medina-Torres *et al.* 2017). Concerning the ultrasonic wave amplitude, higher values of this variable result in a stronger cavitation during the extraction processes (Carrera *et al.* 2012). Therefore, values higher than 50 % would fa-

Table 4

Optimum conditions for the UAE method for TA and TPC

	Total anthocyanins	Total phenolic compounds
Solvent composition (% MeOH)	49.2	73.38
Temperature (ºC)	10.2	10.3
Amplitude (%)	51.1	67.68
Cycle (s)	0.69	0.69
pH	6.94	4.33
Ratio (mL·g^{-1} WL)	10.8:0.5	19.8:0.5

vour TA and TPC extraction. Regarding the solvent methanol percentage, according to both models responses its optimum values is near the maximum methanol concentration tested. The results obtained in this research agree with those found in the literature, where hydroalcoholic mixtures with a polarity similar to that of polyphenols are employed for the extraction (Pasquel-Reátegui *et al.* 2014, Cai *et al.* 2016). In conclusion, TA and TPC maximum extractions were obtained when the solvent used had a high percentage of methanol, the pH was over 4, extraction temperature was low and a high wave cycle was implemented.

Extraction time: Once the optimum values for the factors had been determined, several 'Tempranillo' WL extractions were carried out while the extraction time varied between 2, 5, 10 15, 20, 25 and 30 min in order to study UAE kinetics. The results for TA and TPC are shown in Fig. 4.

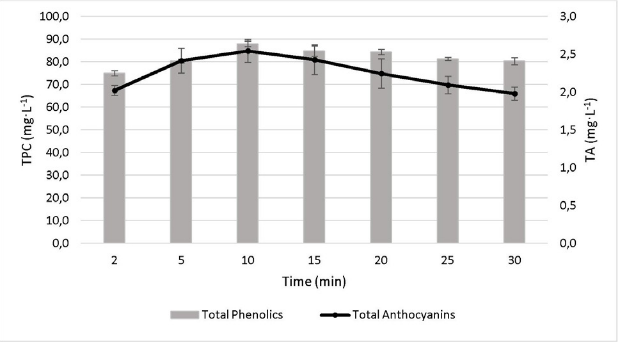

Fig. 4: Total Anthocyanins (TA) and Total Phenolic Compounds (TPC) recovery using different extraction times.

It can be seen how TA and TPC yields were similar through the time, being the 2 min sample the most different when compared to the rest of the samples. Both TA and TPC reached their maximum yield at the 10 min extraction. Longer extraction times showed lower recoveries, probably due to the polyphenolic compounds degradation. More specifically, Tiwari *et al.* (2009) affirmed that ultrasound energy could promote the polyphenolic compounds degradation due to H_2O_2 formation when subjected to high amplitude waves and a low temperature during the extraction. In view of the results obtained and those provided by the literature, 10 min was established as the optimum extraction time. In this way, a reduction of the extraction time and an increase in the TPC extraction yields has been observed compared to previously published results using an ultrasonic bath (Tao *et al.* 2014).

Analytical precision of the UAE method: Once the TA and TPC UAE method had been optimized, the method proposed in this research was analysed in terms of repeatability (precision of the UAE on the same day, =12) and intermediate precision (precision of the UAE on different days, *n*=18). In order to assess those analytical parameters, 30 extractions were performed following the indications described by Espada-Bellido *et al.* (2017). Repeatability results (Coefficient of Variation, CV) were 4.19 % and 4.47 % for TA and TPC respectively. Furthermore, intermediate precision results (CV) were 4.42 % for TA and 4.89 % for TPC. Both CV showed a great precision (< 5 %) of the UAE method for TA and TPC, being at the same time within the acceptance limits (± 10 %) defined by AOAC International 1998).

Conclusions

This work intends to determine a rapid and efficient analytical methodology to maximize the extraction of TA and TPC from red WL. To the best of our knowledge, the study reported here is the first one in which six UAE variables have been modelled and optimized simultaneously for the extraction of either compounds. The optimum conditions for TPC extraction were determined as follows: 73 % MeOH in water solvent with a pH of 4, 10 °C extraction temperature, 20:0.5 mL:g solvent:mass ratio and ultrasonic waves at 0.7 s cycle and 68 % amplitude. Regarding anthocyanins extraction, UAE optimum conditions were as follows: 49 % methanol solvent content, with 6.9 pH, 10 °C temperature, 11:0.5solvent:mass ratio, 0.7 s ultrasonic cycle and 51 % amplitude. Both methods showed high repeatability and intermediate precision levels, either for TA or TPC. These methods, therefore, are appropriate to assess winemaking lees TA and TPC. In addition, this work aims to provide knowledge in the TA and TPC extraction field. However, ethanol must be used instead of methanol, as it is a toxic solvent, and its use would make it impossible to obtain extracts for other purposes than the analysis.

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