Microwave and ultrasound pre-treatments to enhance anthocynins extraction from different wine lees.

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Abstract

Wine lees are rich in anthocyanins (AC), natural colorants with health promoting properties. The extraction kinetics of AC from different wine lees in conventional solid-liquid extraction were studied for the first time. The influence of parameters such as temperature, solid-liquid ratio (R_{S-L}) and type of solvent (hydro-alcoholic mixtures) was also studied. Furthermore, microwaves (MW) and ultrasounds (US) were used as pre-treatments (a prior step to the conventional extraction) in order to increase AC yield. Maximum extraction yield (2.78 $mg_{MALVIDIN-EQUIVALENTS}/g_{DRY-}$

$_{LEES}$) was achieved after 15 minutes at 25ºC, with a R_{S-L} of 1/10 (g/mL) and with a 50%vol. ethanol mixture. When MW were used AC extraction yield was doubled (6.20 $mg_{MALVIDIN-EQUIVALENTS}/g_{DRY-LEES}$) and the required time to achieve a constant yield was reduced (from 15 min to 90s). Meanwhile, US only shortened extraction time in less proportion (from 15 to 5 min). Putative identification of main extract compounds was performed by LC/MS-MS.

Keywords: Wine lees; anthocyanin extraction, kinetic study; microwave pre-treatment; ultrasound pre-treatment

1. Introduction

Wine industry generates huge amounts of wastes and by-products, which are sources of high value compounds, including vine pruning, grape stalks, grape pomace and wine lees (WL) (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015). WL are defined by EEC 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'. Huge amounts of WL are produced per year; they constitute the 14% of the 2-3 million tons of wastes generated in vinification processes, only in Spain. The main components of the solid phase of WL are yeast and bacteria, responsible for the vinification process, tartaric acid salts, precipitated tannins, inorganic matter and free phenolic compounds (Pérez-Serradilla & Luque de Castro, 2011; Dimou et al., 2015). Different types of WL can be found depending on the vinification process. In the case of red wine, it is possible to find first fermentation WL (generated in the alcoholic fermentation) and second fermentation WL, (generated in the malolactic

fermentation). Nevertheless, in the case of a Port wine, first fermentation is stopped by adding extra ethanol (Perestrelo, Silva, Pereira, & Câmara, 2016), and only one type of WL are generated.

Historically, WL have been used for the recovery of tartaric acid (Kontogiannopoulos, Patsios, Mitrouli, & Karabelas, 2017) or as fermentation nutrient supplement (Dimou et al., 2015). However, in recent years the recovery of anthocyanins (AC) from WL has attracted much attention since recent studies showed that the concentration of these colorants is 10 times higher than in grape skins (Peralbo-Molina & Luque de Castro, 2013). Moreover, AC present beneficial effects on human health: its anti-inflammatory, antimicrobial and antioxidant properties are well known (He & Giusti, 2010). Thereby, the exploitation of these dregs would lead to a sustainable growth of the wine industry and would contribute to reducing winery wastes hazards, as they have been classified as pollutants by the European Union (Karpe, Beale, Harding, & Palombo, 2015).

The easiest and the most implemented way to extract compounds from a solid matrix are solid-liquid (S-L) extractions. The most used solvents to recover polyphenols are methanol, ethanol, ethyl acetate and acetone (Muhlack, Potumarthi, & Jeffery, 2017). For example, *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro, 2011) recovered bioactive compounds from WL using mixtures of ethanol and water. Acetone and methanol have been also used for the recovery of polyphenols from WL (Dimou et al., 2015). Nonetheless, substances of interest usually have an intracellular localisation which may represent a problem for the extraction procedure. The movement of those substances of interest from the inside of the cell to the solvent is usually hindered by the mass transfer processes. This is owing to all the mass transfer stages occurring in these types of extractions. In a first step, the solvent should enter

74 the matrix (internal transport), later the dissolution of the compounds in the solvent
75 (solubility) and the release of the solutes to the global phase (external transport). For
76 this reason, cell disruption methods (mechanical, chemical, thermal) can be applied
77 to promote the extraction of valuable intracellular components from diverse raw
78 materials (Kim et al., 2016) by enhancing the mass transfer steps. Within this
79 context, microwave (MW) assisted extraction has been broadly used to enhance the
80 extraction of active compounds from many vegetable matrixes (Rodríguez-Rojo,
81 Visentin, Maestri, & Cocero, 2012; Spigno & De Faveri, 2009), including grape
82 residues such as seeds (Dang, Zhang, & Xiu, 2014) and WL (Pérez-Serradilla &
83 Luque de Castro, 2011) as it has been shown that MW improve the extraction of
84 intracellular compounds as it enhances the internal mass transfer (Rodríguez-Rojo,
85 Visentin, Maestri, & Cocero, 2012) . Prominent among the advantages offered by
86 MW is the double effect of the MW energy. On one hand, the irradiation improves
87 the cellular lysis of materials with large quantities of water due to the rapid heating
88 and evaporation of the intracellular water. On the other hand, a non-thermal effect
89 appears as a result of the alteration of the dielectric camps which could provide a
90 breakdown of the hydrogen bonds of the macromolecules, breaking off their
91 structure (Ganzler, Salgó, & Valkó, 1986). Thanks to the efficiency of the
92 microwave, the heating process takes place in a few seconds. Recently, some authors
93 (Álvarez et al., 2017) suggested using MW pre-treatment as a previous step to the
94 conventional extraction, in which low residence time pre-treatments (below 120s) are
95 employed.
96 Another way to improve the recovery of bioactive compounds from natural matrixes
97 is the use of ultrasounds (US). US assisted extraction has already been applied to an
98 extensive variety of raw materials from vegetable matrixes (Rodríguez-Rojo et al.,

2012) and from WL (Barcia et al., 2014; Tao, Wu, Zhang, & Sun, 2014), showing an increasing of the recovery yield. US increase the external mass transfer due to their mechanical background and 'cavitation effect'. This phenomena takes place due to the high frequency sound waves generated during US application, which generates bubbles in the liquid that collapse. This collapse results in a change in temperature and pressure (Wijngaard, Hossain, Rai, & Brunton, 2012) and the release of cell contents into the medium is enhanced (Rodríguez-Rojo et al.,2012). Similarly, as MW irradiation, US could be used as pre-treatment step before a conventional sold-liquid extraction as an alternative to US assisted extraction to reduce the required extraction time.

The work presented here is a study of the extraction kinetics of AC from different types of WL. The study was focused in the maximization of AC extraction since they are the most abundant polyphenols family present in red grapes and their concentration in wine lees is even higher, as previously, indicated (Peralbo-Molina & Luque de Castro, 2013) . Parameters such as solid-liquid ratio (g/mL), type of solvent (hydro-alcoholic mixtures) and temperature were tested in conventional solid-liquid extractions. Once all the parameters were studied, the best operating conditions were selected. MW and US pre-treatments followed by solid-liquid extraction at selected conditions were also studied for intensifying AC extraction. Furthermore, process parameters for each type of pre-treatment were also investigated such as time, type of solvent (hydro-alcoholic mixtures) and solid:liquid ratio. Additionally, amplitude was studied for US. Optimum extracts were characterized in terms of total polyphenol content, total anthocyanin content and antioxidant activity. Further, putative identification of main component of the selected extracts were identified by LC-MS/MS.

2. Materials and methods

2.1 Raw Material

Port WL were kindly provided by Sogrape Vinhos S.A. (Port, Portugal) in 2015 and immediately stored at 4^{o}C in the absence of light. The lees were centrifuged (Avanti J-26 XPI with a rotor type JA-10) for 90 minutes at 10,000 rpm. The solid phase obtained was freeze-dried (Micro Modulo EDWARDS) at -40ºC for 72 hours, in order to preserve the material and avoid the growth of bacteria. Particle size of dry lees is mainly determined by the nature of solid part of the wine lees, composed mainly by yeast and bacteria, as already mentioned; The freeze dry solid is easily crumbled by hand and it was homogenised by a soft milling step using a chopper (A320R1, Moulinex). Particle size distribution in volume was determined by laser diffraction (Malvern Mastersizer 2000) using a dry disperser accessory (Scirocco 2000); A surface weighted mean particle size value of 13 µm was obtained. Wine lees were stored at room temperature, protected from light.

First (1F) and second fermentation (2F) red WL resulting from the fermentation of *Tempranillo* grapes, from Ribera del Duero Denomination of Origin were kindly provided by Matarromera winery (Valladolid, Spain) in 2015. WL were firstly stored at 4^{o}C, in the absence of light, and then processed as reported above for Port WL.

2.2 Chemicals

Solvents used for extractions were absolute ethanol (99.9% Carlo Erba Reagents, France), bidistilled water (Milli-Q® Integral) and hydrochloric acid (≥37%, puriss. p.a., Riedel-de Haën, France). Chemicals used on the determination of total phenolic content were sodium carbonate (Sigma-Aldrich, France), Folin Ciocalteau reagent (Panreac, Spain) and gallic acid (Fluka, Germany). To determine total anthocyanin

content potassium chloride (Riedel-de Haën, France), sodium acetate trihydrate (≥99.0%, Sigma-Aldrich, France) and hydrochloric acid (≥37%, puriss. p.a., Riedel-de Haën, France) were used to prepare the buffer solutions in bidistilled water. Chemicals used for antioxidant activity assays were: 2‘,2‘-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and disodium fluorescein (FS) from Sigma-Aldrich (France). Sodium chloride (Sigma-Aldrich, France), potassium chloride (Riedel-de Haën, France), sodium phosphate dibasic dihydrate (Sigma-Aldrich, France) and potassium phosphate monobasic anhydrous (Amresco, USA) were used for phosphate buffer solution (PBS) preparation in bidistilled water (Milli-Q® Integral). HPLC analyses were performed using acetonitrile (99.9%, Sigma-Aldrich, France), ultrapure water purified with a Milli-Q water purification system (Merck Millipore, USA), formic acid (99-100%, VWR-CHEM, Spain) and malvidin-3-O-glucoside chloride (Extrasynthese, France) as standard.

2.3 Methods

2.3.1 Anthocyanin Extraction Kinetics

2.3.1.1 Conventional solid-liquid extraction

Conventional S-L extractions were performed by putting in contact the desired solvent with a known amount of dry WL. Parameters such as the $R_{S\text{-}L}$ (0.1, 0.05, 0.033 and 0.025 g/mL), type of solvent (ethanol and hydroalcoholic mixtures varying the percentage of ethanol in 25, 50 and 75%) and temperature (25, 35 and 45ºC) were studied in order to select the best conditions for AC extraction. All the S-L extractions were performed with an agitation of 300 rpm. The pH was adjusted to 2.5 with HCl when the solvent was different from pure ethanol. Samples of 1.5 mL were collected every 5 minutes during a total extraction time of 90 minutes. Total AC

concentrations of each sample was measured in order to build the anthocyanin kinetic extraction curve at different conditions. Conventional S-L extractions were performed in triplicate and data were analysed by t-Student´s test (unpaired samples, unequal variances) with a significance p-value of 0.05.

2.3.1.2 Microwave pre-treatments

MW pre-treatments were carried out in a CEM Discovery One Microwave (CEM Corp.). Power was fixed at 300W since it has been found that energy levels do not have a significant effect on the anthocyanin extraction (Sólyom, Mato, Pérez-Elvira, & Cocero, 2011)**.** A 100 mL QianCap (QLabtech) safe glass pressure reactor was employed to maintain the solvent in a liquid phase. An exact mass of 7.5g of WL was poured inside with a specified amount of solvent in order to reach the desired R_{S-L}. The mixture was homogenised before MW irradiation. Three main parameters were studied in these pre-treatment: R_{S-L} (0.2, 0.15 and 0.1 g/mL), solvent mixture (hydroalcoholic mixtures varying the percentage of water in 100, 50 and 10%) and time of microwaves applied (30, 60 and 90s). Mixtures of ethanol and water were chosen as solvent due to their environmentally friendly and low toxicity properties. Preliminary analysis of the R_{S-L} revealed that higher values of R_{S-L} were needed in comparison with those of literature for grape marc (0.5g/mL) (Álvarez et al., 2017) since freeze dried WL absorbs high amount of solvent due to their powdery nature. When MW pre-treatment was completed, the vessel was cooled down in an ice batch followed by the conventional S-L extraction.

A statistical surface response design was performed using Statgraphics® Centurion XVII software in order to obtain the optimum conditions which maximize the final AC content of the extracts. A central potential composite design 2^3 + stars (CCD), which establish new extremes for the low and high settings for all factors, was

applied. To check the reproducibility, a triplicate of the central point was done. Three variables with three levels of response (-1, 0, 1) were employed. These three levels correspond to the minimum (-1), medium (0) and maximum (1) values of each variable. A total of 17 experiments were obtained.

Responses obtained from the statistical analysis were fitted to a second degree model (Equation 1) that took into account, not only individual interactions, but also quadratic relations between the variables:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \qquad (1)$$

where Y corresponds to the response variable (AC content in this study), β_0, β_j, β_{jj} and β_{ij} are regression coefficients; X stands for each operating variable. The statistical evaluation was performed by analysis of variance (ANOVA) in order to identify which factors contribute the most to the response. Effects with a p-value< 0.05 are statistically significant with a level of confidence of 95%.

2.3.1.3 Sonication pre-treatments

Preliminary experiments were performed with a BRASON (101-147-035) Sonifier®Cell Disruptor Model 450 with a high gain horn of ¾" of diameter. Time of sonication and amplitude were varied in a first attempt between 30s and 90s and between 10 and 100% that correspond to an amplitude value of the sound wave of 19 and 130 μm, respectively. After sonication, samples were submitted to S-L extractions at the best conditions previously studied and the kinetic curves were built by gathering samples along time.

2.3.2 Extract Characterization

2.3.2.1 Total Phenolic Content (TPC)

The total phenolic content was determined by Folin-Ciocalteou method which involves the reduction of the Folin-Ciocalteu reagent to produce a bluish mixture of metal oxides which intensity is proportional to the phenolic content. Protocol was followed as described elsewhere (Waterhouse, Waterhouse, & L., 2003) by putting in contact the sample with the Folin-Ciocalteou reagent and the Na_2CO_3. Absorbance of each sample was measured at 765nm against the blank in a UV 2550 Shimadzu spectrophotometer. TPC values were expressed as milligrams of gallic acid equivalents per gram of dry lees (mg_{GAE}/g_{DL}) and milligrams of gallic acid equivalents per gram of dry extract (mg_{GAE}/g_{DE}).

2.3.2.2 Anthocyanin Content

Monomeric anthocyanin pigments content was evaluated following the AOAC official method 2005.02. This pH differential method is based in the change of color of AC with pH: at pH 1.0 colored oxonium ions are formed, whereas at pH 4.5 predominates the colorless hemiketal form. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Briefly, each sample was properly diluted in pH 1.0 buffer (potassium chloride, 0.025M) and pH 4.5 buffer (sodium acetate, 0.4M) and absorbance was determined at both 520 and 700 nm (Tecan Spark 10M).

$$C_A = \frac{[(A_{520}-A_{700})_{pH1}-(A_{520}-A_{700})]\cdot M_W \cdot DF}{\varepsilon \cdot l} \cdot \frac{1}{R_{S-L}} \qquad (2)$$

where 'C_A' is the anthocyanin content expressed in mg_{MLVE}/g_{DL}; 'A' the absorbance measurements; 'M_w' the molecular weight of malvidin (493.4 $g \cdot mol^{-1}$); 'DF' is the dilution factor; 'ε' represents the molar extinction coefficient (28,000$L \cdot mol^{-1} \cdot cm^{-1}$); 'l' is the path length in cm and 'R_{S-L}' is the solid-liquid ratio ($g \cdot mL^{-1}$) used in the extraction. Anthocyanin (AC) concentration was expressed as milligrams of

malvidin-3-o-glucoside equivalents per gram of dry dry lees (mg_{GAE}/g_{DL}) and milligrams of malvidin-3-o-glucoside equivalents per gram of dry extract (mg_{GAE}/g_{DE}).

2.3.2.3 Antioxidant Activity: ORAC

Oxygen Radical Absorbance Capacity (ORAC) is a method for the evaluation of antioxidant ability of a specific substance based on the fluorescence quenching of disodium fluorescein (FS) salt after exposure to AAPH (2,2-azobis(2-amidino-propane) dihydrochloride), which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate (Garrett et al., 2014). ORAC assay was carried out by the method developed by *Huang et al*. (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002) and modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by *Feliciano et al.* (Feliciano et al., 2009). ORAC results were given in μmol of Trolox equivalents (TE) per gram of dry lees ($\mu mol_{TE}/g_{DL}$) and μmol of TE per gram of dry extract ($\mu mol_{TE}/g_{DE}$) as mean of three replicates.

2.3.2.4 Results basis: yield and richness

AC extraction yield was expressed in terms of milligrams of malvidin equivalents per gram of dry lees (mg_{MLVE}/g_{DL}) in order to maximize AC extraction of from dry WL. In addition, extracts were characterized in terms of richness to have an idea of the purity of the extracts regarding AC. Richness was expresses in milligrams of malvidin equivalents per gram of dry extract (mg_{MLVE}/g_{DE}). ORAC and TPC values were also expressed either in mg_{MLVE}/g_{DL} or mg_{MLVE}/g_{DE}.

2.3.2.5 Solid residue

Sample extracts were evaporated until dryness using a vacuum centrifuge (Centrivap concentrator, Labconco, Kansas City, MO, USA) with a MD 4C NT vacuum pump

(Vacuubrand, Wertheim, Germany) for result expressions per gram of dry extract (g_{DE}).

2.3.2.6 HPLC-DAD-MS/MS (High Performance Liquid chromatography–mass spectrometry)

Main compounds in the WL extracts were identified by LC-MS/MS with a method previously reported (Romero-Díez et al., 2018). The system used was a liquid chromatography Waters Alliance 2695 Separation Module (Waters®, Ireland). The mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Ireland). Chromatographic separation of compounds was carried out in a reversed phase LiChrospher® 100 RP-18 5µm LiChroCART® (250 x 4.0mm) column inside a thermostated oven at 35ºC. A binary mobile phase was used: eluent A consisted of solution formic acid (0.5% v/v) and eluent B was acetonitrile. It was used at a constant flowrate of 0.3 mL/min with the following gradient program: 99:1 A:B for 5 min, from 99:1 A:B to 40:60 A:B in 40 min, from 40:60 A:B to 10:90 A:B in 45 min, held isocratically (90% B) for 10 min, from 10:90 A:B to 99:1 A:B in 10 min, and finally held isocratically (99:1 A:B) for 10 min. The sample injection volume was 20 µL. Absorption spectra were acquired from 210 to 600 nm by a photodiode array detector. AC were monitored at 520 nm, flavonols at 360 nm, phenolic acids at 320 nm, and phenolic compounds in general at 280 nm. Mass spectrometry was performed using an electrospray ion source in negative and positive ion mode (ESI- and ESI+). The ion source temperature was 120°C, the capillary voltage was 2.5 kV, and the source voltage was 30 V. Compounds separated by HPLC were ionized and the mass spectra were recorded in a full scan mode, between m/z 100 and 1500. High purity nitrogen was used as drying and nebulizing gas, and ultrahigh purity argon was used as collision gas. Different collision energy values were used in

fragmentation experiments. For the data acquisition and processing MassLynx® 4.1 software was employed.

2.3.3 Statistical Analysis

All data were expressed as means ± standard deviations (SD). Assays for TPC, AC content and ORAC measurements were performed, at least, in triplicate. A statistical analysis was done using SigmaStat 3.0® software. When homogeneous variances were confirmed, data were analyzed by One Way Analysis of Variance (ANOVA) coupled with the post-hoc Holm–Sidak test ($p<0.05$ was accepted as statistically significant in all cases).

3. Results and Discussion

3.1 Best extraction conditions for AC

3.1.1 Conventional S-L extractions. Extraction kinetics of AC

The selection of the best conditions that influence AC extraction was firstly carried out for Port WL, and later applied for 1F and 2F *Ribera del Duero* WL. Extractions were performed during 90 minutes, but after 15 minutes a steady AC concentration was achieved (Figure 1.A).

Firstly, the effect of R_{S-L} (0.100, 0.050, 0.033 and 0.025 g/mL) was studied. The rest of parameters were kept constant: ethanol was used and a temperature was set at 25ºC. Results revealed that, AC extraction yield slightly increased as R_{S-L} decreased (Figure 1.A). A R_{S-L} of 0.100 yielded 0.61 ± 0.04 mg_{MLVE}/g_{DL}. When R_{S-L} decreased to 0.050 and 0.033, AC content increased to 0.96 ± 0.01 and 0.94 ± 0.03 $mg_{MLVE}/g_{DL,}$ respectively. However no significant differences were found between them. For the case of a R_{S-L} of 0.025, a minor increase in the final AC concentration was observed (1.05 ± 0.10 mg_{MLVE}/g_{DL}). However, this AC extraction yield increase

implied the use of four times more of solvent, which clearly involves economic and environmental issues (Drosou et al., 2015). Thus, it was decided to fix the R_{S-L} in 0.100. Additionally, this ratio has been also used by other authors for recovering of polyphenols from WL with conventional extraction (Pérez-Serradilla & Luque de Castro, 2011) .

Once the R_{S-L} was selected, four different hydro-alcoholic mixtures were studied. The content of ethanol 100%, 75%, 50% and 25% (%vol. ethanol) was varied. In this case parameters which were kept constant were the R_{S-L} (0.100) and temperature (25ºC). As shown in Figure 1.B, AC extraction was significantly enhanced as the amount of ethanol increased from 25% to 75% in the mixture (0.79 ± 0.01 to 3.04 ± 0.38 mg_{MLVE}/g_{DL}, respectively), as it was expected due to the decrease in polarity and dielectric constant values of the solvent mixture that, generally, increases the solubility of polyphenols in hydroalcoholic mixtures as the % ethanol increases (Cacace & Mazza, 2003b; Dimou et al., 2016). However, the use of 100% ethanol did not improve AC extraction (0.51 ± 0.04 mg_{MLVE}/g_{DL}) and the difference between using a 50% (2.78 ± 0.18 mg_{MLVE}/g_{DL}) or a 75% (3.04 ± 0.38 mg_{MLVE}/g_{DL}) aqueous ethanol mixture is not significant. This is mainly due to the fact that at acidic pH, AC remain as ionic molecules (flavilium cation form, AH^+) and maximum AC extraction yield is achieved at approximately 50% ethanol (Cacace & Mazza, 2003b). Therefore, the hydroalcoholic 50% vol. ethanol mixture was selected, also from an economical point of view, since it requires a lower amount of organic solvent.

At the end, the influence of the temperature on the AC yield was investigated. In this context, three temperatures were tested (25, 35 and 45ºC) maintaining, the other parameters constant (50% vol. ethanol mixture, R_{S-L} of 0.100). After 15 minutes of extraction, an AC extraction yield of 2.78 ± 0.18 mg_{MLVE}/g_{DL}, 3.12 ± 0.27 mg_{MLVE}/g

$_{DL}$ and 3.00 ± 0.24 mg_{MLVE}/g_{DL} were achieved for 25, 35 and 45ºC, respectively. Within the studied range, higher temperature led to a slight increase of AC extraction rate, (Figure 1.C). Although it was expected that temperature increases the AC content by increasing the extraction coefficient (Pinelo, Fabbro, Manzocco, Nuñez, & Nicoli, 2005), no significant differences were observed in terms of AC extraction yield. So, to reduce the use of resources and energy, temperature was fixed in 25ºC. Though the use of 45ºC reduces slightly the extraction time (Figure 1.C), it has been demonstrated that low temperatures contribute to prevent anthocyanin degradation, since AC stability is compromised even at 45ºC along time (Cacace & Mazza, 2003a; Sólyom, Solá, Cocero, & Mato, 2014). AC extraction values for the study of each variable are shown in Table S.1 of the *Supplementary Material*.

As a conclusion of the influence of each parameter, best conditions for AC extraction were a R_{S-L} 0.100, a mixture with 50%vol. ethanol and a temperature of 25ºC. Under these conditions, a final AC extraction yield of 2.78 ± 0.18 mg_{MLVE}/g_{DL} was obtained for Port WL. These conditions were also applied to the *Ribera del Duero* WL. Final AC extraction yield of 3.04 ± 0.03 mg_{MLVE}/g_{DL} was obtained for 1F WL, while for 2F WL, lower AC content was achieved, 2.09 ± 0.38 mg_{MLVE}/g_{DL}.

Although there is few available literature regarding AC extraction from WL, the AC recovery from the different WL reached in this work are in accordance to those found in literature. *Tao et al*. (Tao et al., 2014) recovered 5.55 ± 0.19 mg_{MLVE}/g_{DL} of AC from light lees via maceration in an aqueous ethanol solution (51% vol. EtOH) at 60ºC during 36 minutes using a low R_{S-L} (0.0167).

In addition, it has been shown that WL represent a richer source of AC compared to other vinification residues. For example, *Álvarez et al.* (Álvarez et al., 2017) studied the extraction kinetics of AC extraction from grape pomace from the same variety of

grapes and provided by the same winery (Bodegas Matarromera) in year 2014. They performed several S-L extractions, and AC content was also measured along time. For their best conditions, only 1.20 mg_{MLVE}/g_{DL} were extracted and more than 60 minutes were required to attain a steady AC concentration. This can be due to the much lower particle size of WL, which reduces internal mass transfer limitation and, therefore, AC become more accessible.

3.1.2 Microwave pre-treatment

Table 1 collects all MW pre-treatments performed from the statistical analysis, AC concentration just after the pre-treatment and the temperature achieved in each experiment. As the objective was to optimize the MW pre-treatment, AC concentrations were measured just after the pre-treatment for Port WL and, later, applied for the rest of WL. At first glance, it can be observed that AC extraction yield varied a lot depending on the pre-treatment parameters, namely the H_2O percentage (v/v). AC concentration in the extract after pre-treatment was really low (experiment 1 as example) when only water was used. However, higher AC extraction yields were achieved when more ethanol was employed. For example in the experiment 15, AC extraction yield was twice higher (~6 mg_{MLVE}/g_{DL}) than compared with those obtained in the conventional S-L extraction (~3 mg_{MLVE}/g_{DL}). Figure 2 shows the main effect diagram for each variable from the statistical study. There were values for the type of solvent and S-L ratios that maximize the AC extraction yield, which correspond to the optimum point. A different behaviour was observed for the time: the greater the time, the higher the extraction yield of AC. However, if time was increased, temperature would increase during the pre-treatment and the degradation of AC would take place. It is known that AC degrade at temperatures above 100ºC during exposure times of 5-10 minutes (Sólyom et al.,

2014). For this reason, no experiments at higher temperature were proposed. In this work, the highest temperature achieved was 117ºC, but only during a short period of time (90s) avoiding AC degradation. Thus, the optimal values for each parameter were: a hydro-alcoholic mixture of 40% vol. ethanol, a $R_{S\text{-}L}$ of 0.140 (g/mL) and a time pre-treatment of 90s.

From the analysis of variance (Table S.2 in *Supplementary Material*), it could be seen that the percentage of water was the parameter which influences the most in anthocyanin extraction. Furthermore, the pre-treatment time and the interaction between the water percentage and time were also crucial for the extraction.

The regression coefficients of second-order polynomial equation (*Equation 1*) were obtained by fitting experimental results and extraction variables. The final expression for the *Equation 1* is shown below. Some parameters were negligible assuming the p-values from the ANOVA table (Table S.2 in *Supplementary Material*).

$$AC = 5.0166 + 0.624926 \cdot t - 0.00311123 \cdot R_{SL} - 1.74735 \cdot \%H_2O + 0.818187 \cdot t^2 - 0.0485444 \cdot t \cdot R_{SL} - 0.448549 \cdot t \cdot \%H_2O - 0.980728 * R_{SL}^2 + 0.060261 \cdot R_{SL} \cdot \%H_2O - 3.07077 * \%H_2O^2$$

'*AC*' corresponds to the anthocyanin extraction yield, '*t*' is the time of pre-treatment in seconds, '$R_{S\text{-}L}$' is the solid-liquid ratio in g/mL and '$\%H_2O$' is the %vol. of water of the hydroalcoholic mixture.

With the optimal pre-treatment conditions, a MW pre-treatment was performed and, in this case, followed by S-L extraction at the best studied conditions (exact amount of solvent was added in order to obtain a $R_{S\text{-}L}$ of 0.100 and hydro-alcoholic mixture of 50% vol. ethanol) for each type of WL during 30 minutes. AC extraction yields for the optimal conditions obtained for MW pre-treatments after 15 minutes are shown in Table 2. Port WL showed the highest AC content (6.20 ± 0.36 mg_{MLVE}/g_{DL}),

followed by first fermentation lees (4.45 ± 0.30 mg_{MLVE}/g_{DL}) and second fermentation lees (2.88 ± 0.22 mg_{MLVE}/g_{DL}) when MW are applied. The use of MW pre-treatment provided an increment in the AC extraction yield of 2.7, 1.5 and 1.4 times compared with the S-L extraction from Port, 1F and 2F WL, respectively (*Supplementary material)*. The effect of MW was also confirmed by the reached temperature in the optimum conditions (115ºC). High temperatures made possible the breakage of cell walls of the yeast of WL, leading to an improvement on the anthocyanin yield extraction since AC linked to cell walls became more accessible and the internal mass transfer is enhanced (Pérez-Serradilla & de Castro, 2008). Furthermore, the implementation of MW reduces considerably the required extraction time from 15 min to 90s, as the maximum AC extraction yield was achieved just after the pretreatment (*Figure S.2. Supplementary Material*).

No comparison about AC extraction could be done due to the absence of information in literature since previous works measured the TPC instead of AC content when MW are used to recover polyphenols from WL. However, some authors (Pérez-Serradilla & Luque de Castro, 2011) proved the efficiency of MW to enhance polyphenols extraction from WL in a 10% compared to the yields obtained with a Soxhlet extraction. Furthermore, AC yields from WL were higher if comparing to those achieved from grape pomace by *Álvarez et al*. (Álvarez et al., 2017), who achieved AC concentration values up to 1.75 $mg_{MLVE}/g_{\ DRY\ POMACE}$ applying their optimized parameters for MW pre-treatments.

3.1.3 Sonication pre-treatments

First sonication experiments were performed with the lowest (30s and 10% amplitude) and the highest (90s and 100% amplitude) conditions, followed by a conventional S-L extraction for Port WL. No significant differences were observed

in the final AC concentration between the use of ultrasounds (3.02 ± 0.13 mg_{MLVE}/g_{DL} and 3.17 ± 0.08 mg_{MLVE}/g_{DL}) and the conventional S-L extraction (2.78 ± 0.18 mg_{MLVE}/g_{DL}). It was thought that maybe the time of sonication pre-treatment was very short, so a trial with a longer time of processing was carried out. The conditions of 5 minutes and amplitude of 55% were defined for the ultrasounds assisted extraction (USAE) of polyphenols based on a previous work for a different raw material (Rodríguez-Rojo et al., 2012). These conditions were applied to Porto WL and an AC extraction yield of 2.94 ± 0.10 mg_{MLVE}/g_{DL} was achieved. From these results it could be concluded that the AC extraction yield was not enhanced with the use of US. US enhanced the external mass transfer and not the internal mass transfer, which is the limiting step for the AC extraction from WL, so similar yields were achieved. However, US produced a reduction of the required time to achieve a steady AC extraction yield from 15min to 5min as can be seen *in Figure S.2 in Supplementary material.*

3.2 Extracts characterization: yield and richness

3.2.1 Total Phenolic Content (TPC)

TPC quantification was performed only for those extracts obtained at best conditions for the S-L extractions and the optimized MW pre-treatments. Results for the TPC are shown in Table 2. Extracts obtained when MW were used as pre-treatment showed higher yields, being the Port WL extract the one with the highest and significant TPC yield (42.04 ± 0.22 mg_{GAE}/g_{DL}). For the conventional S-L extractions, similar TPC yields were achieved for each type of WL, ranking from 23.42 ± 0.11 to 27.70 ± 0.18 mg_{GAE}/g_{DL}. Nevertheless, these TPC recoveries changed a lot if they were expressed in terms of richness: milligrams of gallic acid equivalents per gram of dry extract (mg_{GAE}/g_{DE}). The richest extract in terms of TPC

were *Ribera del Duero* WL extracts, either for a conventional S-L extraction or with MW pre-treatment. TPC richness for conventional extraction ranging from nearly 196 to 232 mg_{GAE}/g_{DE}. Port WL appeared to be the poorest extract with only 68 ± 7 $mg_{GAE}/g_{DE.}$ Same tendency was observed when MW were applied: 1F WL were the richest extract (294 ± 13 mg_{GAE}/g_{DE}) followed by 2F WL extract (268 ± 11 mg_{GAE}/g_{DE}). The explanation of these differences can be related to the vinification process. Port WL presented a higher sugar concentration because first fermentation is stopped by adding extra ethanol and all sugars were not processed (Perestrelo et al., 2016). These sugars remained linked to WL and were also extracted together with AC reducing the richness of the extract in terms of TPC and AC. Sugars and their degradation compounds concentrations in WL extracts after 15 minutes of extraction are shown in Table S.3 of the *Supplementary material.*

Although TPC values in this work were lower than those achieved when a MAE was applied to 1F WL of syrah grapes (532 mg_{GAE}/g_{DL}), mainly due the use of different grape varieties (Pérez-Serradilla & Luque de Castro, 2011), WL can be considered as a suitable source of polyphenols if compared with the results from others grape residues. *Casazza et al*. (Casazza, Aliakbarian, Mantegna, Cravotto, & Perego, 2010) extracted polyphenols from differences types of *Vitis Vinifera* wastes, in particular grape seeds and skins, using non-conventional techniques such as HPTE (high pressure and temperature extraction), UAE (ultrasound-assisted extraction) and MAE (microwave-assisted extraction). The TPC in grape seeds was far higher (110 to 60 $mg_{GAE}/g_{DRY\ MATTER}$) than in grape skin (20 to 35 $mg_{GAE}/g_{DRY\ MATTER}$) for every type of extraction. Similar TPC values were achieved in literature from grape pomace (261.5 ± 2.5 mg_{GAE}/g_{DE}) (Álvarez et al., 2017) when MW were used as pre-treatment.

3.2.2 Antioxidant Activity

ORAC assay was performed to evaluate the antioxidant ability of the extracts, obtained with or without pre-treatments, as peroxyl radical scavengers. Table 2 shows ORAC values in μmol_{TE} /g_{DL} and μmol_{TE} /g_{DE} for conventional S-L extracts and MW pre-treatments after 15 minutes of extraction. A direct relation between the AC concentrations and the ORAC values was found: the larger the concentration of AC, the greater the ORAC value. When pre-treatments were used, TPC and AC content increased and also ORAC values enhanced proportionally. For example, AC richness from Port WL increased when MW pre-treatment was applied in 2.2 times (6.2 ± 0.4 mg_{GAE}/g_{DL}) compared with the S-L extraction (2.78 ± 0.18 mg_{GAE}/g_{DL}) and the ORAC values did also improved: 2.1 times (1040 ± 107 $\mu mol_{TE}/g_{DE}$) with MW than without them (453 ± 45 $\mu mol_{TE}/g_{DE}$). 1F WL showed the highest ORAC values for both yield and richness as it was expected due to it was the richest extract in terms of TPC and AC. Moreover, the highest increment in ORAC activity between S-L extraction (3201 ± 347) and when MW were applied (4952 ± 480 $\mu mol_{TE}/g_{DE}$) was found for 1F WL extract. Port WL presented almost five times lower antioxidant capacitiy, with ORAC values up to 1040 ± 107 $\mu mol_{TE}/g_{DE}$, when MW pre-treatment was applied. These ORAC values were smaller than those obtained by *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro, 2011), who achieved similar ORAC values when a conventional extraction was performed and a MAE was applied to 1F WL from *Syrah* grape variety, 6100 and 6250 $\mu mol_{TE}/g_{DE}$, respectively, due to the higher content on TPC as previously mentioned. In contrast, if ORAC values obtained from WL were compared with those obtained from grape pomace extracts, it could be said that WL extracts presented higher antioxidant

activities. *Álvarez et al.* (Álvarez et al., 2017) reported ORAC values between 1200-2750 µmol $_{TE}/g_{DE}$ for different MW pre-treatments.

3.2.3 HPLC analysis

HPLC analyses were performed in order to determine the main compounds present in WL extracts after 15 minutes of extraction. Figure S.3 (of the *Supplementary Material*) shows, as an example, the chromatographic profile at 280 nm of a conventional extraction (A) and after the MW pre-treatment (B) from Port WL. From this figure it is possible to corroborate the effect of the MW pre-treatment on the amount of extracted polyphenols.

As this work was focused on the maximization of the extraction of AC, an exhaustive study for their determination was performed. Same AC were found in extracts from 1F and 2F WL, meanwhile for Port WL different compounds appeared. These discrepancies can be seen in Figure 3 where a comparison between chromatograms at 520 nm of the WL 1F extract (A) and the Port WL extract (B) after the MW pre-treatment is displayed.

For the determination of the anthocyanins present in the extracts, the LC-MS/MS was used for the qualitative determination of the main compounds. Putative identification of AC was also tested with other studies already reported in literature (Cantos, Espín, & Tomás-Barberán, 2002; Delgado de la Torre, Priego-Capote, & Luque de Castro, 2015; Sanz et al., 2012; Schwarz, Quast, von Baer, & Winterhalter, 2003; Vallverdú-Queralt et al., 2015; Wu & Prior, 2005) with comparable matrices and databanks ("Database on Polyphenol Content in Foods - Phenol-Explorer," n.d.; "PhytoHub," n.d.).

A total of twelve anthocyanins were identify as Table 3 shows. The respective m/z values, the fragmentations, the putative identification, the phenolic subclass and the

extract in which each anthocyanin appeared is also shown in Table 3. The most interesting finding in this study was the presence of a pyranoanthocyanin, Vitisin A, in both types of extracts at a retention time of 32.13 min. These A-type vitisins are adducts resulting from the cycloaddition of pyruvic acid, a metabolite of the alcoholic fermentation (Marquez, Serratosa, & Merida, 2013) to anthocyanin molecules, usually formed during the maturation of wine. Until now, these types of pyranoanthocyanins had been only identified in the dregs of an old Port wine bottle (Marquez et al., 2013; Oliveira et al., 2010) and in aged red Chilean wine (Schwarz et al., 2003). Additionally, another anthocyanin, petunidin-3-O-glucoside, appeared to be co-eluted at the same retention time (32.13 min) in both Port and Ribera del Duero extracts. However, in Port wine lees, Vitisin A was present in higher amounts than petunidin-3-O-glucoside, whereas in Ribera del Duero wine lees the opposite was observed. This tendency can be seen in Figure S.4 in the *Supplementary Material*, where the signal for the Vitisin A (m/z 561) is much more pronounced than the one for the petunidin-3-O-glucoside (m/z 479), which suggests that Vitisin A is present in higher amounts than petunidin-3-O-glucoside in Port wine lees. In the same way, for Ribera del Duero extracts, the opposite was observed from Figure S.5. Nonetheless, to the best of our knowledge, these types of pyranoanthocyanins have never been identified neither in 1F nor 2F Ribera del Duero extracts. Thus, with the help of the novel extraction technique that has been introduced in this study, it was possible to extract Vitisin-A from Ribera del Duero wine lees.

Apart from these AC, there were anthocyanins identified only in one type of extract, which may be directly correlated to the vinification process. That was the case of compounds such as delphinidin 3-O-(6"-p-acetylglucoside), cyanidin 3-O-(6"-p-acetylglucoside) and delphinidin 3-O-(6"-p-coumaroyl-glucoside) which only

appeared in *Ribera del Duero* WL extracts. Additionally, a derivate from malvidin (malvidin-3-O-6”-acetyl-glucoside) was found only in Porto WL extracts.

Although, the study here presented was focused on the optimization of AC extraction and their identification, chromatograms of major polyphenol families present in the extracts such as flavonoids or hydroxycinnamic acids are displayed in *Supplementary material* in Figure S.6, Figure S.7 and Figure S.8 at 280 nm, 320 nm and 360 nm, respectively. The putative identification of those compounds with higher concentrations is shown in Table S.4 in the *Supplementary material.*

Some of the components were also identified in aging wine lees and correlated with the antioxidant activity of the extract (Romero-Díez et al., 2018) such as myricetin (flavonol) and several antocyanins (3-O-glucoside of delphinidin, petunidin and malvidin, and 3-O-(6"-p-coumaroyl-glucoside) of delphinidin, petunidin and malvidin).

4. Conclusions

WL have been pointed out as an important source of phenolic compounds, namely anthocyanins. AC extraction kinetics from WL were studied. Parameters selected as the best for AC recovery in S-L extractions were: $R_{S\text{-}L}$ of 0.100 g/mL, a hydro-alcoholic mixture with 50% EtOH (% vol.) and at 25ºC. Furthermore, two different pre-treatments have been tested for AC recovery from WL. On the one hand, the use of MW pre-treatment enhanced internal mass transfer, increasing AC extraction yield and reducing the processing time, as well. On the other hand, US only influenced the processing time having no effect on the AC extraction yield. Different origin WL were processed, being red WL (*Ribera del Duero*) those that presented richer extracts and higher antioxidant activity respect to Port WL. Main compounds were identified finding distinctive anthocyanins for both types of lees. Furthermore, a

pyranoanthocyanin, Vitisin A, was identified in both types of lees being predominantly present in Port WL.

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5. Literature

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Nomenclature

Abbreviations

1F: first fermentation

2F: second fermentation

AA: antioxidant activity

AAPH: 2, 2-azobis(2-amidino-propane) dihydrochloride

AC: anthocyanins

CCD: central composite design

CP: central point

DAD: diode array detection

DE: dry extract

DL: dry lees

FS: disodium fluorescein

GAE: gallic acid equivalents

GRAS: general recognize as safe

HPLC: high performance liquid chromatography

HPTE: high pressure and temperature extraction

MAE: microwave assisted extraction

MLVE: malvidin-3-o-glucoside equivalents

MS/MS: mass spectrometer

MW: microwave

OP: optimum point

ORAC: oxygen radical absorbance capacity

SD: standard deviation

S-L: solid-liquid

TE: trolox equivalents

TPC: total phenolic compounds

UAE: ultrasound-assisted extraction

US: ultrasounds

USAE: ultrasound assisted extraction

Greek letters

Y: response variable (anthocyanin concentration, mg_{MLVE}/g_{DL})

$\boldsymbol{\beta_0}$***:*** independent coefficient

$\boldsymbol{\beta_j, \beta_{jj}, \beta_{ij}}$ ***:*** interaction coefficients for variables "i" and "j"

X: stands for each operating variable

Symbols

A: absorbance, nm

DF: dilution factor

l: path length, cm

$\mathbf{M_w}$**:** molecular weight of malvidine, m/mol

$\mathbf{ROO^{\bullet}}$**:** oxygen radicals

$\mathbf{R_{S\text{-}L}}$**:** solid-liquid ratio, g/mL