OPTIMIZING THE EXTRACTION PROCESS OF NATURAL ANTIOXIDANTS FROM CHARDONNAY GRAPE MARC USING MICROWAVE-ASSISTED EXTRACTION

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ABSTRACT

The aim of this work was to extract phenolic compounds from Chardonnay grape marc employing a microwave-assisted extraction (MAE). Firstly, the effect of solvent concentration (30-60%), solid mass (1.0-2.0 g) and extraction time (5-15 min) on the recovery of phenolic content and antioxidant capacity was evaluated using a response surface methodology (RSM). The optimal parameters found by RSM were 48% ethanol for the solvent content, 10 min for the extraction time, and 1.77 g for the solid mass. The extraction was carried out at room temperature to increase scaling-up opportunities at industrial level. It was found that the phenolic profile was mainly composed of flavanols, such as procyanidins, catechin and epicatechin. Furthermore, the polyphenols obtained by MAE showed a DPPH· inhibition value of 87 ± 5 % and the total phenolic content was 1.21 ± 0.04 mg GAE/mL. Finally, it was observed that the degradation temperature of the extract (≈ 200 ºC) was above the temperature commonly used for the manufacture of protein films by thermo-mechanical processes. This highlights the potential use of this extract as a bioactive additive in protein film forming formulations for food and pharmaceutical applications.

Keywords: Grape marc; valorisation; microwave-assisted technology; efficient extraction; phenolic compounds; antioxidant capacity.

1. INTRODUCTION

The wine industry involves the production of a large amount of residues that must be discarded, with some being destined to the distilleries to produce spirits such as grappa (Pasqualone et al., 2014), or used in the production of tartaric acid (Salgado et al., 2010) or compost (Santos et al., 2016). Additionally, it is estimated that 3% of grape marc is used as animal feed (Brenes et al., 2016). However, this waste is characterized by high contents of biodegradable compounds and solid by-products, consisting mainly on grape stalks, wine lees and grape marc (Spigno et al., 2017). These are known to contain a high level of polyphenols that possess antioxidant and radical scavenging activities, with potential health benefits (Bustamante et al., 2008; Granda and de Pascual-Teresa, 2018; Rasines-Perea and Teissedre, 2017). In the food industry, they have been used to enrich vegetal oils in order to improve their oxidative stability (Lafka et al., 2007) or cereal and dairy products (García-Lomillo and González-San José, 2017).

Phenolic compounds vary depending on the type of grape, climatic factors, winemaking techniques and soil type, among others (Bonfante et al., 2017; Di Lorenzo et al., 2016), and they can be classified in two main groups based on their carbon skeleton: flavonoids and non-flavonoids. The former group includes anthocyanidins (malvidin, delphinidin, petunidin, peonidin, and cyanidin), flavonols (quercetin, myricetin, and kaempferol), flavan-3-ols (catechin, epicatechin, epicathecin 3-gallate, and gallocatechin – both as monomers and within larger tannin structures), flavones (luteolin, apigenin), and flavanones (naringenin); and the latter group includes hydroxycinnamic acids (caffeic, p-coumaric, and ferulic acids), benzoic acids (gallic, vanillic, and syringic acids), and stilbenes (resveratrol) (Granato et al., 2011).

In recent years, efficient techniques for the extraction of these bioactive compounds have been assessed. Traditionally, solid-liquid extraction by mechanical agitation and Soxhlet extraction have been employed for polyphenols recovery from grape waste (Bucić-Kojić et al., 2013; Casazza et al., 2012). Nevertheless, the high temperatures used, along with the long times and hazardous solvents, can cause hydrolysis and oxidation of the targeted compounds as well as a high environmental impact (Lončarić et al., 2018). Therefore, other techniques have been evaluated for the extraction of bioactive compounds. For instance, Nayak et al. (2018) employed ultrasound to extract polyphenols from Cabernet grape pomace and Da Porto et al. (2017) considered the supercritical fluid extraction to recover polyphenols from grape seeds.

Microwave-assisted extraction (MAE) has been another technique employed for the extraction of a wide variety of bioactive compounds, and one of its main advantages is a significant reduction of the extraction times (Esquivel-Hernández et al., 2017; Zhang et al., 2008). Furthermore, Nayak et al. (2015) showed that the use of MAE for the extraction of polyphenols from the peel of *Citrus sinensis* was more effective in terms of total phenolic content and antioxidant activity in comparison with conventional, ultrasound-assisted, and accelerated solvent extractions. MAE has also been employed for polyphenols extraction from wine waste; in this regard, Alvarez et al. (2017) employed MAE as a pre-treatment, improving by 57% the efficiency of polyphenols extraction from grape marc; Moreira et al. (2018) explored this technique to extract natural antioxidants from vine shoots, obtaining higher yields than those achieved by the conventional extraction.

When MAE is employed, multiple parameters can affect the extraction process and they should be taken into consideration due to their individual or combined effects on the yield of the extract and its composition. Therefore, the choice of solvent, solvent to solid ratio, power applied and extraction temperatures and times are key factors to maximize the extraction process (Vajic et al., 2015). In order to optimize complex experimental processes that consider many factors, response surface methodology (RSM) is a relevant multivariate technique, which allows a lower number of trials to be undertaken and an efficient interpretation of the optimization (Bezerra et al., 2008).

Taking all the above into consideration, the aim of this study was to analyze the feasibility of employing MAE at room temperature as an efficient technique to recover polyphenols from Chardonnay grape marc. For that purpose, firstly, the optimization of the extraction was carried out using RSM and secondly, the characterization of the extract was accomplished.

2. MATERIALS AND METHODS

2.1 Materials and reagents

Winery grape marc from the Chardonnay variety was obtained from the Marlborough region, New Zealand, during the 2015 vintage. The grape marc was composed of seeds, grape skin and stems and was maintained at -18 ºC prior to utilization. Ethanol and water were employed as solvents for the extraction. Other reagents, such as gallic acid, Folin-Ciocalteu reagent, 2, 2-diphenyl-1–picryhydrazyl (DPPH·), and enkephalin hydrated leucine acetate (95% purity), were purchased from Sigma Aldrich; sodium carbonate, copper sulphate, sodium hydroxide, sodium thiosulphate, and potassium iodide were purchased from ECP; sulphuric acid and

Rochelle salt were purchased from JT Baker; acetic acid was provided by Merck; methanol, acetonitrile and formic acid were purchased from Fisher Scientific.

2.2 Microwave-assisted extraction (MAE) procedure

Prior to the polyphenols extraction, grape marc was defrosted at room temperature and ground for 1 min using a blender (Model 219706, Kensington Food Processor, New Zealand) in order to obtain a fine powder and facilitate the extraction process (Muhamad et al., 2017). Phenolic compounds from grape marc were extracted in a single mode focused CEM reactor (Model Discover, CEM Co., Matthew, NC) operating at 2.45 GHz with the ability to control the output power. The temperature in the system was measured using a fibre optic temperature sensor (RTP-300, CEM Co., Matthew, NC), preventing interactions with the microwaves and their impact on the temperature reading (Gizdavic-Nikolaidis et al., 2010). An external cooling circuit maintained a constant temperature for the mixture and constant irradiation power. All the experiments were run under the same conditions regarding power (93 W), temperature (24 ± 1 °C), and solvent volume (10 mL). Ethanol at different concentrations was employed as a safe and efficient solvent for the extraction of phenolic compounds and the solvent was evaporated by centrifugation at 60 ºC (Dahmoune et al., 2015; Kerton and Marriot, 2013).

2.3 Methods

2.3.1 Determination of total phenolic content (TPC)

TPC was measured by the Folin-Ciocalteu method. In a 4 mL cuvette, 3.16 mL of deionised water was added to 20 µL of the extract. Then, 200 µL of Folin-Ciocalteu reagent was incorporated, mixed well and left to react for 3 min. After that time, 600 µL

of 20% sodium carbonate solution were added. The resulting solutions were shielded from light and left at room temperature for 90 min before the absorbance was measured at 765 nm. Gallic acid was employed to create a standard curve, thus the results were obtained as mg of gallic acid equivalents per mL of extract (mg GAE/mL extract).

2.3.2 Determination of antioxidant activity

The antioxidant activity of the extract was measured using the DPPH radical scavenging assay, as described by Liu et al. (2015) with some modifications. Firstly, a solution of DPPH· in ethanol at a concentration of 63.4 μmol/L was prepared. In a 4 mL cuvette, 3.9 mL of DPPH· solution and 0.1 mL of extract were added, mixed and protected from light at room temperature for 30 min. After that time, the absorbance of each sample was measured at 515 nm using a spectrophotometer (Shimadzu 1700 UV/Vis, China). The percentage of free radicals scavenged by DPPH· was calculated using the following equation:

$$\mathrm{DPPH}\cdot\ (\%\ \text{inhibition}) = \left(\left(A_{\mathrm{DPPH}\cdot} - A_{\mathrm{sample}}\right)/A_{\mathrm{DPPH}\cdot}\right) \times 100$$

where $A_{\mathrm{DPPH}\cdot}$ is the absorbance of the DPPH· solution at 515 nm, and A_{sample} is the absorbance of each extract at 515 nm.

2.3.3 Rebelein sugar method

Sugar content was determined using the Rebelein method (Zoecklein et al., 1995). Firstly, a 2 mg/mL solution was prepared by diluting the grape marc extract in water. Then, 10 mL of copper sulfate (0.168 M) in sulphuric acid (0.005 M), 10 mL of Rochelle salt (0.886 M) in sodium hydroxide (2 M) and 2 mL of the extract solution were combined into a 200 mL Erlenmeyer flask. The mixture was heated until steam was derived and maintained for 1.5 min, after which it was rapidly cooled in an ice bath.

Afterwards, a mixture of 10 mL of potassium iodide (1.81 M) in sodium hydroxide (0.1 M), 10 mL of 16% sulphuric acid, and 10 mL of a 1% starch solution in potassium iodide (0.120 M) in sodium hydroxide (0.01 M) was added to the solution prepared as abovementioned. The resulting solution was titrated using sodium thiosulphate (0.056 M) in sodium hydroxide (0.05 M) until a creamy white solution was obtained. The amount of sugars was calculated using the following equation:

$$\mathrm{RS}\left(\frac{\mathrm{mg}}{\mathrm{g}}\right) = \left(\left(28 - 28\left(\frac{V_S}{V_B}\right)\right) \times 1000\right)/\mathrm{S}$$

where V_s is the amount of titrant used for the sample in mL, V_B is the amount of titrant used for the blank in mL, S is the sample concentration in mg/mL.

2.3.4 High performance liquid chromatography (HPLC)

The determination of monosaccharides was carried out by HPLC with a Jasco LC Net II/ADC chromatograph equipped with a refractive index detector. The analysis was accomplished using a 300 × 7.8 mm CARBOSep CHO-628 LEAD column (Transgenomic Inc., Omaha, NE 68164, USA) operated at 80 ºC (mobile phase: deionised water eluted at 0.4 mL/min). The injection volume was 20 µL.

On the other hand, the determination of the phenolic compounds was carried out by Ultrahigh performance liquid chromatography (UHPLC) with an ACQUITY UPLC™ system from Waters (Milford, MA, USA), equipped with a binary solvent delivery pump, an autosampler, a column compartment and a PDA detector. A reverse phase column (Acquity UPLC BEH C18 1.7 µm, 2.1 mm × 100 mm) and a precolumn (Acquity UPLC BEH C18 1.7 µm VanGuard™) from Waters (Milford, USA) were used at 40 °C for the separation. The flow rate was 0.35 mL/min and the injection volume

was 2.0 μL. Mobile phases consisted of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). Separation was carried out under the following conditions: 0.00-1.6 min, 2% B; 1.6-2.11 min, 15% B; 2.11-8.88 min, 8% B; 8.88-9.80 min, linear gradient from 8 to 10% B; 9.80-17.00 min, 10% B; 17.00-22.00 min, linear gradient from 10 to 20% B; 22.00-23.40 min, linear gradient from 20 to 23% B; 23.40-54.20 min, linear gradient from 23 to 60% B; 54.20-55.20 min, linear gradient from 60 to 100% and finally, washing and re-equilibration of the column prior to the next injection. All samples were kept at 4 °C during the analysis. The wavelength range of the PDA detector was 210-500 nm (20 Hz, 1.2 nm resolution). Benzoic acids were recorded at 240 nm, flavanols at 280 nm, hydroxycinamic acids at 320 nm, and flavonols and dihidroflavonols at 370 nm.

All mass spectrometry (MS) data acquisitions were performed on a SYNAPT™ G2 HDMS with a quadrupole time of flight (Q-TOF) configuration (Waters, Milford, MA, USA), equipped with an electro-spray ionization (ESI) source operating in positive and negative mode. The capillary voltage was set to 1.0 kV for both ESI+ and ESI-. Nitrogen was used as the desolvation and cone gas at flow rates of 1000 L/h and 10 L/h, respectively. The source temperature was 120 °C, and the desolvation temperature was 400 °C. A leucine-enkephalin solution (2 ng/μL) in acetonitrile:water (50:50 (v/v) + 0.1% formic acid) was utilized for the lock mass correction and the ions at mass-to-charge ratio (m/z) 556.2771 and 278.1141 in the positive ionization mode from this solution were monitored (0.3 s scan time, 10 s interval, 3 average scans, ± 0.5 Da mass window, 30 V cone voltage, 10 μL/min flow rate). Data acquisition took place over the 50-1200 m/z mass range in resolution mode (FWHM ≈ 20,000) with a scan time of 0.1 s and an interscan delay of 0.024 s. All the acquired spectra were automatically corrected

during acquisition based on the lock mass. Before analysis, the mass spectrometer was calibrated with a sodium iodide solution.

To perform MS^E mode analysis, the cone voltage was set to 20 V and the first quadrupole (Q1) operated in a wide band RF mode only. Two discrete and independent interleaved acquisition functions were automatically created. The first function, typically set at 6 eV in trap cell of the T-Wave, collected low energy or unfragmented data, while the second function collected high energy or fragmented data, using 6 eV in trap cell and a collision ramp of 10-40 eV in transfer cell. In both cases, argon gas was used for collision-induced dissociation (CID) and data were recorded in centroid mode.

MS^2 product ion spectra was performed using the protonated molecule $[M+H]^+$ as precursor ion at a cone voltage of 20 V. A collision energy ramp from 10 to 40 eV in trap cell and of 6 eV in transfer cell was used with the aim of acquiring spectra with different fragmentation degrees from the precursor ion and, thus, obtaining as much structural information as possible. MS/MS data were collected at a range of 50-2000 m/z in centroid mode in the same conditions as described above.

The identification of the phenolic compounds was carried out using the UV-Vis spectrum to assign the phenolic class (Abad-García et al., 2009), the low collision energy MS^E spectrum in positive mode to determine the molecular weight, the high collision energy MS^E and MS^2 product ion spectra to assign the protonated aglycone $[Y0]^+$ and observed fragmentations in order to elucidate other structural details.

2.3.5 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were performed on a Nicolet Nexus spectrometer employing an ATR Golden Gate (Specac) accessory. Measurements were recorded in the 4000-800 cm^{-1} region using 32 scans at a resolution of 4 cm^{-1}.

2.3.6 Thermogravimetric analysis (TGA)

TGA was carried out using a Mettler Toledo SDTA 851 equipment. All specimens were scanned from room temperature to 800 ºC at a heating rate of 10 ºC/min. All TGA tests were carried out in a nitrogen environment to avoid thermo-oxidative reactions.

2.4 Response surface methodology

2.4.1 Experimental design for formulation optimization

With a view to maximizing the extraction of phenolic compounds, the effect of three independent factors on TPC and DPPH radical scavenging responses was studied under a RSM scheme. The three inputs were ethanol content, solid content, and time, coded at three levels: low (-1), medium (0) and high (+1). The selected values were based on a preliminary study: 30, 45 and 60% for the ethanol concentration, 1.0, 1.5 and 2.0 g for the solid mass, and 5, 10 and 15 min for the extraction time. A Box-Behnken design was applied due to its economy regarding the number of necessary experimental runs. The design variables selected in this study are shown in **Table 1** with actual and coded levels along with response variables. The corresponding Box-Behnken design comprises 12 runs, and 4 additional runs were included at the centre of the design to evaluate the pure error. All runs were made in random order. For each response variable, a full-quadratic polynomial model was created by multiple regression

technique in order to determine subsequently the optimal formulation that maximizes both responses:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum \sum_{i<j=2}^{n} b_{ij} x_i \, x_j$$

where b_0 was the constant coefficient or intercept, b_i were the first order linear coefficients, b_{ii} were the quadratic coefficients, and b_{ij} (with $i \neq j$) were the second order interaction coefficients.

2.4.2 Statistical analysis and response optimization

An analysis of variance (ANOVA) was conducted to evaluate the quality of the fitted quadratic models. The significance ($p < 0.05$) of the regression coefficients was evaluated by determining the F value. For the validation of the model, the coefficient of determination, R^2, as well as the significance values of the model and of the lack of fit were calculated. To be reliable, the model must be statistically significant to a 95% confidence level ($p < 0.05$), while the lack of fit should be non-significant ($p \geq 0.05$). An optimization of the two response variables was then carried out to determine the optimal maximum responses, allowing the same weighting for both. The desirability function approach introduced by Derringer and Suich (1980) was used for this purpose.

Data analysis, ANOVA, and linear regression, including responses optimization, were performed by using Minitab 17 software.

3. RESULTS

3.1 Optimization of MAE parameters

The effects of ethanol concentration, solid mass, and extraction time on TPC and DPPH radical scavenging capacity were studied, and the resulting experimental values are presented in **Table 1**. For each response variable, a quadratic polynomial model was developed by multiple regression. The regression equation coefficients of the obtained models are shown in **Table 2**. The results in **Table 3** revealed that *F* values of the models were significant ($p < 0.05$) for both responses. Furthermore, lack of fit values were non-significant ($p \geq 0.05$), as needed to validate the models.

The following second order polynomial equation was fitted between the non-coded independent factors, ethanol concentration (x_1), solid mass (x_2), and extraction time (x_3), and TPC response variable:

$$\text{TPC} = -3.30 + 0.0311\,x_1 + 2.64\,x_2 + 0.234\,x_3 - 0.000295\,x_1^2 - 0.982\,x_2^2 - 0.00736\,x_3^2 + 0.0094\,x_1x_2 - 0.00201\,x_1x_3 + 0.0374\,x_2x_3$$

As far as TPC is concerned, R^2 amounted to 0.87, while the adjusted R^2 value was 0.68.

In reference to DPPH radical scavenging capacity, the second order equation presented below showed the relation between the studied non-coded independent factors and the inhibition. The corresponding R^2 was 0.96 and the adjusted R^2 value was found to be 0.91.

$$\text{DPPH}\cdot = -127.6 + 1.72\,x_1 + 120.0\,x_2 + 10.89\,x_3 - 0.0175\,x_1^2 - 44.26\,x_2^2 - 0.4184\,x_3^2 + 0.349\,x_1x_2 - 0.0610\,x_1x_3 + 1.920\;x_2x_3$$

In this study, the extraction of phenolic compounds and the inhibition responses were optimized by maximizing TPC and DPPH· inhibition simultaneously, taking into consideration that there is a strong correlation between TPC and scavenging antioxidant

capacity from the DPPH· assay, as reported by Tournour et al. (2015). Without any precondition or constraint, the obtained results for the optimal values of factors were $x_1 = 30;\ x_2 = 1.78;\ x_3 = 15$. As can be seen in **Figure 1** for TPC and in **Figure 2** for DPPH radical scavenging, the longer the time, the better the obtained response. With this combination, the expected theoretical responses were TPC_t^{opt1} = 1.42 mg GAE/mL and $DPPH\cdot_t^{opt1}$ = 93.3%. Nevertheless, with the potential of the industrial scale-up extraction in mind, a maximum time of 10 min was set as a constraint in a second optimization. With this new condition, the values of the factors that allowed a maximum in the responses were x_1 = 48, x_2 = 1.77, x_3 = 10, with predicted optimal responses of TPC_t^{opt2} = 1.22 mg GAE/mL and $DPPH\cdot_t^{opt2}$ = 89.8%. This optimal value was represented with a black dot in **Figure 1** and **Figure 2**. These new values were lower than those without any constraint, and the necessary reduction in time is compensated mainly by an increase in ethanol content. In order to verify the reliability of model and optimization, experiments following the optimal conditions were conducted in the lab. The obtained experimental values were in good agreement with the theoretical ones, with TPC_e^{opt2} = (1.21 ± 0.04) mg GAE/mL and $DPPH\cdot_e^{opt2}$ = (87 ± 5) %.

3.2 Characterization of grape marc extract

Based on the results obtained using RSM for a maximum value of TPC and DPPH· with the restriction of time and temperature, 48% ethanol, 10 min and 1.77 g of sample were found to be the optimal parameters. Consequently, the final extraction was carried out employing the selected conditions. The obtained grape marc extract was characterized by different experimental techniques. Among them, FTIR analysis was performed in order to examine the presence of phenolic compounds in the grape marc extract, and the FTIR spectrum is shown in **Figure 3**. As can be seen, a broad band is

shown at 3290 cm^{-1} associated with the stretching vibration of O-H or O-H wagging of phenolic compounds (Alara et al., 2018). The band at 1723 cm^{-1} is related to the carboxyl group and indicates the presence of some phenolic acids (Lu and Hsieh, 2012). The absorption band at 1675 cm^{-1} could be due to aromatic ring deformations and C=C bonds, which suggested the presence of polyphenols, flavonoids and amino acids (Zhao et al., 2015). The band at 1453 cm^{-1} shows the presence of CH_2, CH_3, aromatic rings and flavonoids. The band around 1261 cm^{-1} is characteristic of the flavonoid-based tannins (De Souza et al., 2015). Meanwhile, the sharp band at around 1020 cm^{-1} corresponds to C-O-H in phenolic compounds and sugar monomers (Saha et al., 2016). Finally, bands at wavenumbers below 919 cm^{-1} are related to C-H bonds in aromatic structures (Sardella et al., 2015).

It is well known that grapes present a high amount of sugars in their composition, as demonstrated by the sharp band shown in the infrared spectrum, and this was indeed confirmed by the Rebelein sugar method and HPLC results. The extracted sample had around 66% (w/v) of sugars, which consisted mainly of glucose (313.1 g/L) and mannose (112.6 g/L). This sugar fraction depends more on the winemaking extraction degree rather than on the grape variety (Llobera and Cañellas, 2007).

Besides sugars, phenolic compounds are also abundant constituents in grape marc. The composition varies depending on the extraction and on the subsequent reactions taking place during the vinification and postfermentation treatments and the wine aging. Normally, white wines are made without aeration to avoid large periods of contact with oxygen, which can cause browning of the wine and deterioration of quality (Salacha et al., 2008). Therefore, the maceration step lasts only few hours and sulphur

dioxide is immediately added in order to protect against enzymatic oxidation. A higher amount of sulphur dioxide affects the content of total phenolics, flavonoids and flavan-3-ols in wines (Ivanova et al., 2011a). The polyphenols are primarily located in seeds and skins of grape (García-Esparza et al., 2018; Nogales-Bueno et al., 2017; Toaldo et al., 2013). In this work, the quantification of individual phenolic compounds was based on the employment of a calibration curve of specific standards and the combination of the retention times using chromatographic analysis by UHPLC (**Figure 4**). Full details on the resulting MS data and their assignments are provided in **Table A.1**. The analysis presented a wide variety of polyphenols, mainly belonging to flavanols, flavonols, dihydroflavonols, hydroxycinnamic acids and hydroxybenzoic acids. 37 peaks were identified in the grape marc extract. Among the flavanols, procyanidins can be highlighted; in fact, the most abundant polyphenol in the sample was a procyanidin trimer, while monomeric catechin and epicatechin were also identified. Specifically, a total of 16 flavanols were determined and procyanidin was the most abundant with 9 of the 37 peaks observed in the grape marc; additionally, quercetin, kaempferol, and gallic acid were identified. Furthermore, glucuronide derivatives, quercetin-3-O-glucuronide and kaempferol-3-O-glucuronide, appeared due to the presence of grape skin in the sample (Ivanova et al., 2011b). Kaempferol derivatives were also observed, since they are usually found in Chardonnay grapes (Ragusa et al., 2017). Among the hydroxycinnamic acids detected, *p*-coumaroyl-tartaric acid, usually located in the skin of white grapes, was found. Regarding the hydroxybenzoic acids, their content is strongly dependant on the grapevine variety. In this case, gallic acid was mainly identified, which is one of the most frequent hydroxybenzoic acids present in wines.

As the grape marc contains polyphenols, which possess antioxidant activity, as demonstrated by the DPPH· assay results shown above, the incorporation of these

compounds into film forming formulations could be of interest for active packaging applications. A grape marc extract has already shown promising properties when included in ethyl cellulose films (Olejar et al. 2014). The incorporation of the grape marc extract into protein-based materials in order to improve antioxidant properties to develop active films could be also a promising alternative to synthetic films. For that reason, the analysis of the degradation temperature of these compounds is of great relevance in order to select the appropriate processing temperatures. The TGA and DTG curves for the grape marc extract are shown in **Figure 5**. It can be noticed that the DTG curve of grape marc was composed of two major weight loss steps. The first mass loss between 30-105 ºC and the peak around 125 ºC were related to the loss of adsorbed and structural water, respectively, accounting approximately 20% weight loss; the second peak around 200 ºC, when the major weight loss occurred, was related to the thermo-degradation of organic compounds present in grape marc (Basso et al., 2016). The presence of tannins could promote the rapid degradation between 200 °C and 300 °C (Anwer et al., 2015). In previous works, it was shown that soy protein-based films can be manufactured at temperatures below 200 ºC when compression-moulding is employed as the processing method (Garrido et al., 2017; Garrido et al., 2019); thus, grape marc extract could be used as a bioactive compound to improve antioxidant properties of films based on proteins.

4. CONCLUSIONS

The microwave-assisted extraction allowed to recover natural antioxidants from grape marc at room temperature in a rapid way, thus, highlighting the potential of this technology for the extraction of bioactive compounds. RSM was successfully employed to determine the optimal parameters of solvent, solid mass and extraction time in order

to obtain the highest values of TPC and DPPH radical scavenging capacity. Using those parameters, phenolic compounds were extracted from grape marc and analysed by UHPLC, which showed that flavanols were the most abundant phenolic compounds in the extract. Since these polyphenols do not degrade thermally up to 200 ºC, this thermal stability facilitates the incorporation of these compounds into biopolymeric formulations to manufacture bioactive products.

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Table 1. Box-Behnken experimental design and responses for total phenolic content (TPC) and DPPH radical scavenging capacity.

Runs	Factors			Coded factors			Responses	
	Ethanol (%)	Solute (g)	Time (min)	Ethanol	Solute	Time	TPC (mg GAE/mL)	DPPH radical scavenging (%)
1	60	1.5	5	1	0	-1	0.581	62.1
2	45	1	5	0	-1	-1	0.322	47.5
3	60	1	10	1	-1	0	0.675	59.7
4	60	2	10	1	1	0	1.278	91.7
5	45	2	15	0	1	1	1.315	92.6
6	30	2	10	-1	1	0	0.869	78.7
7	45	2	5	0	1	-1	0.656	55.4
8	30	1.5	15	-1	0	1	1.528	91.8
9	45	1.5	10	0	0	0	1.192	89.2
10	45	1.5	10	0	0	0	1.268	89.1
11	60	1,5	15	1	0	1	0.870	78.5
12	45	1.5	10	0	0	0	1.071	84.5
13	30	1.5	5	-1	0	-1	0.637	57.1
14	45	1.5	10	0	0	0	1.087	84.4
15	45	1	15	0	-1	1	0.607	65.5
16	30	1	10	-1	-1	0	0.548	57.1

Table 2. Regression analysis for the full quadratic model of total phenolic content (TPC) and DPPH radical scavenging capacity.

	TPC				**DPPH radical scavenging**			
	Coef.	**Std. Δ**	***t*-value**	***p*-value**	**Coef.**	**Std. Δ**	***t*-value**	***p*-value**
b_0 (constant)	1.1545	0.0978	11.80	0.000*	86.78	2.40	36.12	0.000*
b_1 (Etha)	-0.0223	0.0692	-0.32	0.758	0.89	1.70	0.53	0.617
b_2 (Sol)	0.2457	0.0692	3.55	0.012*	11.06	1.70	6.51	0.001*
b_3 (Time)	0.2655	0.0692	3.84	0.009*	13.31	1.70	7.83	0.000*
b_{11} (Etha*Etha)	-0.0665	0.0978	-0.68	0.522	-3.94	2.40	-1.64	0.152
b_{22} (Sol*Sol)	-0.2455	0.0978	-2.51	0.046*	-11.07	2.40	-4.61	0.004*
b_{33} (Time*Time)	-0.1840	0.0978	-1.88	0.109	-10.46	2.40	-4.35	0.005*
b_{12} (Etha*Sol)	0.0706	0.0978	0.72	0.498	2.62	2.40	1.09	0.317
b_{13} (Etha*Time)	-0.1505	0.0978	-1.54	0.175	-4.58	2.40	-1.90	0.106
b_{23} (Sol*Time)	0.0935	0.0978	0.96	0.376	4.80	2.40	2.00	0.093

Coef.: Standardized regression coefficients; Std. Δ: standard error of the coefficients; *t*-value: statistic of the *t*-test; *p*-value: significance value of the *t*-test (*) significant at $p < 0.05$.

Table 3. Analysis of variance (ANOVA) of the full quadratic model.

		TPC				**DPPH radical scavenging**			
	DF	**SS (adj)**	**MS (adj)**	***F*-value**	***p*-value**	**SS (adj)**	**MS (adj)**	***F*-value**	***p*-value**
Model	9	1.59070	0.176744	4.62	0.038*	3593.56	399.28	17.29	0.001*
Linear	3	1.05106	0.350352	9.15	0.012*	2400.74	800.25	34.66	0.000*
Ethanol	1	0.00398	0.003977	0.10	0.758	6.41	6.41	0.28	0.617
Solid	1	0.48308	0.483085	12.62	0.012*	978.15	978.15	42.37	0.001*
Time	1	0.56399	0.563995	14.73	0.009*	1416.18	1416.18	61.34	0.000*
Quadratic	3	0.39411	0.131370	3.43	0.093	989.48	329.83	14.29	0.004*
Etha*Etha	1	0.01768	0.017681	0.46	0.522	62.09	62.09	2.69	0.152
Sol*Sol	1	0.24098	0.240984	6.29	0.046*	489.74	489.74	21.21	0.004*
Time*Time	1	0.13545	0.135446	3.54	0.109	437.65	437.65	18.96	0.005*
Interaction	3	0.14553	0.048509	1.27	0.367	203.34	67.78	2.94	0.121
Etha*Sol	1	0.01993	0.019933	0.52	0.498	27.46	27.46	1.19	0.317
Etha*Time	1	0.09060	0.090601	2.37	0.175	83.72	83.72	3.63	0.106
Sol*Time	1	0.03499	0.034995	0.91	0.376	92.16	92.16	3.99	0.093
Error	6	0.22972	0.038287			138.53	23.09		
Lack of fit	3	0.20390	0.067968	7.90	0.062	116.24	38.75	5.21	0.104
Pure error	3	0.02582	0.008606			22.29	7.43		
Total	15	1.82042				3732.09			
		R^2 (%)		R^2 (adj) (%)		R^2 (%)		R^2 (adj) (%)	
		87.38%		68.45		96.29		90.72	

DF: degrees of freedom; SS: sum of squares; SS (adj): adjusted sum of squares; *F*-value: statistics of the *F*-test; *p*-value: significance value of the *F*-test (*) significant at $p < 0.05$; R^2: coefficient of determination; R^2 (adj): adjusted coefficient of determination.

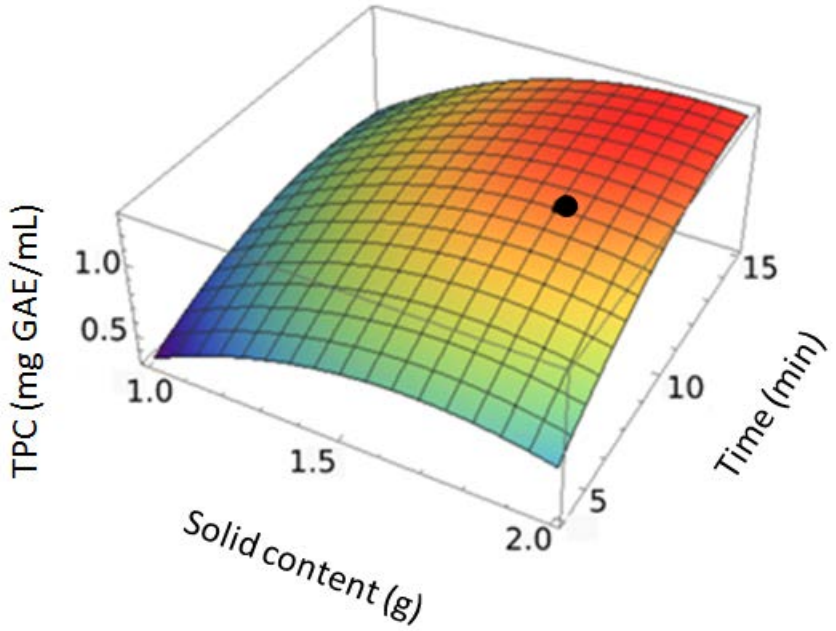

Figure 1. Three-dimensional response plot for TPC model including the combined optimal response with time restriction (black dot).

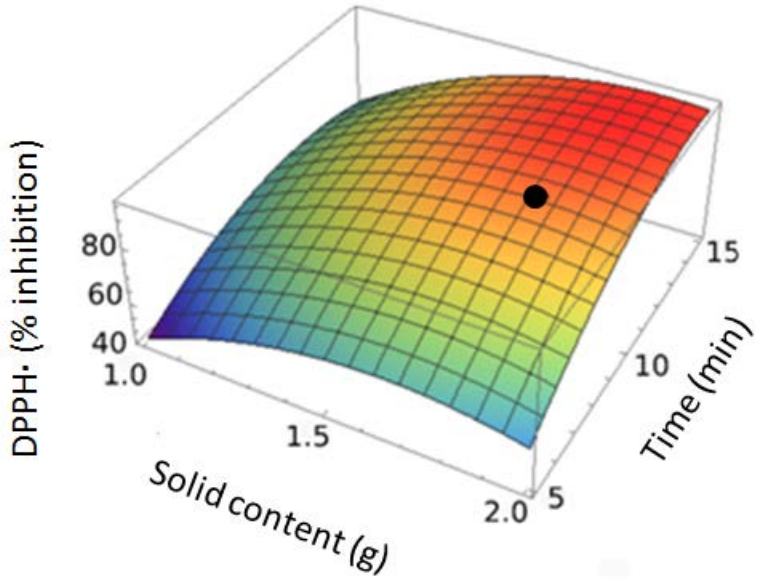

Figure 2. Three-dimensional response plot for DPPH radical scavenging capacity including the combined optimal response with time restriction (black dot).

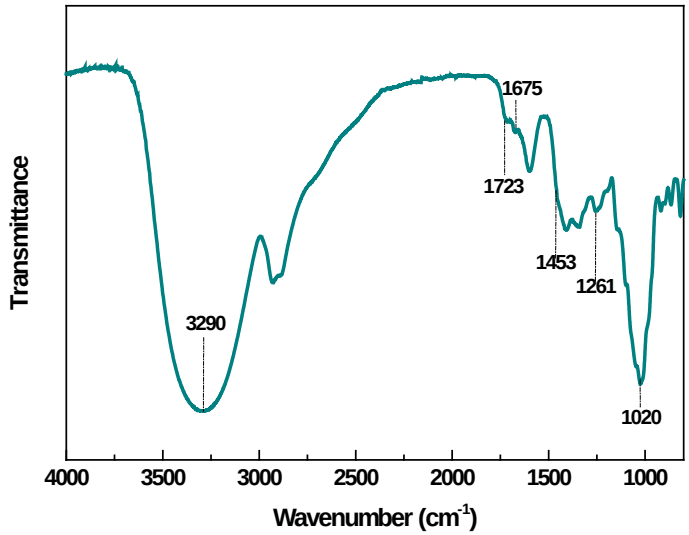

Figure 3. FTIR spectrum of the grape marc extract.

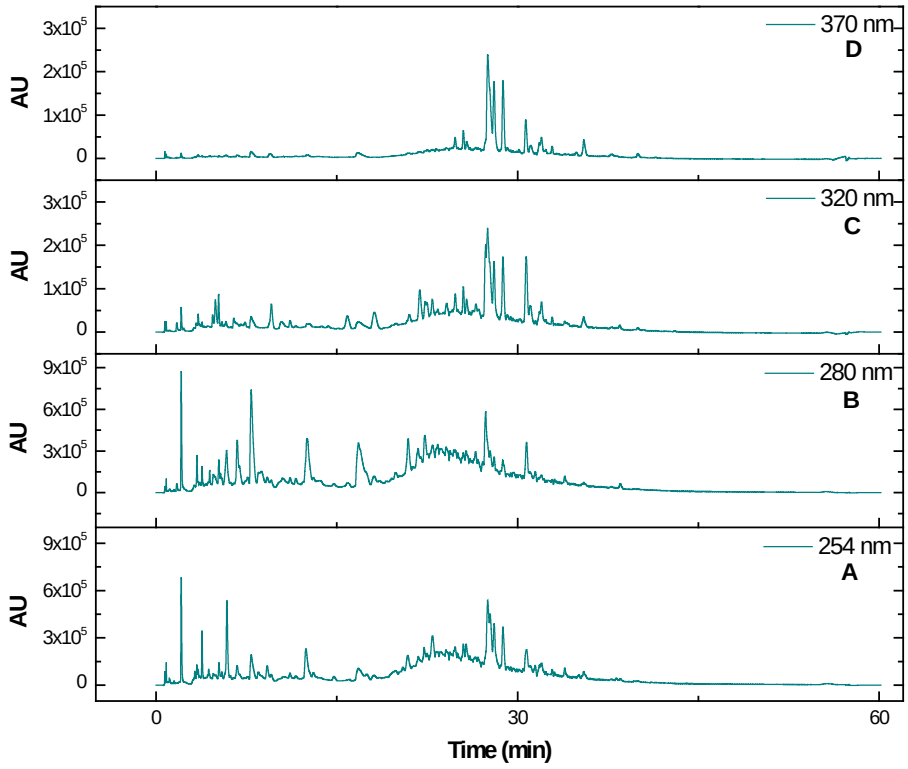

Figure 4. Diode-Array Detection (DAD) chromatogram of grape marc extract at 254 nm (A), 280 nm (B), 320 nm (C) and 370 nm (D).

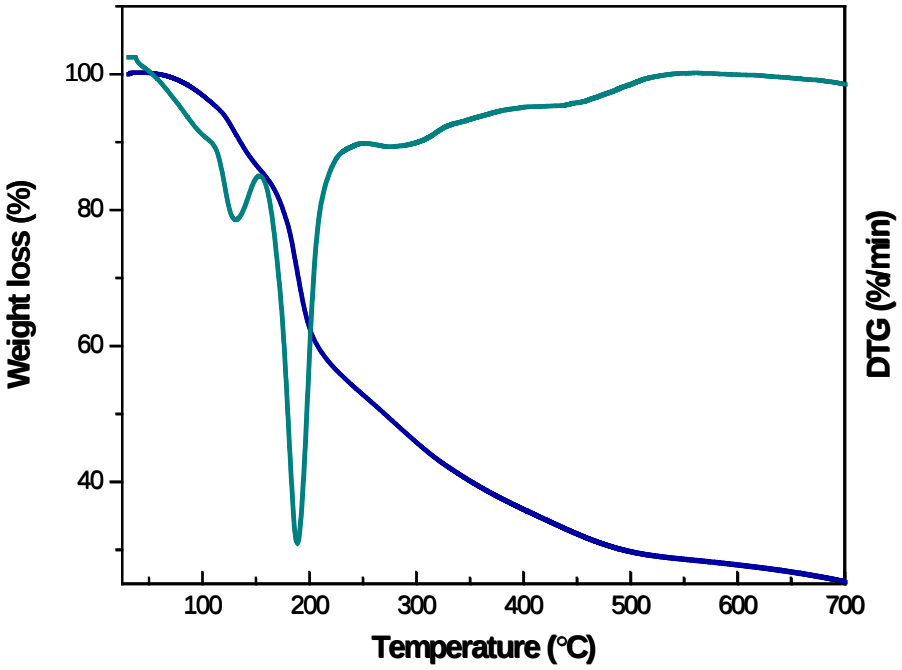

Figure 5. TGA and DTG curves of grape marc extract.