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Microwave-Assisted Extraction of Different Groups of Phenolic Compounds from Grape Skin Pomaces: Modeling and Optimization

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Key words: microwave-assisted extraction (MAE), grape skin pomace, phenolic compounds, antioxidant capacity, artificial neural network (ANN), response surface methodology (RSM)

A microwave-assisted extraction (MAE) technique was employed on grape skin pomaces to enable the extraction of different groups of phenolic compounds (total phenolics, tannins, flavonols, and hydroxycinnamic acids) and to obtain extracts with the highest antioxidant capacity (ORAC). The single-step extraction process was modeled and optimized by means of artificial neural network (ANN) and response surface methodology (RSM) coupled with full factorial design. Methanol concentration (20 to 100%, v/v), temperature (30 to 60°C) and duration (2 to 16 min) were MAE input parameters studied. Optimal parameters were further applied in multistep MAE cycles for the complete recovery of phenolic antioxidants. Results showed that methanol concentration was the most significant parameter influencing the extraction of all groups of phenolics and antioxidant capacity of extracts. Moreover, a significant effect of time and temperature was also noticed, except in the case of total hydroxycinnamic acids. The presented ANN model accurately predicted the effect of the three input parameters simultaneously on the output parameters (training R^2=0.9957; test R^2=0.9945; validation R^2=0.9965). Optimal parameters showed that higher methanol concentrations and lower temperatures (100%, v/v; at 40°C) were more convenient for the extraction of flavonols and hydroxycinnamic acids than for ORAC (78.1%, v/v; at 60°C) or total phenolics and tannins (62.7 and 65.3%, v/v; at 60°C). The number of MAE cycles was found to be a key factor for completing extraction of skin pomace phenolics and should always be considered prior to analytical determination.

LIST OF ABBREVIATIONS

MAE, Microwave assisted extraction; ANN, Artificial neural network; RSM, Response surface methodology; TP, Total phenolics; TT, Total tannins; THCA, Total hydroxycinnamic acids; TF, Total flavonols; ORAC, Oxygen radical absorbance capacity; GAE, Gallic acid equivalents; and TE, Trolox equivalents.

INTRODUCTION

Winemaking is one of the most important agricultural sectors worldwide, and according to the latest data collected by the OIV (International Organisation of Vine and Wine) 73.3 million tons of grapes (around 52% as wine grapes) in 2017, and about 279 million hectoliters of wine were produced in 2018 [OIV, 2018]. Grape pomace is the main solid organic waste from the wine industry, where large quantities are generated after fermentation and pressing, representing about 20% of the initial grape weight [Ky *et al.*, 2014; Laufenberg *et al.*, 2003]. Only 30 to 40% of phenolic compounds are extracted during vinification depending mainly on grape cultivar and applied technology of wine production [Deng *et al.*, 2011; Ky *et al.*, 2014; Tournour *et al.*, 2015; Valls *et al.*, 2017]. This means that grape pomace still exhibits high levels of bioactive compounds (60–70%) with strong antioxidant, antibacterial, and cytotoxic activities as well as favorable pharmacological properties [Bartolomé *et al.*, 2004; Peixoto *et al.*, 2018]. These compounds are known to contribute to human health and are particularly associated with reduced incidence of cardiovascular diseases as atherosclerosis and hypertension, neurodegeneration, and similar medical conditions [Auger *et al.*, 2004; De Sales *et al.*, 2018; Rodriguez-Rodriguez *et al.*, 2012]. Grape skins pomace proved to be a good source of anthocyanins, hydroxycinnamic acids, flavanols and flavonols, whereas flavanols are the most abundant seed polyphenols [Kammerer *et al.*, 2004; Ky *et al.*, 2014]. As a result of the increased concern over the sustainability of agricultural practices, efforts have been made to enable the use of grape pomaces bioactive extracts in various segments of food, pharmaceutical and cosmetic industry, resulting in applications such as natural antioxidant, source of natural pigments, additive in wine production, functional ingredient, *etc*. [Beres *et al.*, 2017; Ky & Teissedre, 2015]. Thus, it is necessary to have efficient extraction methods to achieve high recoveries of phenolic compounds that allow quality control of obtained extracts through their analysis and characterization.

Microwave-assisted extraction (MAE) has been investigated and proposed as better than conventional extraction

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in terms of extraction efficiency, time, and solvent consumption [Chan *et al.*, 2011]. Also, it has been introduced for a wide range of bioactive compounds from food by-products and natural sources, and likewise for the extraction of polyphenols from grape [Hong *et al.*, 2001; Karvela *et al.*, 2009; Krishnaswamy *et al.*, 2013; Liazid *et al.*, 2011] and grape pomace [Casazza *et al.*, 2010; Medouni-Adrar *et al.*, 2015; Pedroza *et al.*, 2015]. Nevertheless, in most of the aforementioned studies conducted on grape or pomace, target compounds were total phenolics, while the mode of operation was focused to the power level of microwave irradiation. This means that extraction was carried out at fixed power usually ranging from 300 to 550 W [Hong *et al.*, 2001; Krishnaswamy *et al.*, 2013; Medouni-Adrar *et al.*, 2015] and in some cases even up to 900 W [Pedroza *et al.*, 2015] and at pre-determined extraction time ranging from 200 to 1003 s [Hong *et al.*, 2001; Krishnaswamy *et al.*, 2013; Medouni-Adrar *et al.*, 2015; Pedroza *et al.*, 2015]. However, the mode of operation that focuses on the extraction temperature rather than microwave power (meaning that extraction temperature is set at desired point by regulating microwave power) is more suitable for the extraction of different groups of thermo-sensitive phenolics [Chan *et al.*, 2011], while temperatures lower than 60°C are recommended in order to avoid possible degradations [Liazid *et al.*, 2011; Pedroza *et al.*, 2015]. In addition, beside the microwave power and temperature, solvent nature and extraction time also showed to be important factors influencing the performance of MAE [Liazid *et al.*, 2011]. Ethanol is, by far, the most used solvent as a good microwave absorber [Chan *et al.*, 2011; Krishnaswamy *et al.*, 2013; Pedroza *et al.*, 2015], while on the other hand, there are only few studies of MAE of grape and pomace phenolics with methanol [Casazza *et al.*, 2010; Hong *et al.*, 2001]. Nevertheless, methanol compared to ethanol extracted higher concentrations of total phenolics, *o*-diphenols and flavonoids, in both grape skin and seed pomace [Casazza *et al.*, 2010], and showed to be a more selective solvent in conventional extraction of phenolic compounds [Pinelo *et al.*, 2005]. In addition, considering the time parameter, earlier studies showed that prolongation of extraction time beyond the optimal conditions was not useful to extract more phenolic compounds [Mané *et al.*, 2007; Medouni-Adrar *et al.*, 2015]. However, prolongation of the extraction time to ensure the completion of extraction and reduced risk of thermal degradation can be achieved through the repeating of the extraction step in multistep MAE [Chan *et al.*, 2011]. The effect of cycle number was only examined by Pedroza *et al*. [2015] revealing that two irradiation cycles (900 W, 1003 s, with temperature fluctuating up to 80°C) were necessary to achieve the equivalent yield of total phenolics with reference solid-liquid extraction; while to the best of our knowledge effects of sequential irradiation cycles with lower power and temperature on the matrix of grape skin pomace were not earlier studied. In addition, modeling and optimization of MAE extraction condition is usually performed through response surface methodology (RSM) [Krishnaswamy *et al.*, 2013; Medouni-Adrar *et al.*, 2015] and artificial neural network (ANN) methods [Ameer *et al.*, 2017]. RSM can demonstrate interaction effects of inherent MAE parameters on target responses, whereas ANN can reliably model the MAE process with better predictive and estimation capabilities.

The aim of the present study was to model and optimize single-step MAE (methanol concentration, temperature and time) of phenolic antioxidants (total phenolics, tannins, hydroxycinnamic acids, flavonols, and ORAC value) from grape skin pomaces by using ANN and RSM, and to further apply optimal parameters in multiple steps in order to study effects of sequential irradiation cycles and to develop a method for the complete recovery of phenolic antioxidants.

MATERIALS AND METHODS

Chemicals

Methanol, ethanol, and hydrochloric acid were purchased from Carlo Erba (Val del Reuil, France). Folin-Ciocalteu's phenol reagent was purchased from Reagecon (Shannon, Ireland), and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) from Acros (Gell, Belgium). Sodium carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate, fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, caffeic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Grape skin pomace sample preparation

This study was conducted on grape skin pomaces from Cabernet Sauvignon, Merlot, and Teran cultivars (*Vitis vinifera* L.). Grape pomaces were provided from Agrolaguna winery (Poreč, Croatia), obtained as wine by-products after alcoholic fermentation and pressing; from grapes harvested in technological maturity in September 2014 and originating out of Istria vine-growing sub-region area (Croatia). Grape pomace samples were first frozen (-80°C) and then freeze-dried (vacuum 0.130 to 0.155 hPa, temperature -30 to 0°C for 24 h, isothermal desorption at 20°C for 12 h) using Christ Alpha 1–4 LSC Plus freeze-dryer (Osterode am Hatz, Germany). Freeze-dried skins were manually separated from seeds and pulp, and ground with an electric grinder. Grape skin powders particle size distributions measured by the laser particle size analyzer (Malvern, Mastersizer 2000, Germany) were: (i) Cabernet Sauvignon d(0.9)≤ 354.31 μm, d(0.5)≤ 123.03 μm, d(0.1)≤ 7.66 μm; (ii) Merlot d(0.9)≤ 376.54 μm, d(0.5)≤ 146.90 μm, d(0.1)≤ 8.37 μm; and (iii) Teran d(0.9)≤ 365.55 μm, d(0.5)≤ 130.70 μm, d(0.1)≤ 8.26 μm. Samples were stored at -20°C before subsequent analyses.

Microwave-Assisted Extraction (MAE)

Phenolic compounds from grape skin pomace were extracted using a professional single-mode microwave reactor (Milestone, Start S Microwave Labstation for Synthesis, Sorisole, Italy), with an adjustable microwave power output, operating at 2.45 GHz; equipped with an air and water reflux condenser and a magnetic stirrer. Parameters that were kept constant during extractions were: stirring (at 80%) and ventilation after extraction (1 min), as well as liquid to solid ratio (50:1), selected based on literature data [Hong *et al.*, 2001; Li *et al.*, 2011; Medouni-Adrar *et al.*, 2015] and preliminary experiment (data not shown). Operating extraction mode was focused to the extraction temperature that was set at the desired point, meaning that power was used to maintain the temperature in the reaction cell, rather than being

applied at continuous level, since this mode reduces the risk of thermal degradation, and is more suitable for the extraction of thermo-sensitive compounds [Chan *et al.*, 2011]. A portion of 0.5 g of freeze-dried grape skin pomace powder and 25 mL of the solvent were added to 50 mL round bottom flask with double neck and a cooling system. MAE was performed at first according to the experimental design shown in Table 1 in order to determine optimal variables (parameters) of single-step MAE. Secondly, the optimal parameters determined were applied in multiple steps (sequential repetitive extraction cycles) in order to define the necessary number of MAE cycles in the final multistep MAE method for the complete extraction of phenolic compounds.

Experimental design for modeling and optimization of single--step MAE

Full factorial design comprising 27 experiments was used to evaluate the effect of three independent variables and to obtain optimal conditions of a single cycle. Independent process variables were: solvent polarity (methanol concentration in methanol-water mixture, v/v), extraction time (min) and temperature (°C); named as X_1, X_2, and X_3, respectively (Table 1). Each experiment in experimental design was run in duplicate [54 (27 × 2) experiments in total]. Ranges of variables were: solvent polarity at 20%, 60% and 100% (v/v) methanol; time at 2, 9 and 16 min; and temperature at 30, 45 and 60°C, as listed in Table 1. The responses (output variables) determined were concentrations of extracted phenolics and antioxidant capacity of skin pomace extracts (Table 1). All experiments were conducted on grape skin pomace of Cabernet Sauvignon. After each MAE experiment (Table 1), the mixture was transferred to a centrifugation tube and centrifuged at 4000 rpm for 5 min on a Rotofix 32 instrument (Hettich Zentrifugen, Germany). Liquid (solvent) was evaporated at 30°C, the residue was dissolved in water and freeze-dried after which grape skin pomace extracts were obtained.

Application of sequential irradiation cycles (multistep MAE)

Optimal parameters of a single MAE cycle were performed in eight sequential consecutive cycles (eight-step MAE) in all three cultivars (Cabernet Sauvignon, Merlot, and Teran), following the earlier described protocol. In total, eight irradiation cycles were tested, since the significant increase in concentrations of phenolic compounds between 7th and 8th cycle was not established, while relative recovery for 8th cycle accounted for less than 1% (w/w, relative recovery was calculated relative to overall amount obtained after eight cycles). Average calculated power of each cycle was 47.3 W. After each MAE cycle, the mixture was transferred to a centrifugation tube and centrifuged as earlier mentioned. The solid part was separated and re-used for MAE with a fresh solvent in a recurring manner. Liquid (solvent) maintained after each cycle was separately evaporated at 30°C, and the residue was dissolved in water and freeze--dried. Concentration and relative recovery (%, w/w) of total phenolics were determined in extracts after each single cycle of multistep MAE and expressed cumulatively. The multistep MAE procedure was run in triplicate for each cultivar.

Spectrophotometric analyses

Spectrophotometric analyses were conducted with a double-beam UV-1600PC spectrophotometer (VWR International, China). Freeze-dried grape pomace skin extracts were solubilized in a wine model solution [Ćurko *et al.*, 2014; Ky & Teissedre, 2015] at concentrations of 3 g/L and 0.25 g/L for analyses of total phenolics (TP) and tannins (TT), respectively; as well as at 7 g/L for analyses of total flavonols (TF) and hydroxycinnamic acids (THCA). Total phenolics were determined with the Folin-Ciocalteu method [Singleton & Rossi, 1965] and results were expressed in mg of gallic acid equivalents (GAE) per g of dry weight (dw) grape skin pomace. Concentrations of total tannins were measured by acid hydrolysis and expressed in mg per g of dry weight (dw) grape skin pomace [Ribéreau-Gayon & Stonestreet, 1966]. Total hydroxycinnamic acids and flavonols concentrations were determined by measuring absorbance at 320 and 360 nm according to the method described by Mazza *et al.* [1999]. Results were expressed in mg of caffeic acid equivalents (CAE) per g of dw; and mg of quercetin equivalents (QE) per g of dw grape skin pomace, for the concentrations of THCA and TF, respectively. All spectrophotometric analyses were conducted in triplicate.

Oxygen Radical Absorbance Capacity (ORAC) Assay

The oxygen radical absorbance capacity (ORAC) was determined according to Ninfali *et al.* [2005], as briefly described by Mazor Jolić *et al.* [2011]. Results were calculated as ORAC values using the differences of areas under fluorescein decay curve between the blank and the sample. The results were expressed as μmol Trolox equivalent (TE) per g of dw grape skin pomace.

Data analysis

Statistical analysis of analytical data was carried out by the Analysis of Variance (ANOVA) using Statistica v.10.0 software (Statsoft Inc., Tulsa, OK, USA). Tukey's HSD Test was used as a comparison test when samples were significantly different after ANOVA ($p<0.05$). To test whether it is possible to predict phenolic and antioxidant characteristics of grape skin pomace based on three input variables (methanol concentration, temperature, and duration of process) artificial neural network modeling was applied. Multiple layer perceptron networks were developed in Statistica v.10.0 software (StatSoft Inc, Tulsa, OK, USA). Response surface methodology (RSM) was used to determine the optimal combination of process parameters which varied at three levels. Experimental data were analyzed using Design-Expert© software (Stat-Ease, Inc., MN, USA) and fitted to an empirical second-order polynomial regression model:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where: y is the predicted response concentration of TP, TT, THCA, TF and ORAC; β_0, β_i, β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms respectively; and X_i and X_j are the actual levels of the independent variables. Based on the regression model, three-dimensional re-

sponse surface methodology (RSM) plots of optimal extraction conditions for TP, TT, THCA, TF and ORAC were designed.

RESULTS AND DISCUSSION

Effect of process variables on the extraction yields of phenolic compounds and antioxidant capacity of grape skin pomace extracts

Systematic study was carried out based on the experimental design presented in Table 1, in order to evaluate the effects of different MAE process variables, *i.e.* solvent (methanol) concentration, temperature and time, on the extraction yields of different groups of phenolics and antioxidant capacity of grape skin pomace extracts.

In addition, polynomial equations and statistical parameters describing the effect of operating process variables on the phenolic and antioxidant characteristics of grape skin pomace extracts are presented in Table 2.

High values of R^2 presented in Table 2 indicated a very good correlation between experimental values and values of models that could explain more than 90% of the variation. Moreover, very low p-values indicated that each generated model was statistically significant, suggesting that MAE could be well described with presented models.

Analyses of experimental data revealed that all three process (input) variables, that is solvent, time and temperature, significantly influenced the MAE of TP and TT ($p<0.05$). In addition, analogous qualitative trends were detected for both output variables (TP, TT) indicating a similar behavior toward variation of solvent concentration and temperature. Experiments performed with 60% (v/v) methanol extracted significantly higher concentrations of TP and TT compared to the ones with the identical time and temperature using 20% (v/v) methanol. Interestingly, an increase in methanol concentration up to 100% (v/v) did not further increase the concentrations of target compounds in both cases. Contrary, concentrations of TP and TT extracted with 100% (v/v) methanol were lower than the ones obtained with 60% (v/v) methanol. A similar type of behavior was observed by Yilmaz & Toledo [2006] for the conventional extraction of grape seeds polyphenols, where the highest concentrations were obtained by 60% or 70% (v/v) methanol. Moreover, 60% (v/v) methanol is most commonly applied in two-step conventional extraction of grape and pomace tannins [Chira *et al.*, 2009; Ky *et al.*, 2014]. Results obtained may be attributed to the changes of solvent polarity, and consequently changes in solubility and diffusivity of TP and TT. In addition, significant differences between the 60% and 100% (v/v) methanol samples of identical extraction time were obtained at 45°C and 60°C, while the same trends among 60% and 100% (v/v) methanol samples of identical extraction time were not observed at the lower temperature (30°C) (Table 1). Furthermore, an increase in the applied temperature (30–45–60°C) resulted in a significant increase of both TP and TT concentrations among the experiments performed under identical conditions of solvent concentration and time. Exceptionally, in the case of 100% (v/v) methanol, an increase of the temperature caused only a slight increase of TP and TT concentrations. Namely, the increase in the temperature favored the extraction by enhancing both the solubility of solute and the diffusion coefficient [Pinelo *et al.*, 2005]; and as a consequence, the highest concentrations of TP and TT were determined at the highest temperature tested (60°C). However, temperature range was kept relatively low (maximum 60°C) and was not further increased in order to avoid possible degradation of phenolic compounds as well as denaturation of membranes [Liazid *et al.*, 2011; Pedroza *et al.*, 2015]. This showed to be particularly important for the extraction of flavonoids that were found to be more sensitive to the degradation caused by high temperature and long extraction time [Casazza *et al.*, 2010]. Furthermore, the time variable differently affected the extraction trends of TP and TT. Prolongation of extraction time from 2 to 9 min among the experiments performed under identical conditions positively affected the extraction of both TP and TT, particularly for the experiments conducted with 60% (v/v) methanol. However, further prolongation from 9 to 16 min only slightly increased concentration of TP, but at the same time negatively affected extraction of TT. Namely, concentrations of TT extracted after 16 min were lower than the ones obtained after 9 min under identical conditions, where a significant decrease was found for 60% (v/v) methanol at higher temperatures (45 and 60°C) as well as for 100% (v/v) methanol at 60°C. As earlier mentioned [Casazza *et al.*, 2010], results obtained confirmed the sensitivity of TT, indicating that temperatures higher than 60°C and extraction time longer than 9 min should be avoided in the case of single-step MAE of TT. This phenomenon could be explained by Fick's second law of diffusion, when the solvent oversaturates, and concentration gradient becomes null after a particular duration; while further augmentation of extraction time may favor degradation reactions and thus decrease in concentration of phenolic compounds [Medouni-Adrar *et al.*, 2015]. Hence, excessive extraction time was not useful to extract more phenolic compounds [Mané *et al.*, 2007; Pinelo *et al.*, 2005]. Finally, the highest values of TP were reached at the conditions of 60% (v/v) methanol and 60°C after 9 or 16 min of extraction, while the highest values of TT were extracted under identical conditions of methanol concentration and temperature but only after 9 min of MAE.

Likewise, methanol concentration also had a significant influence ($p<0.05$) on the extraction of THCA and TF, but trends established were quite different from those noticed for the TP. As it can be seen in Table 1, an increase in methanol concentration significantly promoted the extraction of THCA and TF, and the highest concentrations were thus extracted with 100% (v/v) methanol. In addition, temperature and time had no significant effect on the extraction of THCA, but did affect the extraction of TF. Concentrations of TF extracted at 45°C were higher than those extracted at 30°C or 60°C. Time variable influenced the extraction of TF in similar manner as earlier proposed for TT (Table 1), since the highest concentrations were obtained after 9 min of MAE, while prolongation up to 16 min led to a decrease in TF content. Decreased concentration of phenolic compounds induced by the prolongation of extraction time was previously reported in the literature [Liazid *et al.*, 2011]. This decrease could be ascribed to oxidative degradation of polyphenols, particularly the ones having a greater number of hydroxyl-type substituents in the B ring (like myricetin-3-*O*-

TABLE 1. Operating process variables of microwave-assisted extraction (MAE) experimental design and their effect on phenolic and antioxidant characteristics of grape skin pomace extracts.

Exp. no.	Operating input variables			Output variables				
	X_1	X_2	X_3	TP	TT	THCA	TF	ORAC
	Methanol conc. (%)	Time (min)	Temp (°C)	(mg GAE/g dw skin pomace)	(mg/g dw skin pomace)	(mg/g dw skin pomace)	(mg/g dw skin pomace)	(μmol TE/g dw skin pomace)
1	20	2	30	3.77 ± 0.12^{n}	1.00 ± 0.06^{m}	0.06 ± 0.04^{ef}	0.07 ± 0.01^{g}	70.18 ± 0.84^{n}
2	60	2	30	8.98 ± 0.28^{ij}	7.60 ± 0.20^{gh}	0.44 ± 0.13^{bcde}	0.47 ± 0.05^{f}	127.78 ± 2.77^{jk}
3	100	2	30	8.42 ± 0.17^{jk}	7.38 ± 0.23^{h}	3.22 ± 0.03^{a}	3.22 ± 0.25^{bc}	124.83 ± 3.63^{jk}
4	20	9	30	4.62 ± 0.29^{mn}	2.36 ± 0.09^{lm}	0.30 ± 0.05^{bcdef}	0.16 ± 0.03^{g}	80.71 ± 1.64^{mn}
5	60	9	30	10.68 ± 0.22^{fgh}	9.64 ± 0.21^{ef}	0.34 ± 0.09^{bcdef}	0.50 ± 0.06^{ef}	154.58 ± 7.64^{i}
6	100	9	30	10.08 ± 0.26^{fghi}	8.48 ± 0.24^{fgh}	3.04 ± 0.05^{a}	3.73 ± 0.06^{a}	149.76 ± 2.14^{i}
7	20	16	30	5.13 ± 0.12^{mn}	2.27 ± 0.19^{lm}	0.19 ± 0.11^{def}	0.13 ± 0.05^{g}	97.61 ± 0.47^{lm}
8	60	16	30	11.56 ± 0.44^{def}	9.14 ± 0.47^{efg}	0.33 ± 0.08^{bcdef}	0.47 ± 0.02^{f}	181.71 ± 5.20^{h}
9	100	16	30	10.66 ± 0.84^{fgh}	7.24 ± 0.74^{hi}	2.72 ± 0.11^{a}	3.67 ± 0.07^{a}	178.23 ± 4.06^{h}
10	20	2	45	5.86 ± 0.34^{lm}	2.95 ± 0.08^{kl}	0.02 ± 0.00^{f}	0.11 ± 0.01^{g}	102.44 ± 3.62^{l}
11	60	2	45	13.06 ± 0.44^{d}	12.47 ± 0.34^{d}	0.60 ± 0.05^{bc}	0.69 ± 0.04^{de}	198.85 ± 6.93^{gh}
12	100	2	45	9.79 ± 0.27^{ghij}	8.57 ± 0.35^{efgh}	2.86 ± 0.07^{a}	3.36 ± 0.10^{bc}	185.52 ± 7.59^{gh}
13	20	9	45	7.30 ± 0.48^{kl}	4.99 ± 0.16^{j}	0.11 ± 0.03^{ef}	0.24 ± 0.06^{g}	110.32 ± 1.20^{kl}
14	60	9	45	14.82 ± 0.54^{c}	15.15 ± 0.33^{c}	0.58 ± 0.07^{bcd}	0.75 ± 0.06^{d}	230.52 ± 3.63^{e}
15	100	9	45	10.99 ± 0.41^{efg}	9.15 ± 0.74^{efg}	3.07 ± 0.17^{a}	3.86 ± 0.07^{a}	206.53 ± 6.06^{fg}
16	20	16	45	8.48 ± 0.15^{ijk}	4.39 ± 0.20^{jk}	0.15 ± 0.03^{ef}	0.18 ± 0.04^{g}	115.69 ± 1.00^{kl}
17	60	16	45	16.20 ± 0.29^{c}	12.11 ± 0.23^{d}	0.40 ± 0.10^{bcdef}	0.58 ± 0.06^{def}	269.77 ± 4.43^{bc}
18	100	16	45	11.59 ± 0.45^{def}	8.45 ± 0.10^{fgh}	2.91 ± 0.18^{a}	3.74 ± 0.06^{a}	237.70 ± 1.58^{de}
19	20	2	60	9.30 ± 0.36^{hij}	5.77 ± 0.10^{ij}	0.17 ± 0.07^{ef}	0.16 ± 0.04^{g}	128.48 ± 2.83^{jk}
20	60	2	60	19.19 ± 0.14^{b}	15.93 ± 0.66^{c}	0.61 ± 0.09^{b}	0.61 ± 0.03^{def}	225.74 ± 5.38^{ef}
21	100	2	60	11.01 ± 0.49^{efg}	10.08 ± 0.57^{e}	2.73 ± 0.08^{a}	3.16 ± 0.04^{c}	199.37 ± 7.65^{gh}
22	20	9	60	10.78 ± 0.68^{efgh}	8.21 ± 0.64^{fgh}	0.25 ± 0.08^{bcdef}	0.19 ± 0.03^{g}	137.19 ± 2.33^{ij}
23	60	9	60	21.39 ± 0.29^{a}	21.66 ± 0.37^{a}	0.45 ± 0.05^{bcde}	0.65 ± 0.05^{def}	257.02 ± 5.57^{cd}
24	100	9	60	12.36 ± 0.26^{de}	13.02 ± 0.71^{d}	2.83 ± 0.26^{a}	3.39 ± 0.10^{b}	246.52 ± 9.28^{de}
25	20	16	60	11.54 ± 0.02^{def}	7.69 ± 0.04^{gh}	0.21 ± 0.09^{cdef}	0.17 ± 0.04^{g}	142.65 ± 5.72^{ij}
26	60	16	60	22.16 ± 0.68^{a}	19.61 ± 0.49^{b}	0.61 ± 0.08^{b}	0.59 ± 0.06^{def}	302.25 ± 9.36^{a}
27	100	16	60	12.99 ± 0.44^{d}	9.72 ± 0.14^{ef}	2.77 ± 0.01^{a}	3.28 ± 0.03^{bc}	280.87 ± 7.70^{b}

Data are expressed as average value over two replications ± standard deviation. ANOVA to compare data; different letters indicate significant difference between grape skin pomace extracts (Tukey's test, $p<0.05$). Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

-glucoside and procyanidins) that showed to be less stable to oxidation and more easily degradable [Crupi *et al.*, 2018].

Methanol concentration, time, and temperature significantly influenced the antioxidant activity (ORAC) of grape skin pomace extracts ($p<0.05$), while trends found were close to those earlier established for TP. The highest concentrations were extracted with 60% (v/v) methanol, while significantly lower ORAC values were generally found in experiments using 100% and particularly 20% (v/v) methanol (on average 2-fold lower) under identical conditions of temperature and time. Moreover, an increase in the temperature and time contributed to an increase in ORAC values of grape skin pomace extracts. Namely, the antioxidant activity of extracts significantly increased in the range of 30–45–60°C and 2–9–16 min for temperature and time, respectively. Results obtained are in accordance with findings from an earlier study of Pinelo *et al.* [2005] who reported a higher DPPH inhibition percentage for methanol extracts compared to water or ethanol ones. The same authors showed that temperature had

TABLE 2. Polynomial equations and statistical parameters describing the effect of operating process variables and on the phenolic and antioxidant characteristics of grape skin pomace extracts.

Output variables	2nd-order polynomial equation (quadratic model)	R^2	*p*-value
TP (mg GAE/g dw skin pomace)	$15.26 - 1.73X_1 + 1.16X_2 + 3.16X_3 - 0.02X_1X_2 - 0.91X_1X_3 + 0.08X_2X_3 - 6.19X_1^2 - 0.35X_2^2 + 0.47X_3^2$	0.9191	< 0.0001
TT (mg/g dw skin pomace)	$14.54 + 2.36X_1 + 0.49X_2 + 3.14X_3 - 0.44X_1X_2 - 0.53X_1X_3 + 0.21X_2X_3 - 6.94X_1^2 - 1.83X_2^2 + 0.57X_3^2$	0.9237	< 0.0001
THCA (mg/g dw skin pomace)	$0.55 + 1.35X_1 - 0.01X_2 + 0.02X_3 - 0.03X_1X_2 - 0.03X_1X_3 + 0.02X_2X_3 + 1.03X_1^2 - 0.07X_2^2 + 0.02X_3^2$	0.9911	< 0.0001
TF (mg/g dw skin pomace)	$0.76 + 1.67X_1 + 0.05X_2 - 0.01X_3 + 0.07X_1X_2 - 0.08X_1X_3 - 0.03X_2X_3 + 1.24X_1^2 - 0.13X_2^2 - 0.13X_3^2$	0.9954	< 0.0001
ORAC (μmol TE/g dw skin pomace)	$224.09 + 47.78X_1 + 24.63X_2 + 41.93X_3 + 11.02X_1X_2 + 9.51X_1X_3 + 3.12X_2X_3 - 61.21X_1^2 + 1.30X_2^2 - 12.73X_3^2$	0.9695	< 0.0001

Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

a critical role in the extraction efficiency, where the value of 50°C maximized the antiradical activity of phenolic extracts. Furthermore, solvent concentration, time, and microwave power, and interaction of power with time and solvent concentration, as well as interaction of time and solvent concentration showed to play a significant role in the antioxidant activity of grape seed extracts [Krishnaswamy *et al.*, 2013]. Our results demonstrated that the extract with the highest phenolics content, that was extracted under 60% (v/v) methanol and the highest temperature (60°C) and the longest process duration (16 min), exhibited the highest antioxidant activity.

Modeling single-step MAE by artificial neural network (ANN)

In order to test whether it is possible to predict the concentrations of total phenolics, tannins, flavonols, and hydroxycinnamic acids as well as the antioxidant capacity based on methanol concentration, temperature, and duration of the process, several AANs were developed. In all the cases, three variables were used as input data (methanol concentration, temperature, and duration of the process) and 5 variables were used as output data (TT, TP, THCA, TF and ORAC). The ANN training was performed with random separation of data into training, test, and validation sets as 60:20:20 ratio. Back error propagation algorithm available in Statistica v.10.0 was applied for the model training and model performance was evaluated based on R^2 and Root Mean Squared Error (RMSE) values for training, test, and validation [Benković *et al.*, 2015]. Examples of few developed ANNs are given in Table 3.

Almost all of the developed networks had a high linear correlation coefficient (R^2) for training, test, and validation. The five selected ones (Table 3) had the highest R^2 values for training, test, and validation with lowest RMSE values. It may be observed that there are basically two different ANNs regarding the number of neurons in the hidden layer (8 and 10) since all of them have 3 neurons in the input layer and 5 neurons in the output layer. Also the hidden activation and the output activation of the ANNs with same numbers of neurons in the hidden layer were different. When looking at the correlation coefficients for training, for all of the five networks,

TABLE 3. Characteristics of five selected artificial neural networks (ANNs) based on coefficients of determination and root mean square errors for the prediction of phenolic and antioxidant characteristics of grape skin pomace extracts obtained by microwave-assisted extraction (MAE).

Network number	1	2	3	4	5
Network name*	MLP 3–10–5**	MLP 3–10–5	MLP 3–8-5	MLP 3–10–5	MLP 3–8-5
Training performance	0.9957	0.9947	0.9951	0.9958	0.9919
Training error	0.0016	0.0020	0.0018	0.0015	0.0029
Test performance	0.9945	0.9918	0.9925	0.9939	0.9863
Test error	0.0020	0.0040	0.0022	0.0017	0.0040
Validation performance	0.9965	0.9954	0.9964	0.9965	0.9936
Validation error	0.0026	0.0034	0.0028	0.0031	0.0054
Training algorithm	BFGS103	BFGS66	BFGS100	BFGS128	BFGS69
Hidden activation	Logistic	Tanh	Tanh	Logistic	Logistic
Output activation	Exponential	Exponential	Exponential	Identity	Exponential

*In the network name, the first number describes the number of input variables, the second one the number of neurons in the hidden layer and the third one number of output variables. **The most suitable artificial neural network. Abbreviations: MLP, multi layered perceptron.

the highest value was observed for ANN 4 (R^2=0.9958) which also had the lowest training error (RMSE=0.0015). ANN 1 which had slightly lower training performance (R^2=0.9957) had the highest value for test performance (R^2=0.9945). The highest value for validation performance was observed for ANN 1 (R^2=0.9965) and ANN 4 (R^2=0.9965) which had the same values but the ANN 1 had lower validation error (RMSE=0.0026). Based on these results, ANN 1 was selected as the optimal one. The comparison between experimental and predicted values of TP, TT, THCA, TF, and ORAC for the most suitable ANN 1 (MLP 3–10–5) is presented in Figures 1a-d respectively, while correlation coefficients for prediction of TT, TP, THCA, TF, and ORAC are presented in Table 4.

From Figure 1 it is clearly visible that the ANN managed to achieve a high correlation between experimental data and ANN predictions for each parameter (TP, TT, THCA, TF,

TABLE 4. Correlation coefficients of the most suitable artificial neural network* for the prediction of phenolic and antioxidant characteristics of grape skin pomace extracts.

Output variables	Correlation coefficient (R^2)		
	Training	Testing	Validation
TP	0.9943	0.9893	0.9981
TT	0.9928	0.9900	0.9981
THCA	0.9973	0.9978	0.9916
TF	0.9991	0.9978	0.9988
ORAC	0.9952	0.9979	0.9962

*The most suitable artificial neural network: ANN 1 (MLP 3–10–5). Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity.

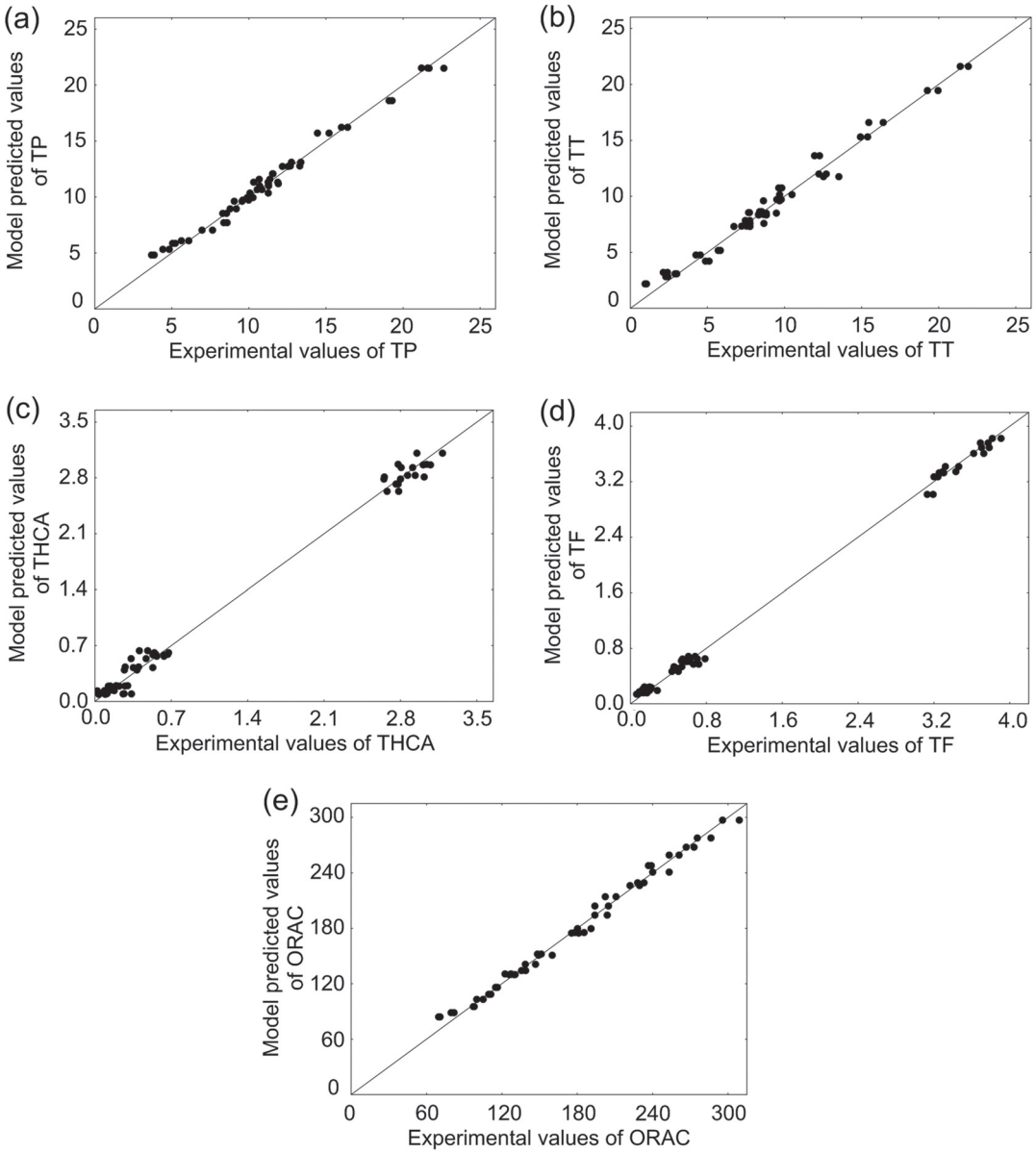

FIGURE 1. Comparison between experimental and predicted values of the most suitable ANN 1 (MLP 3–10–5) for: (a) total phenolics (TP), (b) total tannins (TT), (c) total hydroxycinnamic acids (THCA), (d) total flavonols (TF), and (e) oxygen radical absorbance capacity (ORAC).

and ORAC). From Table 4 it is visible that the best correlations between experimental data and the ANN predictions were obtained for TF with the R^2 values of 0.9991, 0.9978, and 0.9988 for training, test, and validation, respectively. The second highest value for validation was observed for TP (R^2=0.9981) and TT (R^2=0.9981) which had the same value, followed by ORAC (R^2=0.9962) and THCA (R^2=0.9916). Considering that those are very high values obtained for validations for all the tested parameters, these models could easily be used to monitor extraction processes since a good-fitting model or quantitative model would have R^2 values above 0.90 and quantitative models are compact representations where a single differential or difference equation may describe the performance of the system for a large set of input functions and initial states [Lunze, 1998]. This is not surprising since ANNs were proven to be one of the most useful tools in extraction processes for monitoring, predicting, and optimizing different compounds in microwave-assisted or ultrasound-assisted extractions like phenolic compounds from *Achillea berbresteinii* [Salarbashi *et al.*, 2014]; total polyphenolic compounds from chokeberries [Simić *et al.*, 2016]; as well as total extract, stevioside, and rebaudioside A from *Stevia rebaudiana* (Bertoni) leaves [Ameer *et al.*, 2017].

Optimization of single-step MAE by response surface methodology (RSM) and effects of sequential irradiation cycles

In order to provide overall optimal conditions of simultaneous and maximum extraction of phenolic antioxidants from grape skins, various output responses were first considered at the same time. However, desirability function (D), which is the most important and applied multicriteria methodology in optimization procedures [Bezerra *et al.*, 2008], of this joint model was importantly lower (D=0.7870) compared to D values obtained by each individual model. Also, earlier it has been shown that optimal conditions can vary significantly among the different phenolic groups [Karvela *et al.*, 2009]. Hence, the individual models developed (Table 2) were used to optimize the parameters of MAE process. Optimal conditions were selected using desirability for the maximum concentrations of phenolic compounds and antioxidant capacity of grape skin pomace extracts. Three-dimensional

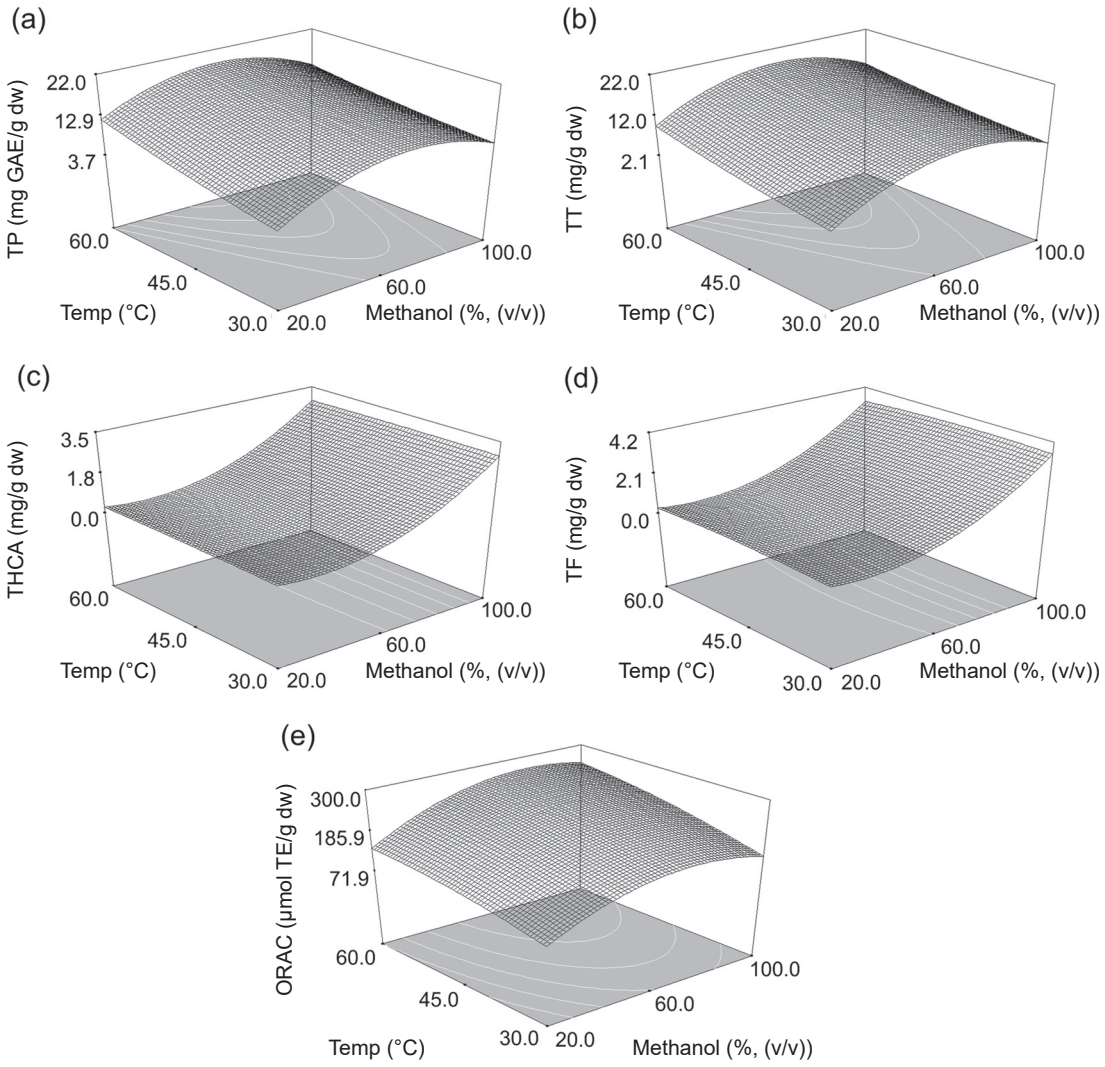

FIGURE 2. RSM plots of the models developed for single-step MAE of: (a) total phenolics (TP), (b) total tannins (TT), (c) total hydroxycinnamic acids (THCA), (d) total flavonols (TF), and (e) oxygen radical absorbance capacity (ORAC).

response surface plots were created for each variable individually and optimum conditions of single-step MAE for maximum response of TP, TT, THCA and TF, as well maximum antioxidant capacity of extracts (ORAC) are presented in Figures 2a-e. Given to the results that prolongation of single-step extraction time was not useful to extract more polyphenols as well as to optimize the overall time of multistep final method, all plots were generated by keeping the time variable to the fixed value (9 min) and plotting it against two other variables (methanol concentration and temperature). Optimal conditions for single-step MAE were 62.7% and 65.3% (v/v) methanol for TP and TT, respectively, at 60°C for 9 min with the predicted yields of 18.91 and 18.38 mg/g dw skin pomace for the first and the latter (Figures 2a and b). Optimal conditions for single-step MAE of THCA and TF were 100% (v/v) methanol, at 40°C for 9 min with the predicted yields of 2.94 and 3.68 mg/g dw skin pomace for THCA and TF, respectively (Figures 2c and d). Optimal conditions for the maximum antioxidant capacity of extracts (ORAC) by single-step MAE were 78.1% (v/v) methanol, at 60°C for 9 min with the predicted yields of 265.77 μmol TE/g dw skin pomace (Figure 2e). Validity of predicted optimal values for each output variable were experimentally confirmed. Experimental and predicted values of optimal conditions were given in Table 5. Experimental data were in accordance with the predicted ones, since predicted and experimental values were not significantly different within the 95% confidence interval.

Effects of sequential irradiation cycles were further studied on three different cultivars (Cabernet Sauvignon, Merlot, and Teran) by application of optimal conditions of a single MAE cycle in multiple steps (cycles) on the model of TP covering all phenolic compounds. Application of sequential irradiation cycles allowed the prolongation of the extraction time [Chan *et al.*, 2011] but without risk of degradation due to the joint effects of temperature and longer extraction time [Medouni-Adrar *et al.*, 2015; Pinelo *et al.*, 2005]. Also, it is important to note that this was manipulated by the addition of fresh solvent to the residue and repeating the extraction step. This procedure allowed us to avoid the solvent evaporation earlier reported [Pedroza *et al.*, 2015], and ensured the completion of extraction, so that the MAE method could be applied prior to analytical determination. Effects of eight sequential irradiation extraction cycles on the extraction of total phenolics (concentration and relative recovery (%, w/w) – calculated relative to the overall amount obtained after eight cycles) are presented in Table 6.

TABLE 5. Phenolic and antioxidant characteristics of grape skin pomace extracts obtained by optimized single-step microwave-assisted extraction conditions.

Output variables	Experimental concentrations	Predicted concentrations
TP (mg GAE/g dw skin pomace)	19.05±0.27	18.91
TT (mg/g dw skin pomace)	18.18±0.35	18.38
THCA (mg/g dw skin pomace)	3.02±0.09	2.94
TF (mg/g dw skin pomace)	3.70±0.05	3.68
ORAC (μmol TE/g dw skin pomace)	261.04±5.32	265.77

Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

The major part of TP was extracted in the first extraction step (1st), and then less and less TP were extracted in each successive individual cycle. Concentrations in each cycle decreased in the order: Cabernet Sauvignon, Teran, Merlot. On the other hand, very similar values of relative recovery were found after the second cycle independently of cultivar, meaning that % (w/w) of extracted TP were quite similar for all three cultivars. For example, ~ 83% (w/w) of TP were cumulatively extracted after three cycles in all three cultivars, and around 90% and 94% (w/w) of TP, after four and five cycles, respec-

TABLE 6. Cumulative effect of sequential irradiation cycles on the extraction of total phenolics (concentration and relative recovery) from Cabernet Sauvignon, Merlot, and Teran grape skin pomaces.

Cycle number	Cabernet Sauvignon		Merlot		Teran	
	TP (mg GAE/g dw skin pomace)	TP (%)	TP (mg GAE/g dw skin pomace)	TP (%)	TP (mg GAE/g dw skin pomace)	TP (%)
1st	19.05^{m}	46.4^{j}	15.18^{o}	51.6^{h}	17.82^{n}	49.3^{i}
2nd	28.66^{hi}	69.7^{g}	21.17^{l}	71.9^{f}	25.96^{j}	71.8^{f}
3rd	34.05^{e}	82.9^{e}	24.30^{k}	82.5^{e}	30.22^{g}	83.6^{e}
4th	37.07^{c}	90.2^{d}	26.58^{j}	90.3^{d}	32.70^{f}	90.4^{d}
5th	38.66^{b}	94.1^{c}	27.82^{i}	94.5^{c}	34.14^{e}	94.4^{c}
6th	40.35^{a}	98.2^{ab}	29.03^{h}	98.6^{ab}	35.44^{d}	98.0^{ab}
7th	40.73^{a}	99.1^{ab}	29.21^{gh}	99.2^{ab}	35.89^{cd}	99.3^{ab}
8th	41.09^{a}	100.0^{a}	29.45^{gh}	100.0^{a}	36.16^{cd}	100.0^{a}

Data are expressed as average value of three replications ± standard deviation. ANOVA to compare data among three cultivars; different letters indicate statistical differences between extracts (Tukey's test, $p<0.05$). Abbreviations: TP, total phenolics; GAE, gallic acid equivalents.

tively (Table 6). In addition, results in Table 6 showed that concentrations cumulatively extracted by sequential irradiation cycles showed a significant increase with the prolongation of extraction cycle number up to six. Further extraction only slightly contributed to the concentrations of TP. Hence, there were no significant differences among the last three cycles. According to these results, the extraction process can finally be limited to six cycles for all three cultivars, that approximately extracted more than 98% (w/w) of total phenolics. Nevertheless, it is important to note that the number of cycles should not be considered as fixed. Namely, wide ranges in concentrations of different phenolics were detected in extensive studies of grape pomace over the years, comprising variations of cultivar and vintage as well as geographical origin, maturity, and winemaking technology [Deng *et al.*, 2011; Kammerer *et al.*, 2004; Ky *et al.*, 2014; Ky & Teissedre, 2015]. For instance, these differences can lead even up to ten times lower/higher concentrations of phenolic compounds and antioxidant activity of grape skin pomaces [Ky *et al.*, 2014; Ky & Teissedre, 2015]. Hence, our results demonstrated the importance of MAE with successive irradiation cycles, particularly for the conditions operating under lower power and temperature, where the exact number should always be examined in order to ensure the completion of the extraction process.

Comparison of our final MAE conditions to literature data for grape skin and pomace was difficult, due to great variation considering the operating systems and parameters studied (extraction solvent, temperature range, power, time, number of irradiation cycles, *etc*.), thus reflecting to the differences in selected or optimal conditions for extraction of TP [Chan *et al.*, 2011]. For instance, Pedroza *et al.* [2015] proposed extraction from Chardonnay grape skin pomace with 60% (v/v) aqueous ethanol solution, and liquid to solid ratio of 4 mL/g for 1033 s at 900 W, in two cycles; while successive irradiation under these conditions caused solvent evaporation and imbibition, and led to decreasing recovery. In our study, in order to avoid these negative effects of sequential irradiation cycles, multistep MAE was performed by the addition of fresh solvent in each repetitive cycle, while extraction was conducted with significantly lower irradiation power and longer time, as well as different solvent. Furthermore, optimal conditions for single-step MAE of TP from Ahmar Bou-Amar grape skin pomace obtained by optimization modeling were 51.45% (v/v) acetone, with solid to liquid ratio of 0.1 g/32.25 mL for 113.74 s and 384.44 W [Medouni-Adrar *et al.*, 2015]. Hong *et al.* [2001] also proposed single-step MAE of TP from grape skin with 90% (v/v) methanol, solid to liquid ratio of 1 g/15 mL for 200 s and 540 W. Overall extraction time (6×9 min) of our MAE methods was comparable to the study of Casazza *et al.* [2010] who also worked with a similar operating system, as well as lower power for longer time. Namely, Casazza *et al.* [2010] performed single-step MAE from Pinot noir grape skin pomace using 100% (v/v) methanol, with solid-liquid ratio of 0.2 g dw/mL for 60 min at 110°C and 60 W. Concentrations extracted with this single-step extraction at higher temperatures (110°C) in Pinot Noir skin pomace were slightly lower than those shown in Table 6, probably due to the differences in MAE parameters but also to grape cultivar, maturity, vintage, winemaking technology, *etc.* [Deng *et al.*, 2011; Kammerer *et al.*, 2004; Ky *et al.*, 2014; Valls *et al.*, 2017]. Concentrations of TP determined in grape skin pomaces of three cultivars decreased in the order: Cabernet Sauvignon, Teran, and Merlot, and were comparable with other studies regardless of the extraction method prior to the analysis. For instance, concentrations of TP found were in line with the values previously reported for Cabernet Sauvignon and Merlot or other red grape cultivars (11.8–54.8 mg GAE/g dw grape skin pomace) [Casazza *et al.*, 2010; Deng *et al.*, 2011; Ky *et al.*, 2014; Medouni-Adrar *et al.*, 2015; Yilmaz & Toledo, 2006]. Finally, results showed high efficiency of MAE method, which allowed completion of extraction in shorter time compared to time consumed during conventional solid-liquid extraction methods, that for processes with temperatures under 60°C can take from 6 to 24 h [Casazza *et al.*, 2010; Ky *et al.*, 2014].

CONCLUSIONS

The effects of methanol concentration (20, 60, and 100%, v/v), time (2, 9, and 16 min), and temperature (30, 45, and 60°C) on the extraction of phenolic antioxidants from grape skin pomace were studied using modeling and optimization by ANN and RSM. All input parameters significantly influenced the MAE of total phenolics, tannins, flavonols, and antioxidant capacity of extracts (ORAC), while extraction yields of hydroxycinnamic acids was markedly influenced only by methanol concentration. The ANN model was accurate to predict the extraction yields of phenolic antioxidants with high correlation coefficients for training (R^2=0.9957), test (R^2=0.9945), and validation (R^2=0.9965), thus confirming that ANN could be successfully used in MAE experiments for monitoring or prediction. The optimal parameters of a single-step MAE cycle for maximum yields of phenolic compounds and antioxidant capacity obtained by RSM were: (i) 62.7% and 65.3% (v/v) methanol for total phenolics and tannins, respectively, at 60°C for 9 min; (ii) 100% (v/v) methanol, at 40°C for 9 min for total flavonols and hydroxycinnamic acids; and (iii) 78.1% (v/v) methanol, at 60 °C for 9 min for ORAC. The number of extraction steps showed to be an important factor influencing extraction yields of phenolic compounds. Relative recovery of total phenolics (%, w/w) showed to be rather constant extraction parameter for all three cultivars, where six MAE cycles significantly contributed to the concentration of TP and extracted more than 98% (w/w) of total phenolics. Multistep MAE by optimal parameters proved to be a highly efficient method for the extraction of grape skin pomace phenolics prior to analytical determination.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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