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Optimization of Ultrasound-Assisted Extraction of Phenols From Seeds of Grape Pomace

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Abstract

The aim of this research was to optimize the extraction condition of ultrasound-assisted extraction (UAE) of phenols from the red grape of Vranac variety (*Vitis vinifera L.*) pomace seeds. The minimum experiments needed for optimization of UAE by response surface methodology (RSM) were obtained by spectrophotometric and HPLC analyses of seed extracts. UAE greatly depends on three independent variables: extraction temperature, time and liquid/solid ratio. The RSM can be used for optimization of UAE conditions to obtain maximum responses such as extraction yield, TPC, (+)-catechin, (-)-epicatechin and proanthocyanidin content. The predicted values of the model were in accordance with experimental data under the same conditions (RSD was 0.74%). Experimental data also confirmed that UAE gives a better yield of phenolics than conventional solvent extraction (23.76% increase). The UAE under optimal extraction conditions is suitable for obtaining extracts that are rich in phenolic content, and have strong antioxidant activity which could be used as additives in food and medicaments.

Keywords: Grape pomace seeds, ultrasound assisted extraction, phenolic compounds, radical scavenging activity, response surface methodology.

1. Introduction

Grapes are rich in phenol compounds, which are very important for human health as compounds with antioxidant,[1–7] anti-cancer,[8] anti-inflationary,[9] antimicrobial activities.[3,4] There are also studies on the beneficial effects of these compounds on the heart and other chronic diseases.[8–11]

In 2012 world vineyards reached a total area surface (which includes areas not yet in production or harvested) of 7,528,000 ha, global grape production of 69,200,000 tonnes and world wine production (excluding juice and musts) of 252,000,000 hL.[12] Wine production generated significant quantities of waste.

These wine wastes present the raw residual biomass wastes (*i.e.* solid extraction residues or wine leaves). The raw biomass can be used as a solid fuel, or transformed to the product gas with various uses like district heating, the electricity production in gas engines, and chemical syntheses, e.g. the production of methanol, ammonia and platform chemicals. The most often applied emerging thermochemical processes such as gasification, pyrolysis and catalytic treatment of liquefied biomass were well documented.[13,14]

After processing of grape into wine, certain amounts of phenolic compounds remain in the grape residues, especially from grape seeds due to the fact that their extraction is less efficient compared to other parts of the grape, such as grape skin and pulp. Phenolic content, composition and antioxidant activity of extracts from seeds of grape pomace, and from whole pomace obtain by conventional solvent extraction, have been well documented.[3,15,16] This extraction technique requires long extraction time by using certain grades of organic solvents, usually at higher temperature. The long extraction time or higher temperature can have negative effects on the target components. They may cause the degradation of phenolic compounds. Several alternative techniques, such as ultrasonic waves, supercritical fluids or microwaves, have been development to extract high added components for a shorter time

and less solvent requirements.[17,18] The ultrasound-assisted extraction (UAE) is widely used in the extraction of natural products.[19–23] The UAE is an inexpensive, simple, and efficient extraction technique.[23] There were many studies about optimization of the extraction process by either empiric or statistical methods. One of the most popular statistical methods which can be applied for optimization of extraction to maximize extraction yield and/or phenolic content of extracts is RSM – response surface methodology.[21–27] This methodology usually includes optimization of extraction responses such as extraction yield and/or total phenolic content (TPC), and there are no research studies that include other responses such as (+)-catechin, (-)-epicatechin and proanthocyanidin content.

The object of this research was to optimize ultrasound-assisted extraction by RSM to maximize extraction yield, TPC and individual phenol content in extracts obtained from seeds of grape pomace. Further, this technique was compared with conventional solvent extraction by estimated phenol composition and the radical scavenging activity of these extracts.

2. Materials and Methods

2. 1. Chemicals

HPLC-grade solvents (methanol, acetonitrile and formic acid) were obtained from Merck (Darmstadt, Germany). The standards of investigated compounds ((+)-catechin, (-)-epicatechin, (-)-epicatechin gallate and proanthocyanidin B2), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical and all other chemicals (methanol, acetone, acetic acid) were of analytical quality and supplied from Sigma Chemical Co. (St. Louis, MO).

2. 2. Samples

The grape pomace of the red grape variety Vranac (southern Serbia vineyard region) was taken from a local winery, immediately after the vinification process. The grape seeds were separated manually from the rest of wine pomace. The seeds were washed, dried at 60 °C and crushed in a grinder for 2 min to an average particle size of 0.5 mm in diameter and then used for extractions.

2. 3. Conventional Solvent Extraction

The seeds were weighed and extracted with a solvent system of methanol/acetone/water/acetic acid (30/42/27.5/0.5), by stirring continuously at 200 RPM, at a determined temperature in the dark, for a determined time, and then centrifuged for 10 min at 2500 × g. The extract was evaporated to dryness under a vacuum rotary evaporator, and diluted in methanol to a concentration of 0.1 g/mL.

2. 4. Ultrasound-Assisted Extraction

An ultrasound instrument (EI, Nis, Serbia) with a volume of 3 L, frequency of 40 kHz and input power of 500 W, was used in the experiments. The frequency and input power were chosen on the basis of preliminary experiments based on the TPC of extracts (the TPC was increased by increasing of these parameters). All UAE experiments were carried out by the same solvent system as for conventional solvent extraction. The suspension of sample with solvent system, in a determined liquid/solid ratio, was irradiated for the predetermined extraction time and temperature. After treatment, extracts were centrifuged for 10 min at 2500 × g, and evaporated to dryness under a vacuum rotary evaporator, and diluted in methanol to a concentration of 0.1 g/mL. All extracts were filtered before analysis.

2. 5. Yield Determination

The yield of extraction Y, expressed in percentage, was calculated from sample and extract dry weight (m_S and m_E, respectively) after removing solvent according to the formula:

$$Y\,(\%) = (m_E / m_S) \times 100 \quad (1)$$

2. 6. Determination of Total Phenol Content (TPC)

TPC in selected extracts were spectrophotometrically determined[5] by reading absorbance at 280 nm on an UV–Vis spectrophotometer (Agilent 8453 device, Agilent Technologies, Santa Clara, CA, USA). Results were expressed as mg of gallic acid equivalents (GAE) /g of extract dry matter (DM).

2. 7. Determination of Flavan-3-ol Monomers and Proanthocyanidins

Phenol composition of selected extracts was analyzed by reverse phase high performance liquid chromatography (RP-HPLC) of the extracts, on an Agilent 1200 chromatographic system equipped with a photodiode array (DAD) and fluorescence detectors (FD). An Agilent-Eclipse XDB C-18 4.6 × 150 mm column, thermostated at 30 °C, was used. The solvents A: formic acid/water (5:95 v/v) and B: acetonitrile/formic acid/water (80:5:15 v/v) were used and the elution gradient were previously described.[7] The injection volume was 5 μL, and the flow rate was 0.9 mL/min. The detection wavelengths were 280 nm for DAD and 275/322 nm ($\lambda_{Ex}/\lambda_{Em}$) for FD. The (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate and proanthocyanidins were identified by comparing their spectral characteristics and retention times with data of original reference standard compounds, and with data given in the lite-

rature.[1,2,7] The calibration curves of standard phenolic compounds (five data points, n = 2) were linear with R^2 = 0.99. Results were expressed as mg/g extract DM. The reproducibility of the HPLC analyses, performed for the same sample, n = 3, in terms of peak areas, was very good (RSD were <7%).

2. 8. Experimental Design

The UAE was optimized using response surface methodology (RSM). The Design Expert software (Version 7.1.6, Stat-Ease, Inc., Minneapolis, MN, USA) was used in this regard. The statistical models and its graphical representation were constructed using central composite design (CCD). Three independent variables: extraction time (X_1), extraction temperature (X_2) and liquid/solid ratio (X_3) were selected as variables which potentially could affect on extraction efficiency. The preliminary single factor experiments were used for determination of values of the ranges and center points (Table 1).

Twenty different experiments, including six replicates of the central point, were performed. The extraction yield, TPC, (+)-catechin, (-)-epicatechin and total proanthocyanidin content were chosen as the responses for the combination of the independent variables (Table 2). All experiments were carried out in triplicate (n = 3), except for the central point.

Table 1. Indepenent variables and their levels

Variables		Levels		
coded	decoded	–1	0	1
X1	Extraction time (minute)	5	10	15
X2	Temperature (°C)	30	40	50
X3	Liquid/solid ratio (mL/g)	40	50	60

2. 9. Determination of Antioxidant Activity

Antioxidant activity of all investigated extracts was estimated by the DPPH test previously described.[7] The antioxidant activity of investigated extracts was expressed as median efficient concentrations (EC_{50}). This is the concentration of extract needed for a decrease in absorbance of DPPH solution to 50%.

2. 10. Statistical Analysis

All the experiments were carried out in triplicate. Values are presented as means ± standard deviation. Significant differences were determined by analysis of variance (ANOVA) followed by Tukey test.

3. Results and Discussion

The input data of three independent variables and five responses needed for CCD experiments are shown in Table 2. The adequate responses were obtained by spec-

Table 2. Central composite design (CCD) of three variables and adequate responses

Exp. No.	Coded variables X1	X2	X3	Responses Yield (%)	TPC (mg GAE[a]/ g)	(+)-catechin (mg/g)	(-)-epicatechin (mg/g)	Total proanthocyanidins (mg CE[b]/g)
1	1	–1	1	9.87	149.99	8.17	9.78	68.22
2	0	0	0	9.45	144.77	8.08	9.64	66.07
3	1	1	–1	7.91	129.67	7.43	7.41	63.06
4	0	0	0	9.45	144.77	8.08	9.64	66.07
5	1	–1	–1	7.78	122.04	6.44	6.97	62.15
6	–1	1	–1	6.41	119.42	6.09	6.64	60.97
7	0	0	0	9.44	144.68	8.02	9.6	66.01
8	0	1	0	9.44	144.73	8.05	9.67	66.06
9	–1	–1	–1	6.04	119.04	6.02	6.42	58.6
10	–1	0	1	7.4	130.07	6.72	6.64	64.51
11	–1	–1	1	7.56	129.28	6.48	6.79	62.14
12	1	1	1	9.36	153.06	8.45	9.81	64.37
13	0	1	0	9.87	147.11	8.01	9.70	60.22
14	0	0	–1	8.69	137.01	7.8	8.84	61.07
15	–1	0	0	7.31	127.12	6.34	6.51	61.29
16	0	0	0	9.45	144.77	8.08	9.64	66.07
17	1	0	0	9.85	145.02	7.07	9.69	62.05
18	0	–1	0	9.57	141.20	8.01	8.71	64.78
19	0	0	0	9.44	144.12	8.11	9.58	66.11
20	0	0	0	9.45	144.72	8.06	9.64	66.05

[a] gallic acid equivalents; [b] catechin equivalents; The values of responses are represented as mean, SD are not given.

trophotometric and HPLC analyses of grape pomace seed extracts obtained by UAE. The HPLC analysis showed that the main compounds from the grape pomace seed extracts were flavan-3-ol monomers and their oligomers (Figure 1).

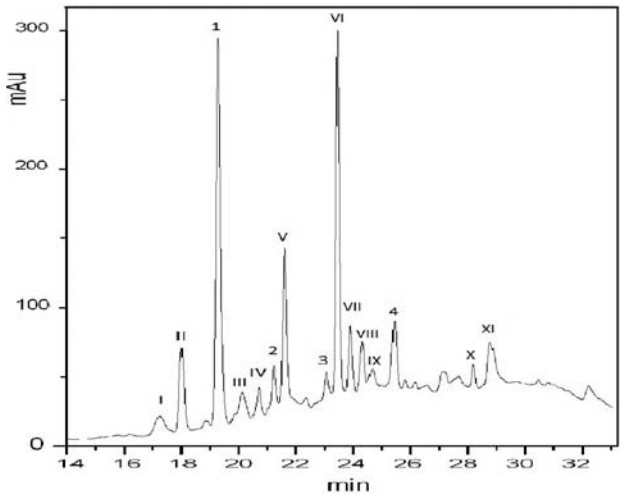

Figure 1. HPLC chromatogram of grape pomace seed extract recorded on fluorescence detector – 275/322 nm (l_{Ex}/l_{Em}); compounds: 1 – (+)-catechin, 2 – (-)-procyanidin B2, 3 – (-)-epicatechin, 4 – (-)-epicatechin gallate; compounds I to XI are flavan-3-ol oligomers.

Three flavan-3-ol monomers, (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate and a dimmer, procyanidin B2, were detected in the seed extract. The content of these compounds per total extracts was 22.12%. We also detected numbers of flavan-3-ol oligomers, numbered as compounds I –XI (Figure 1), in significant quantity. They showed similar UV absorbance spectra to these presented by (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate, and may be assigned to nonacylated procyanidins derived from (+)-catechin and (-)-epicatechin, and esterified procyanidins with gallic acid derived from (-)-epicatechin gallate.[1,2] Due to the lack of standards for these compounds, we have not been able to identify them individually. Their content was expressed as (+)-catechin equivalents (CE) and was 40.01% per total extracts.

After evaluation of 20 trials (Table 2) by fitting all the responses as a function of extraction time (X_1), temperature (X_2) and liquid/solid ratio (X_3), the following polynomial equations were obtained:

$$Y = 9.39 + 1.05X_1 + 0.057X_2 + 0.75X_3 + 0.18X_1X_3 - 0.63X_1^2 - X_2^2 - 0.068X_3^2 \quad (2)$$

$$TPC = 142.94 + 7.37X_1 + 1.18X_2 + 8.37X_3 + 3.67X_1X_3 - 3.93X_1^2 - 10.15X_2 \quad (3)$$

$$(+)\text{-catechin content} = 7.95 + 0.61X_1 + 0.11X_2 + 0.33X_3 + 0.006X_1X_2 - 1.3X_2^2 + 0.51X_3^2 \quad (4)$$

$$(-)\text{-epicatechin content} = 8.35 + 1.01X_1 + 0.036X_2 + 0.8X_3 \quad (5)$$

$$\text{Total proanthocyanidins content} = 65.92 + 2.21X_1 - 0.099X_2 + 1.83X_3 - 0.49X_1X_2 - 0.84X_2X_3 - 1.12X_1^2 - 0.96X_2^2 \quad (6)$$

These polynomial equations corresponding to quadratic (Eq. 2–4, 6) and linear models (Eq. 5) that were highly significant as suggested by the ANOVA results (Table 3). All non-significant coefficients were eliminated to simplify the models.

By optimizing design, the following optimal conditions (extraction time, temperature and liquid/solid ratio) were obtained: 14 min, 43 °C, 54 mL/g for extraction yield; 14 min, 40 °C, 60 mL/g for TPC and (+)-catechin content; 15 min, 50 °C, 60 mL/g for (-)-epicatechin; 13 min, 40 °C, 60 mL/g for total proanthocyanidin content.

Table 3. Analysis of variance (ANOVA) of the chosen models and their coefficients

Source	Response									
	Yield		TPC		(+)-catechin		(-)-epicatechin		Total procyanidins	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Model	64.96	<0.0001	36.34	< 0.0001	4.57	0.0168	5.35	0.0105	15.81	0.0002
X_1	229.17	< 0.0001	76.37	< 0.0001	12.29	0.0067	8.20	0.0118	51.06	< 0.0001
X_2	656.96	< 0.0001	19.2	0.0029	5.34	0.0127	11	0.0094	10	0.0017
X_3	127.79	< 0.0001	105.74	< 0.0001	6.90	0.0101	6.14	0.0156	37.91	0.0002
X_1X_2	0.080 ns	0.7834	1.26 ns	0.2913	9.81	0.0094			5.08	0.0494
X_1X_3	5.54	0.0431	15.29	0.0036	0.23 ns	0.6420			2.15 ns	0.1766
X_2X_3	2.97ns	0.1189	0.34 ns	0.5759	1.37 ns	0.2724			7.29	0.0244
X_1^2	22.10	0.0011	5.77	0.0398	2.59 ns	0.1421			5.49	0.0447
X_2^2	20.31	0.0015	14.12	0.0045	5.40	0.0452			9.4	0.0013
X_3^2	122.34	< 0.0001	0.069 ns	0.7981	10.5	0.0083			2.44 ns	0.1527
Lack of Fit	0.02 ns	0.3255	0.585 ns	0.3314	0.18 ns	0. 2913	0.57ns	0.1121	0.22 ns	0.3547

ns- not significant.

The values of all investigated responses increased by increasing UAE parameters, especially extraction time and liquid/solid ratio. The maximum values of responses were obtained with the extraction temperature between 40 °C and 50 °C. This can be explained by the fact that some of the phenolic compounds are affected by processes oxidation and degradation at the higher temperature. After taking into account all responses for design optimisation calculation, the optimal conditions were extraction time of 15 min, temperature of 40 °C and liquid/solid ratio of 58 mL/g as an optimal for UAE. Desirability bar graph for five responses is shown on Figure 2.

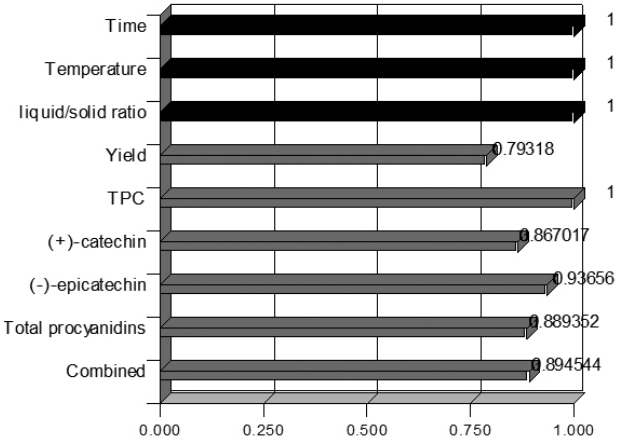

Figure 2. Solution to multiple response optimization - desirability bar graph.

It shows how well each variable satisfies the criteria: values near unity are good. All responses, individually and also their combined effect showed significant desirability.

Under these UAE conditions the response surface prediction was as follows: Y = 9.78%, TPC = 152.14 mg GAE/g, (+)-catechin content = 8.46 mg/g, (-)-epicatechin content = 9.85 mg/g and total proanthocyanidin content = 67.33 mg CE/g. Three-dimensional response surface plots of five response variables at different extraction time, temperatures and liquid/solid ratio, are shown in Figures 3–7. They represent the value of response as a function of two factors while the third stays constant.

Finally, the extracts, obtained under these conditions, by UAE and conventional solvent extraction, were subjected to the spectrophotometric, HPLC analyses and DPPH test. The results of these experiments for UAE (Table 4) showed good agreement with the prediction results obtained by optimization design (RSD was 0.74%). Furthermore, these results showed significantly higher values of all responses than the results obtained by conventional solvent extraction, for prolonged extraction time (60 min). The increasing in extraction yield, TPC, (+)-catechin, (-)-epicatechin and proanthocyanidin content were 16.90, 23.76, 15.41, 21.24 and 23.10%, respectively.

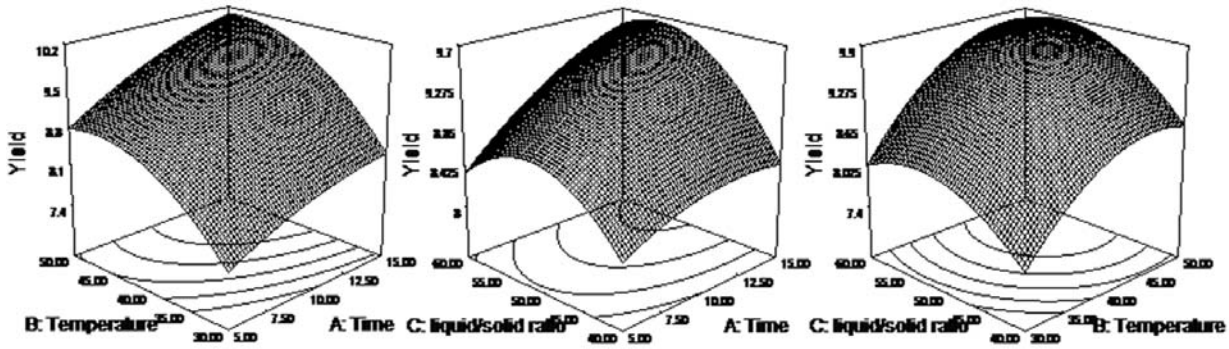

Figure 3. 3-D response surface for combined effect of UAE extraction time, temperature and liquid/solid ratio on extraction yield.

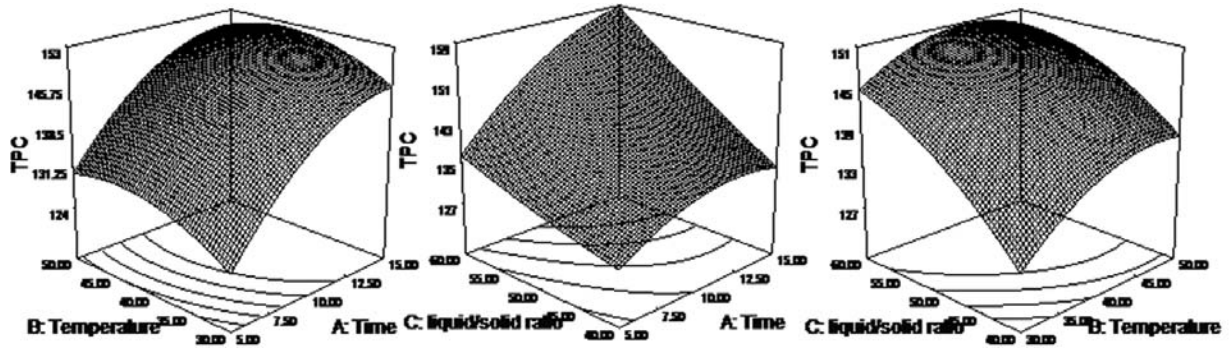

Figure 4. 3-D response surface for combined effect of UAE extraction time, temperature and liquid/solid ratio on total phenol content**.**

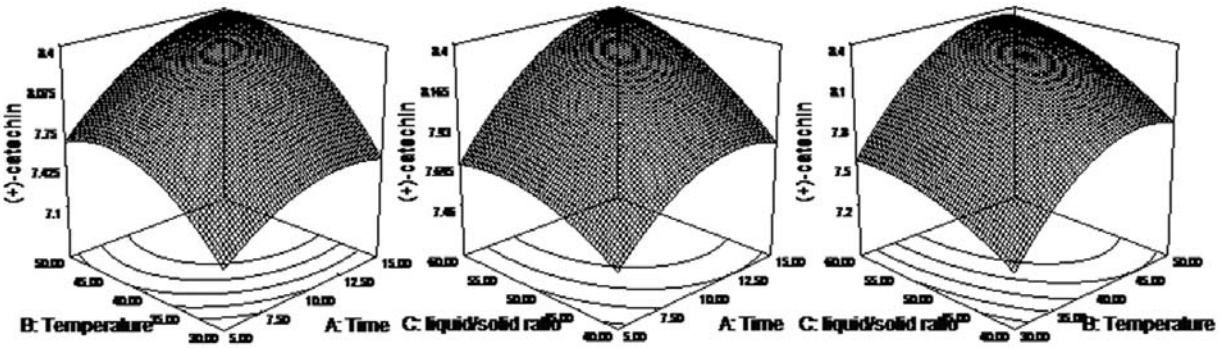

Figure 5. 3-D response surface for combined effect of UAE extraction time, temperature and liquid/solid ratio on (+)-catechin content**.**

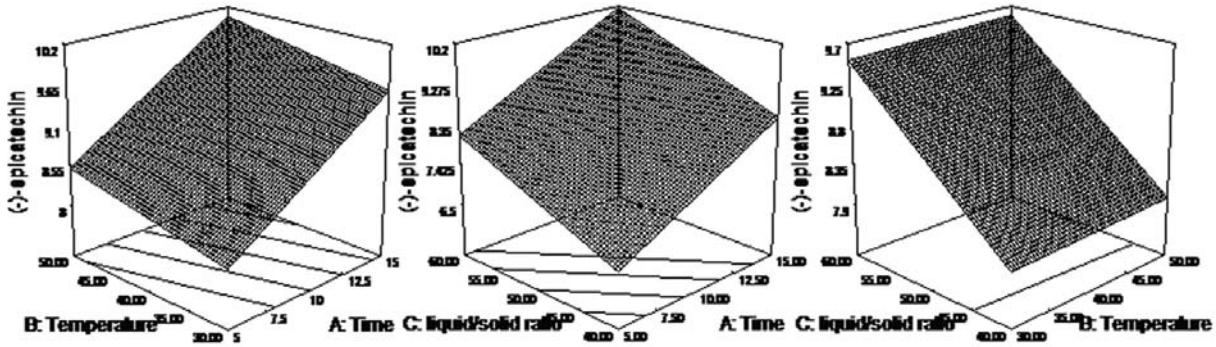

Figure 6. 3-D response surface for combined effect of UAE extraction time, temperature and liquid/solid ratio on (-)-epicatechin content**.**

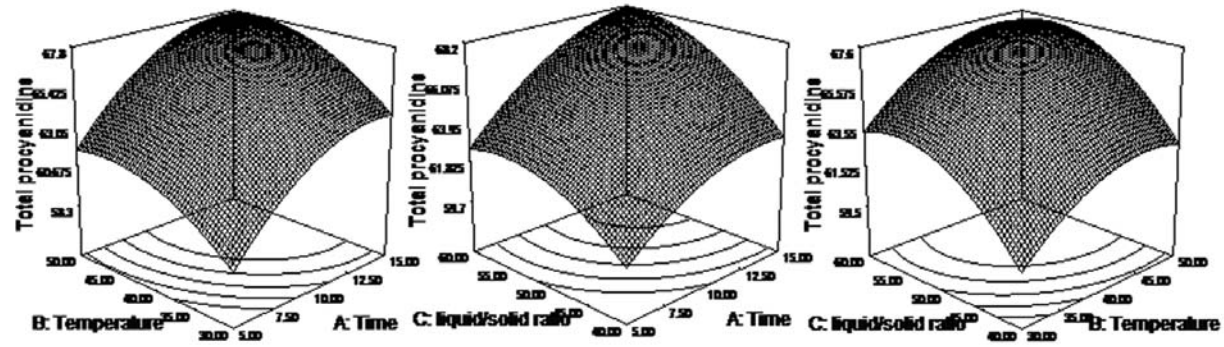

Figure 7. 3-D response surface for combined effect of UAE extraction time, temperature and liquid/solid ratio on proanthocyanidin content**.**

In addition, the results of antioxidant activities of these extracts, expressed as EC_{50} values (mg/g DM), estimated by DPPH test, are shown in Table 3. Lower EC_{50} values correspond to higher antioxidant activity of extracts. The UAE extracts showed significantly higher antioxidant activity than extracts obtained by a conventional solvent extraction (34.54% increase). The antioxidant activity of seed extracts from UAE corresponds to higher phenol content and suggests that the phenolic compounds at least partially are responsible for the strong antioxidant activity of these extracts. The literature data also confirm the strong antioxidant activity of seed extracts and high correlation with total phenol content.[4,6] Although flavan-3-ols greatly contributed to the antioxidant activity of the extracts, other classes of phenolic compounds also effect on the total antioxidant activity of the extracts. Their influence on the antioxidant activity may be explain by the synergetic effects that occur among different phenolic compound in complex mixtures such as investigated extracts.[4] Significantly lower antioxidant activity of extracts obtained by conventional solvent extraction can be explained by the fact that degradation of the antioxidant substances occurs during their exposure to prolonged extraction time compared to the UAE time. Overall, if we want to obtain extracts that showed strong antioxidant activity, we must select the extraction conditions that ensure a high content of TPC and maximizing as much as possible the other responses.

Table 4. The extraction yield (Y), total phenol content (TPC) and radical scavenging activity (EC_{50}) of Vranac grape pomace seed extracts obtained by ultrasound assisted extraction (UAE) on extraction time of 15 min, temperature of 40 °C and liquid/solid ratio of 58 mL/g and conventional solvent extraction on extraction time of 60 min, temperature of 40 °C and liquid/solid ratio of 58 mL/g

Investigated parameters	UAE	Conventional solvent extraction
Y (%)	9.64 ± 0.36[a]	8.01 ± 0.37[b]
TPC (mg GAE/g)	148.55 ± 1.89[a]	113.25 ± 1.91[b]
(+)-Catechin (mg/g)	8.37 ± 0.39[a]	7.08 ± 0.11[b]
(-)-Epicatechin (mg/g)	9.70 ± 0.34[a]	7.64 ± 0.41[b]
Proanthocyanidins (mg CE/g)	65.94 ± 0.76[a]	50.71 ± 0.73[b]
EC_{50} (mg/g)	0.36 ± 0.31[b]	0.55 ± 0.05[a]

Data are expressed as mean ± SD (n = 3). Means in the same row bearing different letters are significantly different ($p < 0.05$), as analyzed by the Tukey test.

4. Conclusion

The ultrasound-assisted extraction (UAE) has proved to be a very good technique for the extraction of phenolic compounds. The extracts obtained by UAE had values of TPC for 23.76% higher compared to conventional solvent extraction, thereby covering the additional energetic input, needed for ultrasound application. Secondly, UAE permits higher extraction yields in shorter periods of time, thereby reducing the energy input and degradation of phenolic compounds. Thirdly, the extracts obtained by UAE showed significantly stronger antioxidant activity than extracts obtained by conventional extraction techniques (34.54% stronger).

The response surface methodology (RSM) proved to be a very powerful statistical method for optimization of extraction conditions. The prediction by central composite design (CCD) is suitable for obtaining extracts that are rich in phenolic content. These extracts, with strong antioxidant activity, could be used as additives in food and medicaments.

5. Acknowledgments

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Povzetek

Namen raziskav je optimiranje pogojev ultrazvočne ekstrakcije (UAE) fenolov iz pulpe pešk grozdja sorte Vranac (*Vitis vinifera L.).* Vzorci so bili analizirani spektrofotometrično in s pomočjo HPLC. Najmanjše število potrebnih eksperimentov je bilo določeno s pomočjo metode površinskih odzivov (response surface method; RSM). UAE je zelo odvisna od treh neodvisnih spremenljivk: temperature, časa in razmerja tekoče/trdno. RSM je bila uporabljena tudi za optimiranje pogojev ekstrakcije glede na maksimalni izkoristek in vsebnosti posameznih komponent. Vrednosti, napovedane z modelom se ujemajo z eksperimentalnimi podatki, ki potrjujejo tudi boljši izkoristek UAE v primerjavi s klasično solventno ekstrakcijo. Z uporabo UAE pri optimalnih pogojih dobimo ekstrakte bogate s fenoli in močno antioksidacijsko aktivnostjo, kar se lahko uporablja v prehranskih dodatkih in medikamentih.