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Solid-liquid Extraction of Phenolics from Red Grape Skins

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Abstract

For the characterization of grape cultivars, the profile and content of flavonoids are important because these compounds have an impact on grape and wine quality. A new extraction method for the recovery of flavonoids, e.g. anthocyanins, flavonols and flavan-3-ols from grape skins was developed. The optimization of solid-liquid extraction of flavonoids was conducted, with respect to the type of the organic solvent and its percentage in the extraction solvent as well as the extraction temperature and extraction time, using response surface methodology. Optimal conditions were obtained by using extraction solvent composed from acetonitrile:water:formic acid (20:79:1; *v/v/v*), at an extraction temperature of 50 °C, an extraction time of 1 h in a single-step extraction and with a solid-to-solvent ratio of 1:80 g mL^{-1} (125 mg of grape skin powder and 10 mL of extraction solvent). The new optimal extraction method is inexpensive, simple, fast, accurate and selective for the recovery of simple flavonoids.

Keywords: Grape skins; solid-liquid extraction; flavonoids; multi-response optimization

1. Introduction

Phenolics are a large and structurally diverse class of molecules present in different plant spacies which could be divided in many subgroups based on their structure. Flavonoids are one of the largest phenolic subgroup which play a very important role in growth, reproduction and in various defense reactions in plants. Based on the oxidation state of phenol rings in the flavonoid structure, they can be divided to anthocyanins, flavonols, flavan-3-ols, flavanones, isoflavones, chalcones, flavananols and xanthones. Many of these compounds have biological activities like antioxidative, antifungal, antimicrobial and anti-inflammatory properties thus they have a positive effect on human health.[1,2] Grapes contain large amount of different flavonoids such as anthocyanins, flavonols and flavan-3-ols.[3]

In the last decade a variety of new extraction techniques have been developed such as ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and many others. These techniques may be applied for the extraction of phenolics from different plant matrices. Based on the literature, it is evident that application of these techniques for extraction of phenolics from grape skins is very rare. There is only a few studies concerning UAE,[4,5] MAE[6] and SFE.[7] Application of SFE for extraction of phenolics is limited due to their polarity. In our last study UAE method was optimized for the recovery of flavonoids from grape skins,[4] but nowadays SLE is the most used technique for extraction of phenolics from the grape skins.[8–16] This extraction technique has many advantages, the most important being no need to invest in new equipment and the possibility of extracting multiple samples at once. Thus, these facts greatly reduce the price of the analysis, and increase the efficiency of labor in cases where it is necessary to analyze large numbers of samples. Over the past six decades, hundreds of publications on analysis of grape phenolic compounds have been published, but there is still no available standardized procedure for the extraction.[17] Different authors used different extraction conditions for recovery of

flavonoids from grape skins. Solvents, like methanol,[8–10] acetone,[18] ethyl acetate,[19] ethanol[11] and their combination with different portions of water with or without addition of acid,[12–15,20] are the common extraction solvents for recovery of flavonoids from grape berry skin. Portion of water can be from 0%[8–10] to 50%.[11–15,20] One of the most commonly used extraction solvent is a mixture of ethanol:water:formic acid (70:29:1, *v/v/v*).[12,15,21] Extraction time can be less than 5 min[14,15] to up to few days[9] while the temperature can be from 4 °C[11] to 70 °C.[22] Solid-to-solvent ratio and number of extraction steps are parameters that have great influence on extraction yield and they can be in the range from 1:2[16] to 1:80 g mL^{-1} [22] and from 1[8,12,14,21] to 9,[10] respectively. From the quantitative point of view, results obtained by different extraction methods could not be compared, because different extraction conditions would have different extraction efficiency.

Thus the aim of the present study was to optimize the most popular extraction technique, SLE, to obtain the best extraction conditions for the recovery of individual flavonoids from grape berry skins. Several parameters: the type of organic solvent, the percentage of organic phase in the extraction solvent, the extraction temperature and the extraction time, which could affect the extraction efficiency were evaluated and optimized using a response surface methodology (RSM) and employing a Box-Behnken experimental design (BBD).

2. Experimental

2. 1. Chemicals

Acetonitrile and methanol of HPLC grade were purchased from J. T. Baker (Deventer, Netherlands). Formic acid and 85% orthophosphoric acid were obtained from Fluka (Buchs, Switzerland). Ethanol and acetone were provided from Kemika (Zagreb, Croatia).

The standards used for identification and quantification purposes were as follows: delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin-3,5-*O*-diglucoside, cyanidin-3,5-*O*-diglucoside, malvidin-3,5-*O*-diglucoside, epigallocatechin, procyanidin B1, procyanidin B2, rutin, and myricetin (Extrasynthese, Genay Cedex, France); (–)-epicatechin and (+)-catechin (Sigma-Aldrich, St. Louis, MO, USA); kaempferol, quercetin-3-*O*-glusoside and isorhamnetin (Fluka, Steinheim, Germany); quercetin-3-*O*-glucoside (Sigma, St. Louis, MO, USA). All standards were analytical standard grade.

2. 2. Grape Preparation

Grape samples ('Regent') were obtained in 2012 and 2013 from the vineyard located at the Experimental station Jazbina, Faculty of Agriculture, University of Zagreb, Croatia. Grapes were harvested in a state of full ripeness and immediately separated from the stalk. To obtain homogenous samples of the berries at a similar level of ripeness (sugar and flavonoid content), a simple flotation method was used with sucrose water solutions of different densities. Grape berries with a density range of 1.088 to 1.099 g cm^{-3} were selected for further analysis. The berry skins were manually removed from the pulp and freeze-dried. Dry skins were ground (Coffee Grinder SMK150, Gorenje, Slovenia) and powder obtained was stored (2 °C) in a glass container.

2. 3. Extraction of Flavonoids from Grape Skin

All experiments were completed on a magnetic stirrer (RCT basic, IKA, Staufen, Germany) at 400 rpm. After extractions were complete, supernatants were collected, concentrated under a vacuum to remove organic modifier (40 °C) on a Hei-Vap Adventage G3 rotary evaporator (Heidolph, Schwabach, Germany) and brought to a final volume of 10 mL with eluent A (water:phosphoric acid, 99.5:0.5, *v/v*). The extracts were filtered with a Phenex-PTFE (polytetrafluorethylene) 0.20 μm syringe filter (Phenomenex, Torrance, USA) and analyzed by HPLC.

Before setting the levels of the studied factors some preliminary experiments using the one-factor-at-the-time methodology were necessary. For determination of appropriate organic phase in extraction solvent the following extraction solvents composed of ethanol:water (70:30; *v/v*), methanol:water (70:30 *v/v*) and ethanol:water:formic acid (70:29:1; *v/v/v*) were used. Temperature and time of extraction was 30 °C and 3 h, respectively, while the extractions were performed in a single step with the solid-to-solvent ratio of 1:80 g mL^{-1} (125 mg of grape skins powder and 10 mL of the extraction solvent).

The optimal range of extraction time (30 min–24 h) was determined in a single-step extraction by an extraction solvent composed of ethanol:water:formic acid (70:29:1; *v/v/v*). The solid-to-solvent ratio was 1:80 g mL^{-1} (125 mg of grape skins powder and 10 mL of the extraction solvent) while the temperature was 30 °C. The extract was centrifuged in a LC-321 centrifuge (Tehtnica, Železniki, Slovenia) for 20 min at 5000 rpm at room temperature. All experiments were performed in triplicate.

2. 4. Experimental Design and Statistical Analysis

After determining the optimal conditions of the extraction factors through the single-factor tests, the effect of the three numerical factors (the percentage of organic phase in the extraction solvent, the extraction temperature and the extraction time) and one categorical factor (the type of organic solvent) on the content of individual anthocyanins, flavonol glycosides and flavan-3-ols were studied through the Box-Behnken experimental design and response surface methodology. The BBD is very efficient for studies with a high number of factors.[23] These four independent factors

Table 1. Independent factors and their levels used in the response surface design

Factors	Factor level		
Coded levels	–1	0	1
A: Percentage of organic solvent (%)	20	50	80
B: Extraction temperature (°C)	30	45	60
C: Extraction time (h)	1	2	3
D: Type of organic solvent	Acetonitrile	Ethanol	Acetone

were investigated at three different coded levels (Table 1). Extraction solvents were composed of appropriate percentage of organic solvent (20, 50 and 80%), water (79, 49 and 19%) and 1% of formic acid. In all experiments solid-to-solvent ratio was fixed at 1:80 g mL^{-1} which means that mass of grape skin powder was 125 mg while the volume of appropriate extraction solvent was 10 mL.

The resulting contents of the 3-*O*-glucoside and 3,5-*O*-diglucoside of delphinidin, cyanidin, peonidin and malvidin; those of 3-*O*-glycoside quercetin, myricetin, kaempferol and isorhamnetin and those of gallocatechin, procyanidin B1, procyanidin B2, catechin, epicatechin and epigallocatechin were used as responses (*Y*, dependent variables). The results of the BBD experiments were analyzed by non-linear multiple regression with backward elimination to fit the following second-order equation to the dependent *Y* variables:

$$Y = \beta_0 + \sum\beta_i x_i + \sum\beta_{ij} x_i x_j + \sum\beta_{ii} x_i^2 \quad (I = 1, 2\ldots k) \qquad (1)$$

β_0, β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction effects, respectively, x_i and x_j are the levels of independent factors in the coded values. Coefficients were interpreted using an *F*-test. To establish the optimum conditions for individual anthocyanin, flavonol glycoside and flavan-3-ol contents, analysis of variance (ANOVA), regression analysis and plotting of the response surface plot were conducted. For optimization multicriteria methodology (Derringer function or desirability function) was used. This methodology is applied when various responses must be considered at the same time, and it is necessary to find optimal compromises between the total number of considered responses. The optimal experimental conditions were based on the maximal content of the individual flavonoids.[24]

The analysis of the experimental design and calculation of the predicted data was completed using the Design Expert software (Trial Version 9.0.3.1, Stat-Ease Inc., Minneapolis, USA).

The mean values, standard deviations and significant differences of the data were calculated and reported using OriginPro 8 (OriginLab Corporation, Northampton, USA). The results were analyzed with ANOVA and the differences between the means were evaluated by Tukey's post-hoc test at a confidence level of 95% ($p < 0.05$). The data reported in all of the tables were the average of triplicate observation.

2. 5. HPLC Analysis

Separation, identification and quantification of flavonoids from grape skin extracts were performed according to the method described by Tomaz and Maslov[25] on an Agilent 1100 Series system (Agilent, Germany), equipped with autosampler, column thermostat, diode array detector (DAD) and fluorescence detector (FLD). The separation was performed with a reversed-phase column Luna Phenyl-Hexyl (4.6 × 250 mm; 5 μm particle (Phenomenex, Torrance, USA)) heated at 50 °C. The solvents were water:phosphoric acid (99.5:0.5, *v/v*, eluent A) and acetonitrile:water:phosphoric acid; 50:49.5:0.5, *v/v/v*, eluent B), and the flow rate was 0.9 mL min^{-1}. The gradient for eluent B was: 0 min, 0%; 7 min, 20%; 35 min, 40%; 40 min, 40%; 45 min, 80%; 50 min, 100%; 60 min 0%. The injection volume for all samples was 20 μL. Flavonol glycosides were detected at 360 nm while anthocyanins at 518 nm using DAD, while flavan-3-ols were detected at λ_{ex} = 225 nm and λ_{em} = 320 nm by means of FLD.

2. 6. LC-MS Analysis

For peak assignment, grape skin extracts were analyzed with Agilent 1200 Series system (Agilent, Germany) coupled on-line to an Agilent model 6410 mass spectrometer fitted with ESI source. The separation was performed with column described in the previous section with the solvents water:formic acid (99.5:0.5, *v/v*, eluent A) and acetonitrile:water:formic acid; 50:49.5:0.5, *v/v/v*, eluent B). Eluation gradient was same as previously described while the flow rate was 0.5 mL min^{-1}. The mass spectra of flavan-3-ols and flavonols were recorded in the negative mode while those of anthocyanins in the positive mode. Negative and positive ion mass spectra of column eluate were recorded in the range *m/z* 100–1000. The electrospray ionization (ESI) parameters were as following; drying gas (N_2) flow and temperature, 8 L min^{-1} and 300 °C, nebulizer pressure 30 psi, capillary voltage 4500 V for negative ion mode or –4500 V for positive ion mode. Fragmentation voltage was 135 V.[25]

2. 7. Quantification of Individual Compounds from Grape Skin Extracts

Individual flavonoids in grape berry skin extracts were identified by matching the retention time of each chro-

matographic peak with external standards and DAD spectrum. Quantification of individual flavonoid peaks was done by using a calibration curve of the corresponding standard compound which was based on the peak area. Range for calibration curves and related regression equation together with limits of detection and limits of quantification were described in our previous study.[25] When reference compounds were not available, the calibration by a structurally related compound was used. For quantification of myricetin-3-*O*-glucoside, kaempferol-3-*O*-glucuronide and isorhamnetin-3-*O*-glucoside appropriate aglycons were used. Contents of gallocatechin and peonidin-3,5-*O*-diglucoside were expressed in epigallocatechin and peonidin-3-*O*-glusoside equivalents. Results are expressed in mg kg^{-1} of dry weight (d.w.) of grape skin. In preliminary tests contents of particular class of flavonoids, namely anthocyanin contents (AC), flavonol glycoside contents (FGC) and flavan-3-ol contents (FC), were expressed as sum of content of individual compounds determined by HPLC.

3. Results and Discussion

Cultivar 'Regent' is one of the successful newly bred varieties obtained by back-crossing hybrids of *Vitis vinifera* L. and some other *Vitis* species possessing fungal resistance, with high quality grapevine cultivars. Species *V. vinifera* contains only anthocyanin-3-monoglucosides while most of other *Vitis* species together with anthocyanin-3-monoglucosides contain dominant allele for synthesis of anthocyanin-3,5-diglucosides. Thus, this characteri-

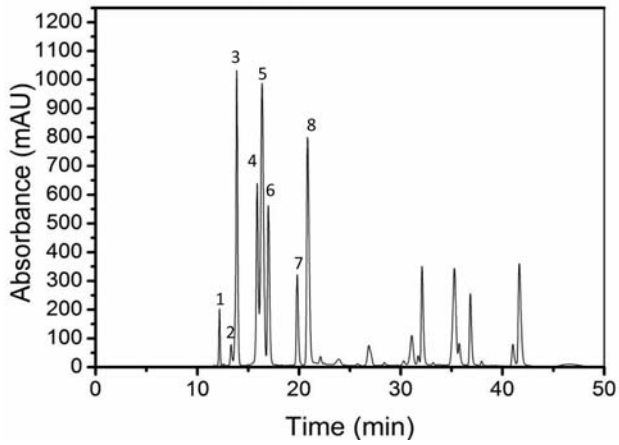

Fig. 1. Chromatogram of anthocyanins recorded at 518 nm. Peak assignation: 1. Delphinidin-3,5-*O*-diglucoside, 2. Cyanidin-3,5-*O*-diglucoside, 3. Delphinidin-3-*O*-glucoside, 4. Peonidin-3,5-*O*-diglucoside, 5. Malvidin-3,5-*O*-diglucoside, 6. Cyanidin-3-*O*-glucoside, 7. Peonidin-3-*O*-glucoside, 8. Malvidin -3-*O*-glucoside

Table 2. Effect of the extraction solvent composition on the contents of individual flavonoids from grape skins. Results are expressed as mg kg^{-1} dry weight of grape skin

Compound	70% Methanol $\tilde{Y}$ ± SD	70% Ethanol $\tilde{Y}$ ± SD	70% Ethanol + 1% formic acid $\tilde{Y}$ ± SD
Delphinidin-3,5-*O*-diglucoside	864.36 ± 24.73[a]	1040.47 ± 10.71[b]	1340.47 ± 4.01[c]
Cyanidin-3,5-*O*-diglucoside	1122.89 ± 29.73[a]	1171.23 ± 7.46[a]	1347.41 ± 7.50[b]
Delphinidin-3-*O*-glucoside	9284.13 ± 71.73[a]	9006.86 ± 73.90[b]	10960.35 ± 61.00[c]
Peonidin-3,5-*O*-diglucoside	954.63 ± 4.51[a]	940.65 ± 5.27[a]	1127.25 ± 14.01[b]
Malvidin-3,5-*O*-diglucoside	13630.26 ± 30.00[a]	13909.08 ± 44.76[b]	15766.92 ± 57.85[c]
Cyanidin-3-*O*-glucoside	1796.35 ± 12.91[a]	1716.18 ± 13.86[b]	2053.23 ± 10.01[c]
Peonidin-3-*O*-glucoside	485.82 ± 7.51[a]	508.18 ± 8.83[a]	590.17 ± 11.00[b]
Malvidin -3-*O*-glucoside	6783.82 ± 10.71[a]	6988.87 ± 50.23[b]	7832.98 ± 30.00 [c]
Total anthocyanins	**34922.26 ± 105.34[a]**	**35281.52 ± 100.76[a]**	**41018.78 ± 110.32[b]**
Myricetin-3-*O*-glucoside	481.91 ± 5.54[a]	528.04 ± 7.00[b]	550.44 ± 11.83[c]
Rutin	245.11 ± 5.02[a]	250.71 ± 4.27[a]	255.11 ± 5.03[a]
Quercetin-3-*O*-glucoside	1587.82 ± 15.27[a]	1661.67 ± 10.50[b]	1720.70 ± 16.91[c]
Kaempferol-3-*O*-glucuronide	54.06 ± 5.49[a]	69.61 ± 9.51[b]	111.01 ± 10.03[c]
Isorhamnetin-3-*O*-glucoside	92.57 ± 2.44[a]	106.11 ± 4.47[b]	110.14 ± 2.02[b]
Total flavonol glycosides	**2462.47 ± 9.37[a]**	**2616.14 ± 10.32[b]**	**2747.40 ± 18.82[c]**
Gallocatechin	6.06 ± 1.01[a]	7.27 ± 1.94[a]	11.02 ± 0.97[b]
Procyanidin B1	49.16 ± 2.02[a]	53.18 ± 3.04[a,b]	59.05 ± 2.09[b]
Epigallocatechin	25.32 ± 2.08[a]	24.04 ± 3.12[a]	36.71 ± 1.03[b]
Catechin	7.73 ± 2.52[a]	14.25 ± 3.03[b]	12.48 ± 2.49[b]
Procyanidin B2	0.73 ± 0.64[a]	0.66 ± 0.57[a]	17.68 ± 0.64[b]
Epicatechin	20.54 ± 1.53[a]	20.77 ± 3.68[a]	26.93 ± 0.91[b]
Total flavan-3-ols	**109.54 ± 3.22[a]**	**120.17 ± 5.65[b]**	**163.87 ± 4.87[c]**

$\tilde{Y}$ mean value (n = 3). SD standard deviation. Superscript letters a, b, and c indicate grouping within a row. Different letters show statistical difference $p < 0.05$

stic was retained in 'Regent' and it contains anthocyanin-3,5-diglucosides together with anthocyanin-3-monoglucosides (Fig. 1). This cultivar contains very high content of flavonols and flavan-3-ols, as well.[26]

3. 1. Effect of the Extraction Solvent Composition on the Recovery of Flavonoids from Grape Skins

The extraction efficiency is strongly dependent on the type of solvent. Solubility of flavonoids is governed by the polarity of the solvents used. Depending on the solvent system used for the extraction, a mixture of different compounds, such as flavonoids and non-phenolic compounds (sugars, organic acids and fats) soluble in the solvent will be extracted from grape berry skins. Selection of the appropriate extraction solvent is necessary to achieve excellent effectiveness and good selectivity. Methanol and ethanol have similar physico-chemical properties. Some experiments were conducted to make a choice which alcohol will be used in further studies. In these experiments, extraction solvents were composed of 70% of appropriate alcohol in water. For the most of the examined compounds from grape skins, ethanol was better organic solvent (Table 2). This finding could be explained by the viscosity of alcoholic solutions. Water solutions of methanol are more viscous than equivalent ethanolic solutions.[27,28] The diffusion of the analytes from the solid samples to the bulk solvent is facilitated in the media of low viscosity. This observation is in accordance with results obtained by Lapornik et al.[16] So based on obtained results and the fact that ethanol is environmentally benign and relatively safe for human health, it was chosen as an organic modifier in the following experiments. According to the literature, acetone is an excellent solvent for the recovery of flavan-3-ols and particularly for the extraction of oligomeric and polymeric forms from grape skins. Based on the experience gained during development of the HPLC method for the separation of phenolics,[25] acetonitrile was selected as a third organic phase. Flavonoids, especially anthocyanins, are the most stable at very low pH when they are in flavylium form; so it is necessary to conduct extraction in an acidic environment. Acylated anthocyanins and flavonol glycosides are labile in solutions containing mineral acid thus it is mandatory to add a weaker acid such as formic acid to the extraction solvent. By adding formic acid in the extraction solvent, a significant recovery increase was observed for all of the examined flavonoids from grape skins (Table 2). Bakker et al.[29] showed that artefacts such as formylated derivates of anthocyanins can be obtained using an extraction solvent containing 2% formic acid. By close inspection of the chromatograms recorded at 518 nm after injection of the extract obtained by extraction solvent containing 1% formic acid, these artefacts were not observed. Based on the obtained results extraction solvents with the appropriate organic phases and 1% of formic acid were selected for further experiments.

3. 2. Effect of the Extraction Time on the Recovery of Flavonoids From Grape Skins

The contents of anthocyanins, flavonol glycosides and flavan-3-ols from grape skins at different extraction times are presented in Fig. 2. Extraction was conducted

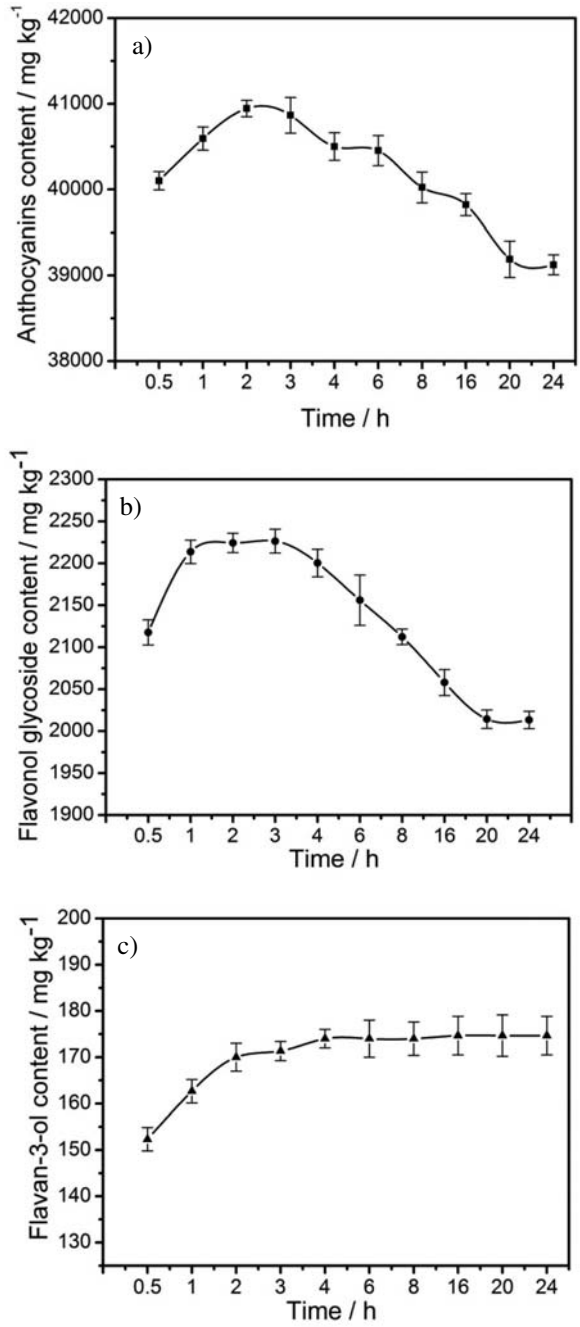

Fig. 2. Effect of extraction time on a) anthocyanin contents; b) flavonol glycoside contents and c) flavan-3-ol contents from grape skins. Other extraction conditions were as follows: single-step extraction by an extraction solvent composed of ethanol:water:formic acid (70:29:1; *v/v/v*), the solid-to-solvent ratio of 1:80 g mL^{-1} and temperature of 30 °C.

for different time periods (30 min, 1, 2, 3, 4, 6, 8, 16, 18, 20 and 24 h) while the following factors were constant: solid-to-solvent ratio 1:80 g mL^{-1}, single-step extraction and temperature of 30 °C. A significant increase of AC was observed between 1 and 3 h. The same trend follows FGC and FC. Longer extraction times led to a slight decrease of AC and FGC but FC remained at a steady value. Therefore, in the experimental design time periods between 1 and 3 h were used.

3. 3. Optimization of Extraction Conditions by Response Surface Methodology

For the optimization of the best extraction conditions of flavonoids from grape skins, research was focused on the effect of the extracting solvent (percentage of organic modifier), extraction time and temperature. The 45 experimental conditions, including three replicates at the center point, were chosen (Supplemental Table 1).

All of the target compounds were best described by a quadratic polynomial model. Catechin was best described by a linear model. Parameters describing a model are presented in Supplemental Table 2.

The effect of solvent type was the most significant factor. The effect of acetonitrile, ethanol and acetone are described in Table 4. The highest contents of the great majority of the examined compounds from grape skins were obtained using acetonitrile as organic modifier (Table 3). Different percentage of organic modifier was also examined, and the best recoveries were at 20%.

An enhancement in the extraction temperature increased anthocyanins and flavonol glycosides recoveries. The optimal temperature for extraction of these phenolic compounds was up to 50 °C. At higher temperatures significant decomposition of anthocyanins and flavonol glycosides was noticed. The optimum extraction time of 1 h was sufficient extraction time for almost all of the target analytes. A prolonged time of extraction showed to have a positive effect on the recovery of flavan-3-ols.

The interactions of the extraction factors were studied from the contour plots generated by the model. It was observed that for the most compounds from grape skins, a clear interaction between the various extraction factors exists (Figs. 3–5). The most frequent interaction effect was observed between the percentage of the organic phase in the extraction solvent and the extraction temperature. Raising the temperature reduces the viscosity of the extraction solvent and enhances extraction. The percentage of organic modifier in the extraction solvent had a great effect on the polarity of the extraction media. According to the well-known principle for predicting solubility »like dissolves like«, the optimal ex-

Table 3. Optimal extraction conditions for the recovery of individual and all together flavonoids determined by response surface methodology. Predicted and experimental values are expressed in mg kg^{-1} dry weight of grape skin

Compound	Organic modifier	Percentage of organic phase (%)	Temperature (°C)	Time (h:min)	Desirability	Predicted values	Experimental values $\tilde{Y}$ ± SD
Delphinidin-3,5-*O*-diglucoside	Ethanol	64	46	1:02	1.000	1596.26	1589.37 ± 9.27
Cyanidin-3,5-*O*-diglucoside	Ethanol	20	45	1:00	1.000	1559.22	1548.48 ± 16.34
Delphinidin-3-*O*-glucoside	Acetonitrile	20	43	1:00	1.000	12346.60	12362.42 ± 55.31
Peonidin-3,5-*O*-diglucoside	Ethanol	20	49	1:00	1.000	1158.68	1143.67 ± 12.23
Malvidi-3,5-*O*-diglucoside	Acetonitrile	20	45	1:00	1.000	16772.28	16738.07 ± 44.03
Cyanidin-3-*O*-glucoside	Acetonitrile	20	39	1:00	1.000	2499.56	2471.87 ± 22.66
Peonidin-3-*O*-glucoside	Acetonitrile	20	42	1:00	1.000	664.81	658.22 ± 7.19
Malvidin -3-*O*-glucoside	Acetonitrile	20	45	1:00	1.000	8947.09	8930.59 ± 40.18
Total anthocyanins	**Acetonitrile**	**21**	**44**	**1:00**	**1.000**	**44524.03**	**44987.32 ± 59.32**
Myricetin-3-*O*-glucoside	Ethanol	20	45	1:00	1.000	658.19	637.76 ± 12.81
Rutin	Ethanol	20	45	1:00	1.000	310.58	307.21 ± 16.01
Quercetin-3-*O*-glucoside	Acetonitrile	80	45	1:00	1.000	1944.26	1951.61 ± 9.75
Kaempferol-3-*O*-glucuronide	Acetonitrile	20	50	1:36	1.000	83.05	81.30 ± 5.15
Isorhamnetin-3-*O*-glucoside	Acetonitrile	20	41	1:18	1.000	122.83	119.06 ± 7.42
Total flavonol glycosides	**Acetonitrile**	**21**	**45**	**1:04**	**1.000**	**3249.22**	**3265.32 ± 15.38**
Gallocatechin	Acetonitrile	70	40	1:00	1.000	16.71	15.84 ± 1.32
Procyanidin B1	Acetone	80	60	1:37	1.000	92.11	93.47 ± 3.97
Epigallocatechin	Acetonitrile	21	59	3:00	1.000	43.23	40.94 ± 2.07
Catechin	Acetonitrile	80	60	3:00	1.000	28.78	28.57 ± 1.25
Procyanidin B2	Acetone	20	60	1:00	1.000	43.83	42.03 ± 1.97
Epicatechin	Acetronitrile	20	60	1:00	1.000	32.35	34.72 ± 3.06
Total flavan-3-ols	**Acetonitrile**	**20**	**60**	**2:00**	**1.000**	**229.58**	**220.34 ± 8.25**
All together	**Acetonitrile**	**20**	**50**	**1:00**	**0.797**		

traction yield may be achieved when the polarity of the extraction solvent and analytes coincide. When the optimum viscosity and polarity are fulfilled, the maximum extraction yield was achieved. For nearly all of the studied anthocyanins from grape skins optimum temperature is around 45 °C with 20% acetonitrile in the extraction solvent (Fig. 3.).

In the case of some compounds, namely delphinidin-3-*O*-glucoside, delphinidin-3,5-*O*-diglucoside (Fig. 3.) and procyanidin B2 (Fig. 4.) the interaction between the extraction time and the percentage of organic modifier in the extraction solvent are the most important ones. This observation could be explained by the polarity. The dissolution of the target analytes is faster in the

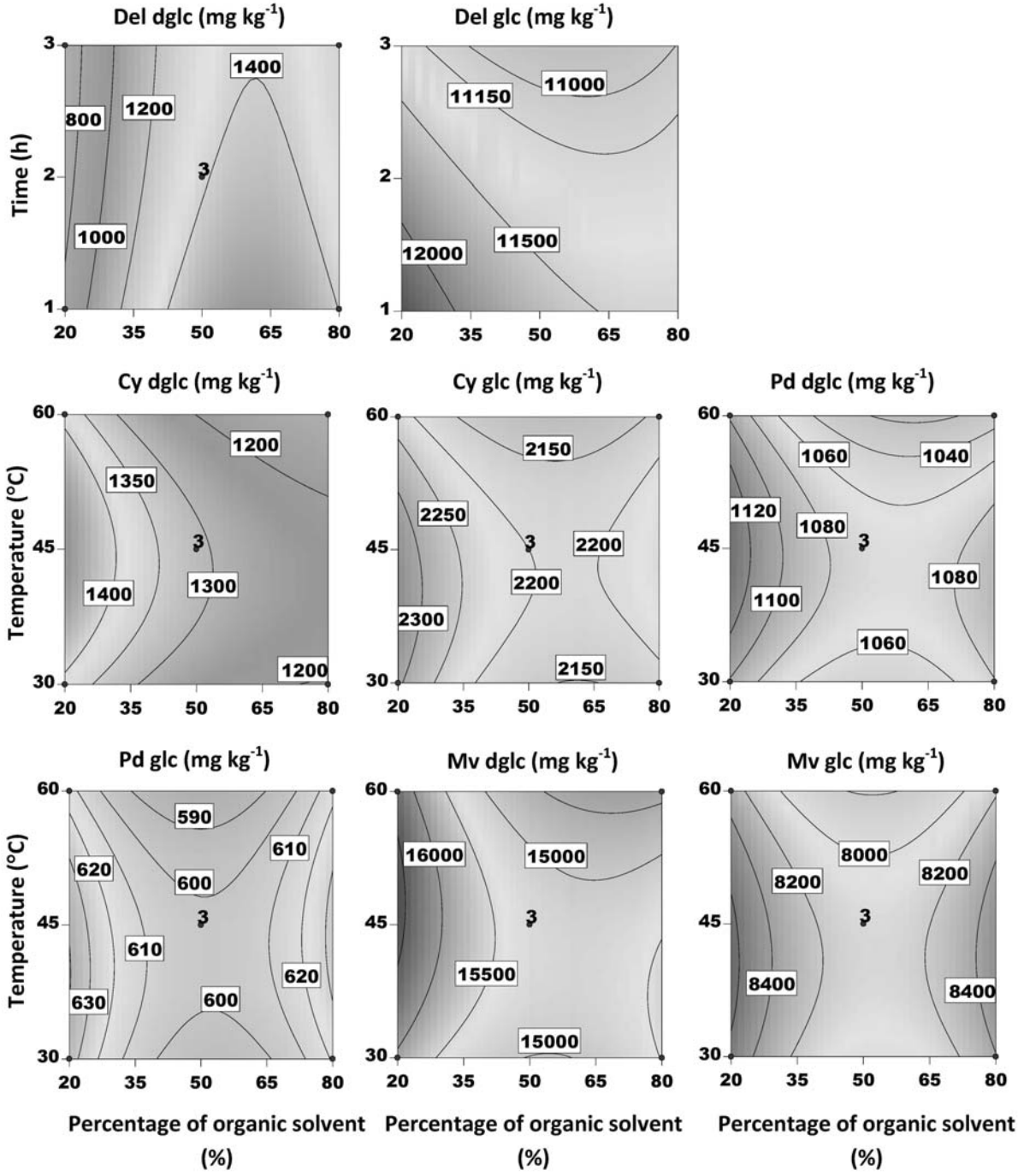

Fig. 3. Contour plots of most prominent interaction factors for individual anthocyanins from grape skins using acetonitrile as organic modifier. Cy dglc – Cyanidin-3,5-*O*-diglucoside; Cy glc – Cyanidin-3-*O*-glucoside; Del glc – Delphinidin-3-*O*-glucoside; Del dglc – Delphinidin-3,5-*O*-diglucoside; Mv dglc – Malvidin-3,5-*O*-diglucoside; Mv glc – Malvidin-3-*O*-glucoside; Pd dglc – Peonidin-3,5-*O*-diglucoside; Pd glc – Peonidin-3-*O*-glucoside.

solvent of suitable polarity so the extraction time is shorter.

Interaction between the time and the temperature had a significant effect on the extraction of the majority of examined flavan-3-ols (Fig. 4.) and quercetin-3-*O*-glucoside (Fig. 5.). The increase in the extraction time and the extraction temperature caused an increase in the recovery of flavan-3-ols from grape skins. These findings could be explained by the position of these compounds in the grape skin cells. They could be bound to the cell wall of grape skin so for the effective extraction of these compounds it is necessary to disrupt cell wall. Disruption of cell walls is enhanced in an acidic environment and at a higher temperature.[30,31] The decrease in the extraction temperature and time had a positive effect on quercetin-3-*O*-glucoside recovery. This compound is thermally unstable and prolonged extraction at higher temperature led to its decomposition.

The optimization of extraction conditions for individual responses as well as for all individual compounds together from grape skins is presented in Table 4. Multicriteria methodology (Derringer function or desirability function) was used. The examination of optimal extraction conditions was based on the maximum recovery of individual anthocyanins, flavonol glycosides, and flavan-3-ol from grape skins (Supplemental Fig.1). The optimized conditions were as follows: extraction solvent composed of acetonitrile:water:formic acid (20:79:1; *v/v/v*), at an extraction temperature of 50 °C, extraction time of 1 h and in a single-step extraction with a solid-to-solvent ratio of 1:80 g mL^{-1} (125 mg of grape skin powder and 10 mL of extraction solvent).

The optimized conditions obtained by RSM were used to verificate the predictive model of extraction of phenolics from the red grape skin. The results (Table 3 and Table 4) showed that the experimental and predicted values differentiate for less than 1.5%. To validate the new optimized SLE method, the reproducibility and precision were determined. The reproducibility and the precision of the optimized SLE method were satisfactory. The calculated RSD values were less than 5% for all of the examined compounds from grape skins. The

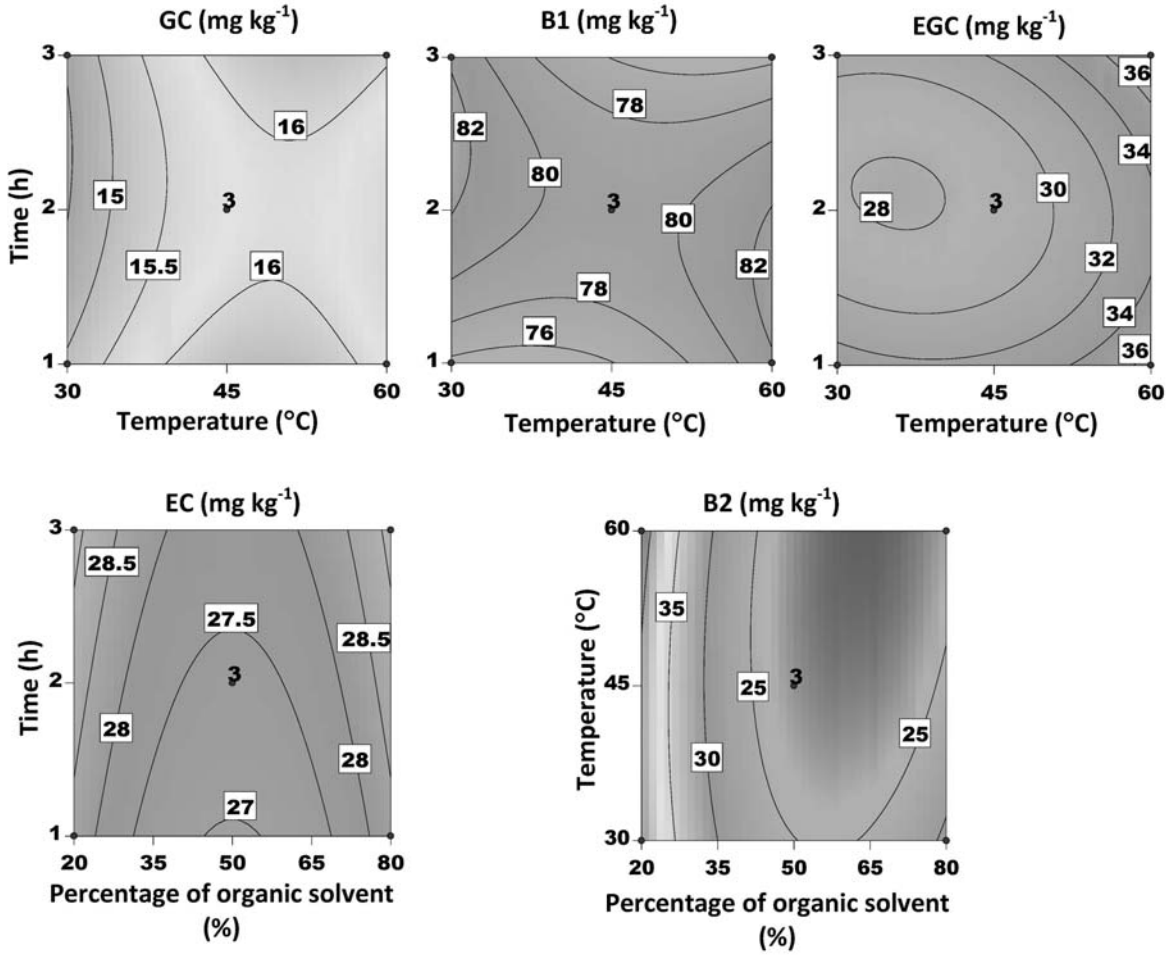

Fig. 4. Contour plots of most prominent interaction factors for individual flavan-3-ols from grape skins using acetonitrile as organic modifier. EC – Epicatechin; EGC – Epigallocatechin; GC – Gallocatechin; B1 –Procyanidin B1; B2 – Procyanidin B2.

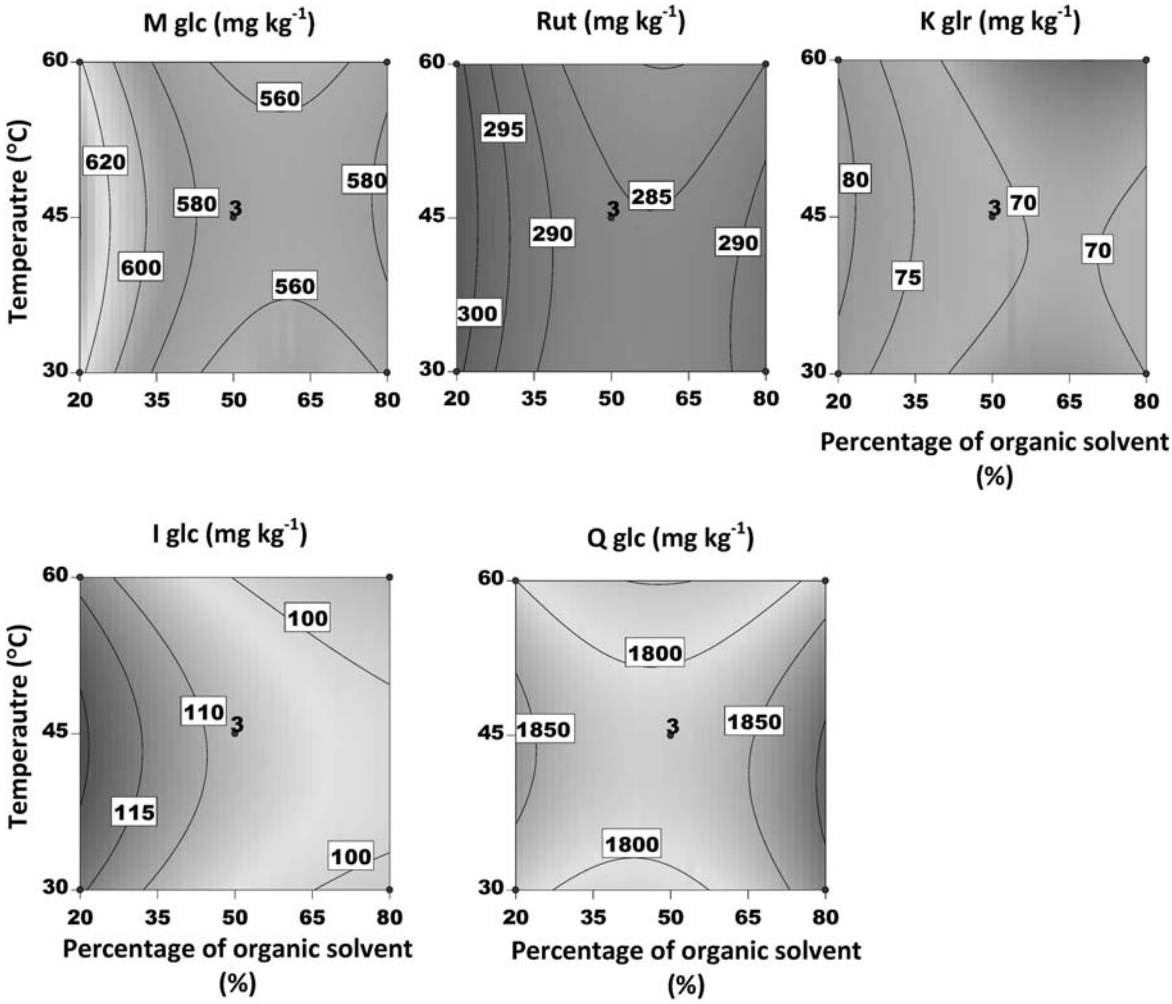

Fig. 5. Contour plots of most prominent interaction factors for individual flavonols from grape skins using acetonitrile as organic modifier. I glc – Isorhamnetin-3-*O*-glucoside; K glr – Kaempferol-3-*O*-glucuronide; My glc – Myricetin-3-*O*-glucoside; Q glc – Quercetin-3-*O*-glucoside; Rut – Rutin.

highest RSD values were calculated for individual flavan-3-ols. This can be explained by their low content and chemical nature (Table 4). During extraction, these compounds can hydrolyze, isomerize or can arise as hydrolysis products of tannins. A low percentage of acetonitrile exists in the extraction solvent so there is no need for it to be removed by evaporation in a vacuum before HPLC analysis, which greatly improves the precision and accuracy of the method and also contributes to a low RSD value.

4. Conclusion

The RSM was successfully employed to optimize solid-liquid extraction of flavonoids from grape berry skins. For the first time, the effect of different types of organic modifier in the extraction solvent on the extraction efficiency is considered. For optimization of extraction conditions, a multi-response methodology was applied for the first time. This study clearly demonstrates that numerous factors have great effect on extraction efficiency, such as the type of organic modifier and its percentage in the extraction solvent, the extraction time and temperature and in particular the nature of analytes and their position within the grape skin cell, have a great effect. Optimal extraction conditions may vary significantly, even for the members of the same group of flavonoids. The results revealed that SLE using extraction solvent composed from acetonitrile:water:formic acid (20:79:1; *v/v/v*), at the extraction temperature of 50 °C, extraction time of 1 h in single-step extraction and with the solid-to-solvent ratio of 1:80 g mL^{-1} (125 mg of grape skin powder and 10 mL of extraction solvent) is an effective method for the recovery of flavonoids from grape skins. The great advantage of applying this new optimized SLE method is the reduction of the number of operations throughout the extraction process; there is no longer a need for the organic modifier

Table 4. The content of individual flavonoids extracted from grape skins by using optimal conditions. Results are expressed in mg kg^{-1} dry weight of grape skin

Compound	Predicted values	Experimental values (n = 3)	
		$\tilde{Y}$ ± SD	RSD
Delphinidin-3,5-*O*-diglucoside	795.88	793.70 ± 1.59	0.20
Cyanidin-3,5-*O*-diglucoside	1452.81	1448.29 ± 7.10	0.49
Delphinidin-3-*O*-glucoside	12256.80	12270.98 ± 49.95	0.41
Peonidin-3,5-*O*-diglucoside	1153.01	1162.60 ± 7.33	0.63
Malvidin-3,5-*O*-diglucoside	16803.90	16735.50 ± 34.33	0.21
Cyanidin-3-*O*-glucoside	2413.40	2401.35 ± 12.16	0.51
Peonidin-3-*O*-glucoside	664.81	658.22 ± 7.19	1.09
Malvidin -3-*O*-glucoside	8888.49	8936.18 ± 43.01	0.48
Total anthocyanins	**44522.20**	**44406.82 ± 110.34**	**0.25**
Myricetin-3-*O*-glucoside	647.05	639.17 ± 4.74	0.74
Rutin	304.76	307.32 ± 6.40	2.08
Quercetin-3-*O*-glucoside	1864.52	1852.36 ± 13.05	0.70
Kaempferol-3-*O*-glucuronide	82.35	84.34 ± 3.31	3.91
Isorhamnetin-3-*O*-glucoside	121.59	119.66 ± 3.84	3.21
Total flavonol glycosides	**3229.92**	**3302.85 ± 20.67**	**0.63**
Gallocatechin	12.99	12.14 ± 0.32	2.64
Procyanidin B1	80.75	83.24 ± 3.82	4.59
Epigallocatechin	40.33	39.79 ± 1.37	4.44
Catechin	18.68	18.05 ± 0.74	4.07
Procyanidin B2	41.40	42.48 ± 2.12	4.99
Epicatechin	30.92	29.94 ± 0.86	2.87
Total flavan-3-ols	**225.13**	**223.64 ± 5.43**	**2.42**

$\tilde{Y}$ mean value (n = 3). SD standard deviation. RSD relative standard deviation

in the extraction solvent to be removed. Therefore any possible errors that may arise during frequent sample transfer from one vessel to another can be avoided.

5. References

1. K. B. Pandey, S. I. Rizvi, *Oxidative Medicine and Cellular Longevity*, **2009**, *2*, 270–278. http://dx.doi.org/10.4161/oxim.2.5.9498
2. G. Agati, E. Azzarello, S. Pollastri,M. Tattini, *Plant science*, **2012**, *196*, 67–76. http://dx.doi.org/10.1016/j.plantsci.2012.07.014
3. A. P. B. Gollücke, *Recent Patents on Food, Nutrition and Agriculture*, **2010**, *2*, 105–109. http://dx.doi.org/10.2174/2212798411002020105
4. I. Tomaz, L. Maslov, D. Stupić, D. Preiner, D. Ašperger, J. Karoglan Kontić, *Phytochemical Analysis*, **2015**, *27*, 13–22. http://dx.doi.org/10.1002/pca.2582
5. C. Carrera, A. Ruiz-Rodriguez, M. Palma,C. G. Barroso, *Analytica Chimica Acta*, **2012**, *732*, 100–104. http://dx.doi.org/10.1016/j.aca.2011.11.032
6. A. Liazid, R. F. Guerrero, E. Cantos, M. Palma,C. G. Barroso, *Food Chemistry*, **2011**, *124*, 1238–1243. http://dx.doi.org/10.1016/j.foodchem.2010.07.053
7. Z. Ju,L. R. Howard, *Journal of Food Science*, **2005**, *70*, S270–S276. http://dx.doi.org/10.1111/j.1365-2621.2005.tb07202.x
8. P. Mazzuca, P. Ferranti, G. Picariello, L. Chianese, F. Addeo, *Journal of Mass Spectrometry*, **2005**, *40*, 83–90. http://dx.doi.org/10.1002/jms.778
9. I. Revilla, S. Perez-Magarino, M. L. Gonzalez-SanJose,S. Beltran, *Journal of Chromatography A*, **1999**, *847*, 83–90. http://dx.doi.org/10.1016/S0021-9673(99)00256-3
10. J. Cacho, P. Fernandez, V. Ferreira, J. E. Castells, *American journal of enology and viticulture*, **1992**, *43*, 244–248.
11. G. Mazza, L. Fukumoto, P. Delaquis, B. Girard, B. Ewert, *Journal of Agricultural and Food Chemistry*, **1999**, *47*, 4009–4017. http://dx.doi.org/10.1021/jf990449f
12. D. Favretto,R. Flamini, *American Journal of Enology and Viticulture*, **2000**, *51*, 55–64.
13. R. R. Montealegre, R. R. Peces, J. L. C. Vozmediano, J. M. Gascuena,E. G. Romero, *Journal of Food Composition and Analysis*, **2006**, *19*, 687–693. http://dx.doi.org/10.1016/j.jfca.2005.05.003
14. S. Gomez-Alonso, E. Garcia-Romero,I. Hermosin-Gutierrez, *Journal of Food Composition and Analysis*, **2007**, *20*, 618–626. http://dx.doi.org/10.1016/j.jfca.2007.03.002
15. P. Iacopini, M. Baldi, P. Storchi, L. Sebastiani, *Journal of Food Composition and Analysis*, **2008**, *21*, 589–598. http://dx.doi.org/10.1016/j.jfca.2008.03.011
16. B. Lapornik, M. Prosek, A. G. Wondra, *Journal of Food Engineering*, **2005**, *71*, 214–222. http://dx.doi.org/10.1016/j.jfoodeng.2004.10.036

17. B. Lorrain, I. Ky, L. Pechamat, P. L. Teissedre, *Molecules*, **2013**, *18*, 1076–1100. http://dx.doi.org/10.3390/molecules18011076
18. V. Ivanova, M. Stefova, B. Vojnoski, A. Dornyei, L. Mark, V. Dimovska, T. Stafilov, F. Kilar, *Food Research International*, **2011**, *44*, 2851–2860. http://dx.doi.org/10.1016/j.foodres.2011.06.046
19. Z. M. Jin, J. J. He, H. Q. Bi, X. Y. Cui, C. Q. Duan, *Molecules*, **2009**, *14*, 4922–4935. http://dx.doi.org/10.3390/molecules14124922
20. R. Tsao,R. Yang, *Journal of Chromatography A*, **2003**, *1018*, 29–40. http://dx.doi.org/10.1016/j.chroma.2003.08.034
21. R. Flamini, D. Tomasi, *Vitis*, **2000**, *39*, 79–81.
22. J. E. Cacace, G. Mazza, *Journal of Food Engineering*, **2003**, *59*, 379–389. http://dx.doi.org/10.1016/S0260-8774(02)00497-1
23. S. L. C. Ferreira, R. E. Bruns, H. S. Ferreira, G. D. Matos, J. M. David, G. C. Brandao, E. G. P. da Silva, L. A. Portugal, P. S. Reis, A. S. Souza, W. N. L. dos Santos, *Analytica Chimica Acta*, **2007**, *597*, 179–186. http://dx.doi.org/10.1016/j.aca.2007.07.011
24. M. A. Bezerra, R. E. Santelli, E. P. Oliveira, L. S. Villar, L. A. Escaleira, *Talanta*, **2008**, *76*, 965–977. http://dx.doi.org/10.1016/j.talanta.2008.05.019
25. I. Tomaz, L. Maslov, *Food Analytical Methods*, **2016**, *9*, 401–410. http://dx.doi.org/10.1007/s12161-015-0206-7
26. J. Karoglan Kontić, I. Rendulić Jelušić, I. Tomaz, D. Preiner, Z. Marković, D. Stupić, Ž. Andabaka, E. Maletić, *International Journal of Food Properties*, **2016**, *19,* 1806–1824.
27. S. Z. Mikhail, W. R. Kimel, *Journal of Chememical & Engeenering Data* **1961**, *6*, 533–537. http://dx.doi.org/10.1021/je60011a015
28. I. S. Khattab, F. Bandarkar, M. A. A. Fakhree, A. Jouyban, *Korean Journal of Chemical Engineering*, **2012**, *29*, 812–817. http://dx.doi.org/10.1007/s11814-011-0239-6
29. J. Bakker, C. F. Timberlake, *Journal of the Science of Food and Agriculture*, **1985**, *36*, 1315–1324. http://dx.doi.org/10.1002/jsfa.2740361217
30. M. Pinelo, A. Arnous, A. S. Meyer, *Trends in Food Science & Technology*, **2006**, *17*, 579–590. http://dx.doi.org/10.1016/j.tifs.2006.05.003
31. B. A. Acosta-Estrada, J. A. Gutierrez-Uribe, S. O. Serna-Saldivar, *Food Chemistry*, **2014**, *152*, 46–55. http://dx.doi.org/10.1016/j.foodchem.2013.11.093

Povzetek

Sestava in koncentracija flavonoidov sta pomembna za karakterizacijo grozdnih sort, saj te spojine vplivajo na kakovost grozdja in vina. Razvili smo novo ekstrakcijsko metodo za pridobivanje flavonoidov, npr. antocianinov, flavonolov in flavan-3-olov iz grozdnih kožic. Optimizacijo ekstrakcije flavonoidov tekoče-trdno smo izvajali z uporabo modeliranja odzivne površine (RSM) ob upoštevanju tipa organskega topila in njegovega deleža, kakor tudi temperature in časa ekstrakcije. Optimalni pogoji so bili ekstrakcijsko topilo acetonitril:voda:mravljična kislina (20:79:1; *v/v/v*), temperatura ekstrakcije 50 °C in čas ekstrakcije 1 h, ekstrakcija v enem koraku z razmerjem med trdnim vzorcem in topilom 1:80 g mL^{-1} (125 mg grozdnih kožic v prahu in 10 mL ekstrakcijskega topila). Nova optimalna ekstrakcijska metoda je poceni, preprosta, hitra, točna in selektivna za ekstrakcijo preprostih flavonoidov.