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Phenolic composition and free radical scavenging activity of wine produced from the Serbian autochthonous grape variety Prokupac – A model approach

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Abstract: Phenolic compounds are very important quality parameters of wine because of their impact on colour, taste and health properties. The present study was aimed at evaluating the general phenolic composition and free radical scavenging activity of aqueous and organic fractions obtained using liquid–liquid extraction of red wine produced from the Serbian autochthonous grape variety Prokupac. The total phenolic contents in the different fractions ranged from 48.22 to 289.12 mg GAE per g dry fraction. Phenolic acids (mainly hydroxycinnamic acids) and quercetin 3-*O*-glucuronide were the main components in the EtOAc fraction at pH 2.0; catechins, phenolic acids (mainly hydroxybenzoic acids) and quercetin were found in the EtOAc fraction at pH 7.0, while anthocyanins were identified in the aqueous residue after EtOAc extraction. The major anthocyanin extracted into the aqueous fraction was malvidin-3-glucoside, while the most abundant non-anthocyanin phenolic compounds in the organic fractions were ethyl gallate and *trans*-caftaric acid. The radical scavenging activities of the fraction differed significantly and the IC_{50} values were 138.58 μg mL^{-1} for the aqueous fraction, 17.83 and 3.47 μg mL^{-1} for the EtOAc fractions at pH 2.0 and 7.0, respectively. As the EtOAc fractions were found to be more potent radical scavengers, it could be assumed that non-anthocyanin phenolic compounds were responsible for such activity in Prokupac wine.

Keywords: anthocyanins; flavonoids; phenolic acids.

INTRODUCTION

The southern region of Serbia has a long-standing tradition of viticulture and winemaking since the dominant soil types and climatic conditions of the region

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are very favourable for the cultivation of vines. The vineyards of southern Serbia are focused on the old autochthonous vine varieties such as Prokupac.[1] According to the literature, there is only limited information about the chemical composition of wines produced from the Prokupac variety cultured in southern Serbia.

Based on their carbon ring structure, wine polyphenols are divided into flavonoids (anthocyanins, flavan-3-ols, flavonols, dihydroflavonols) and non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and derivatives, stilbenes and volatile phenols).[2] The quantities of these phenolic compounds vary considerably in different types of wines depending on the grape variety, environmental factors in the vineyard, the wine processing techniques, soil and atmospheric conditions during ripening and fruit maturation.[3] The ageing of the wine could also modify the phenolic composition because phenolic compounds undergo different transformations, such as oxidation processes, condensation and polymerisation reactions, and extraction from wood.[4] Therefore, each type of grape presents a distinct sensory appeal, chemical composition and biological activity.

The anti-oxidant activity of wines has been related to their polyphenolic constituents and is mainly based on their free radical scavenging capacity.[5] Wine phenolics show beneficial physiological properties, *e.g.*, cardioprotective, anticarcinogenic and anti-inflammatory activities, due to their ideal chemical structure for free radical scavenging activities.[6] As oxidative stress arises from an imbalance in the human antioxidant status, it contributes to the pathology of chronic diseases.[7] Reactive oxygen species (ROS), naturally formed during normal metabolism, can damage biological structures, such as proteins, lipids or DNA. Human metabolism counts on an antioxidant defensive system involving enzymes and proteins to prevent these effects.[8] Since these protective mechanisms can be disrupted by various pathological phenomena, antioxidant supplements are essential to counter oxidative damage.[9] It is recognised that besides a role in endogenous defence in plants, the consumption of dietary polyphenols plays an important role in protecting against some pathological events.[10]

In addition to the importance of wine polyphenols as antioxidants, their study may also contribute to wine grape taxonomic characterisation and for certification of wine quality and origin.[11] In fact, both antioxidant activity and sensory properties depend on not only the amount, but also the type and structural features of polyphenols.[12]

The aim of this work was to determine the phenolic composition and free radical-scavenging activity in different fractions of red wine produced from the Serbian autochthonous grape variety Prokupac. For this purpose, three fractions of Prokupac wine containing different classes of phenolics were obtained by liquid–liquid extractions. Chemical analyses were realized using HPLC and LC-MS, while antiradical activity was tested using the DPPH (2,2-diphenyl-1-(2,4,6--trinitrophenyl)hydazinyl) method.

EXPERIMENTAL

Wine sample

The wine produced from the autochthonous grape variety Prokupac (2010 vintage) was obtained from the Braća Rajković vinery in the southwest region of Serbia. Prokupac grape was cultivated in Gornje Zleginje (altitude 359 m, 43°26′15″N, 21°10′01″E).

Standards

Standard compounds: delphinidin 3-*O*-*β*-glucoside chloride, malvidin 3-*O*-*β*-glucoside chloride, gentisic acid, caffeic acid, ellagic acid, catechin, proanthocyanidin B1 and B2, epicatechin, protocatechuic acid and quercetin were obtained from Extrasynthese (France).

Fractionation of Prokupac wine

Liquid–liquid extraction methods according to Ghiselli *et al.*[13] were performed to obtain several fractions containing different classes of polyphenolic compounds. In brief, ethanol removal was realized by vacuum evaporation. Special attention was paid to control the evaporation process, monitoring the pH and the volume of the solution to avoid complexation and precipitation processes. The de-alcoholised wine (100 mL) was first extracted with ethyl acetate (three times with 100 mL of EtOAc each), whereby an aqueous residue and an organic phase were obtained. The organic phase was evaporated, re-dissolved in water at pH 7.0 and further extracted with EtOAc (three times with 100 mL of EtOAc each) The aqueous residue from this extraction was adjusted at pH 2.0 and extracted again with EtOAc (three times with 100 mL of EtOAc each). The obtained fractions were then evaporated under reduced pressure.

Determination of the total phenolics

The concentration of total phenolic compounds in the fractions was determined spectrophotometrically using the Folin–Ciocalteu method with slight modifications.[14] Two hundred microlitres of the fractions (5 mg mL^{-1} 50 % EtOH) were added to 1 mL of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 µl of sodium carbonate (75 g L^{-1}) were added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0–100 mg L^{-1}) was used for the construction of a calibration curve. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight of fraction (mg GAE g^{-1} DW). Triplicate measurements were taken and the mean values were calculated.

Fingerprint HPLC-DAD analysis

Analyses of phenolic compounds from the aqueous and organic fractions were performed using HPLC Agilent 1200 Series with UV–Vis diode-array detector (DAD) for multi-wavelength detection. The aqueous fraction was separated on a Zorbax SB-Aq column (250 mm×4.6 mm, 5µm) according to the Compendium of International Methods OIV.[15] A gradient consisting of solvent A (H_2O/HCOOH/CH_3CN, 87:10:3, v/v/v) and solvent B (H_2O/ /HCOOH/CH_3CN, 40:10:50, v/v/v) was applied at a flow rate 0.8 mL min^{-1} as follows: 6 to 30 % B linear in 0 to15 min, 30 to 50 % B linear in 15 to 30 min, 50 to 60 % B linear in 30 to 35 min, and 60 to 6 % B linear in 35 to 41 min. The column was thermostated at 40 °C. Fifty microlitres of wine, previously filtered through a 0.45-µm membrane, was injected onto the column. Identification was possible by monitoring the anthocyanins at 520 nm and by comparing their spectra and retention times with those of commercial standards.

Analysis of the EtOAc wine fractions obtained at pH 7.0 and 2.0 was performed on a reversed-phase Zorbax SB-Aq column (250 mm×4.6 mm, 5µm) at 40 °C. A gradient consisting of solvent A (H_2O/CH_3COOH, 95:5, v/v) and solvent B (CH_3CN) was applied at a flow rate of 0.5 mL min^{-1} as follows: 0 to 60 % B linear from 0 to 35 min. Fifty microlitres of

wine, previously filtered through a 0.45 μm membrane, was injected onto the column. Chromatograms were acquired at 280–330 nm. The content of an individual phenolic compound was determined by comparing the area of the appropriate peak against the total peak area of the phenolics and the data are expressed in percentages.

LC–MS analysis

LC–MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC. For the analysis of the EtOAc wine fractions, the same column and gradient program was used as for the HPLC-DAD analysis. For the analysis of anthocyanins, mobile phase A was 10 % formic acid in water and mobile phase B was acetonitrile. The injection volume was 10 μL, and elution was at 1 mL min^{-1} with gradient program (0–1 min, 1–7 % B; 1–4 min, 7 % B; 4–7.5 min, 7–10 % B; 7.5–11.5 min, 10–14 % B; 11.5–15.5 min, 14–25 % B; 15.5–18.5 min, 25–40 % B; 18.5–22 min 40–75 % B; 22–25 min 75% B; 25–26 min 75–99 % B; 26–27 min, 99–1 % B) using the same column as that employed for the HPLC-DAD analysis. Mass spectra were acquired using an Agilent ESI-MSD TOF. The drying gas (N_2) flow was 12 L min^{-1}; the nebulizer pressure was 45 psig; the drying gas temperature was 350 °C. For ESI analysis, the parameters were: capillary voltage, 4000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V 250 V, for the negative (EtOAc wine fractions) and positive modes (anthocyanins). The mass range was from 100 to 2000 *m*/*z*. Processing of data was realized with the software Molecular Feature Extractor.

Free radical scavenging activity

The free radical scavenging activity of the fractions were analysed using the DPPH assay.[16] This antioxidant assay is based on the measurement of the DPPH colour loss at 517 nm caused by the reaction of DPPH with the test sample. Fractions diluted in appropriate solvents (10–100 μL) were dispensed into a set of test tubes and the final volume was adjusted to 5 mL. Finally, 0.5 ml of a 0.5 mM methanolic DPPH solution was transferred into each test tube. The absorbances were recorded at 517 nm after 30 min incubation at room temperature in the dark, against methanol as the blank. The percent inhibition was calculated against the control solution, containing methanol instead of a test solution.

Statistical analysis

All data are expressed as the mean values ± standard deviation from three replicates (n = 3). For statistical analysis, one-way analysis of variance (ANOVA) was applied, followed by the Student's t test. The correlation coefficient between antioxidant activity values and the content of total phenolic compounds were measured using the Pearson correlation coefficient (r) and the Origin 8.0 software program. Correlations were considered statistically significant, if the p-value was less than 0.05.

RESULTS AND DISCUSSION

Total phenolics

In this study, the total amount of polyphenols was measured using the Folin–Ciocalteu method, referred to gallic acid. Total phenolic contents in different fractions ranged from 48.22±2.03 to 289.12±5.05 mg GAE g^{-1} dry fraction (Table I). The highest level was observed in the EtOAc fraction obtained at pH 7.0, while the aqueous fraction showed the lowest amount of total polyphenols. Although Ghiselli *et al.*[13] also showed that the EtOAc fraction obtained at pH 7.0

of the wine produced from the Sangiovese R10 grape clone from Italy was more abundant in phenolics than the EtOAc fraction obtained at 2.0, they found significantly bigger amounts of polyphenolics in the aqueous residue.

TABLE I. Total phenolic content and free radical-scavenging activity of different fractions obtained from Prokupac wine

Fraction	Total phenolic content mg GAE g^{-1} dry fraction[a]	DPPH-IC_{50}[a] µg mL^{-1}
Aqueous	48.22±2.03	138.58±3.33
EtOAc at pH 2.0	118.36±3.12	17.83±0.97
EtOAc at pH 7.0	289.12±5.05	3.47±0.34

[a]Values are significantly different, $p < 0.05$

According to Atanacković *et al.*,[17] of the wines produced from the cultivars Prokupac, Merlot, Cabernet Sauvignon and Pinot noir, the lowest phenolic content was found in the wine from the native cultivar Prokupac.

Phenolic compounds of Prokupac wine

Phenolic compounds are very important quality parameters of wine because of their impact on colour, taste and health properties,[18] as well as from chemotaxonomic point of view. The application of the liquid–liquid extraction method allowed the separation of the phenolics of Prokupac wine into fractions containing compounds with similar characteristics. This method made the identification of individual compounds easier and allowed an estimation of which classes of compounds were mainly responsible for the radical scavenging activity of the wine.

HPLC-DAD and LC–MS were applied to analyze the compounds in the obtained fractions and the results are presented in Tables II–IV and Figs. 1–3. The exact mass measurements of the pseudo-molecular ions of analytes performed by the time-of-flight (TOF) mass spectrometer in the negative polarity mode enabled the determination of the molecular formula of the phenolic acids, flavonoids, and procyanidins. Molecular formula determinations were performed by Molecular Feature Extractor program, taking into account *m*/*z* values and isotopic abundance patterns for all ion species noticed for respective compound. Molecular formulas of anthocyanins were determined using MS spectra in positive polarity mode, from corresponding molecular ions.

Complete identification of the compounds was achieved by comparing the UV spectra and molecular formula obtained from accurate mass measurements, with those from the literature, together with comparison of the HPLC retention times with those of authentic standards.

Generally, phenolic acids (mainly hydroxycinnamic acids) and quercetin 3--*O*-glucuronide were the main components of the EtOAc fraction at pH 2.0. Some procyanidins, catechin, epicatechin, phenolic acids (mainly hydroxyben-

zoic acids) and quercetin were found in the EtOAc extract at pH 7.0. Several anthocyanins were identified in the aqueous residue after EtOAc extraction.

TABLE II. Anthocyanin compounds detected in the aqueous fraction of Prokupac wine

Peak	*Rt* min	Compound	DAD λ_{max} / nm	Accurate mass g mol^{-1}	Total phenolics content in the fraction, %
1	12.6	Delphinidin 3-*O*-glucoside	524	465.1022	5.1
2	15.4	Petunidin 3-*O*-hexoside	526	479.1191	7.4
3	16.5	Peonidin 3-*O*-hexoside	520	463.1276	8.6
4	17.1	Malvidin 3-*O*-glucoside	528	493.1371	49.1
5	17.6	Vitisin A	510	561.1259	6.0
6	19.5	Peonidin 3-*O*-(6-*O*-acetyl)hexoside	520	505.1401	1.0
7	19.6	Malvidin 3-*O*-(6-*O*-acetyl)hexoside	520	535.1423	2.6
8	20.3	Malvidin 3-*O*-(6-*O*-coumaroyl)-hexoside	534	639.1719	1.2
9	20.6	Pionitin A	510	625.1588	2.1
10	21.2	(*p*-Hydroxyphenyl)pyranomalvidin glucoside	504	625.1588	3.0

TABLE III. Phenolic compounds detected in EtOAc fraction at pH 2.0

Peak	*Rt* min	Compound	DAD λ_{max} / nm	MS species	Accurate mass g mol^{-1}	Total phenolics content in the fraction, %
1	6.0	*cis*-Caftaric acid	302*sh*, 324	M–H, M+HCOOH, M+Cl, 2M–H	312.0525	4.3
2	6.2	Gentisic acid	260, 296	M–H, M+HCOOH, M+Cl, 2M–H	154.0283	11.8
3	6.8	*trans*-Caftaric acid	302*sh*, 330	M–H, M+HCOOH, M+Cl, 2M–H	312.0528	13.2
4	8.4	*trans*-Fertaric acid	302*sh*, 330	M–H, M+HCOO, M+Cl	326.0671	3.4
5	8.9	Coutaric acid	302*sh*, 314	M–H, M+HCOO, M+Cl	296.0564	5.4
6	10.0	Caffeic acid	302*sh*, 322	M–H, M+HCOO, M+Cl	180.0437	6.4
7	10.5	*cis*-Fertaric acid	302*sh*, 330	M–H, M+HCOO, M+Cl	326.0670	5.5
8	16.3	Quercetin 3-*O*-glucuronide	256, 264*sh*, 296*sh*, 346	M–H, M+HCOO, M+Cl	478.0790	4.3
9	17.0	Ellagic acid	254, 366	M–H, M+HCOO, M+Cl	302.0097	6.2

The principal source of the red colour in wine comes from its anthocyanin content. Nevertheless, free anthocyanins are not particularly stable.[19] Their extraction and stability are affected by vinery production practices. Monomeric anthocyanins are subject to hydrolysis, oxidation, and polymerization in wines. Their concentration usually decreases during the fermentation and maceration but this process may continue throughout the life of a wine.[1] Wine anthocyanins are the 3-*O*-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Glucosylated derivatives of these anthocyanins esterified at the C6 of the glucose with acetyl or cumaroyl groups are also usually found in wine samples, generally in low concentrations.[20]

TABLE IV. Phenolic compounds detected in the EtOAc fraction at pH 7.0

Peak	*Rt* min	Compound	DAD λ_{max} / nm	MS species	Accurate mass g mol^{-1}	Total phenolics content in the fraction, %
1	7.5	Catechin	280	M–H, M+HCOO, M+Cl	290.0821	6.8
2	7.9	Proanthocyanidin dimer	280	M–H, M+HCOO, M+Cl, 2M–H	578.1462	9.7
3	8.4	Epicatechin	280	M–H, M+HCOO, M+Cl	290.0824	7.3
4	9.7	Proanthocyanidin dimer	280	M–H, M+HCOO, M+Cl, 2M–H	578.1462	2.8
5	10.4	Protocatechuic acid	290	M–H, M+HCOO, M+Cl	154.0273	6.4
6	11.6	Ethyl gallate	272	M–H, M+HCOO, M+Cl, 2M–H, 2M–Cl	198.0549	19.7
7	13.7	Proanthocyanidin dimer	280	M–H, M+HCOO, M+Cl, 2M–H	578.1462	1.5
8	24.6	Ethyl caffeate	300, 324	M–H, M+HCOO, M+Cl	208.0737	3.0
9	31.8	Quercetin	254, 264, 292*sh*, 370	M–H, M+HCOO, M+Cl	302.0457	2.4

In the present work, the analysis of the aqueous fraction by HPLC-DAD and LC–MS allowed the identification of 10 anthocyanin compounds (Table II). Malvidin glucoside was the predominant anthocyanin (49.1 % of the total), as it is usual for *Vitis vinifera* wines,[21] followed by peonidin hexoside (8.6 % of the total) and petunidin hexoside (7.4 % of the total). Mitić *et al*.[22] showed that malvidin glucoside was the most abundant among anthocyanins detected in Prokupac grapes. Furthermore, they detected some flavan-3-ols and hydroxycinnamic acids in grapes, which were also found in the Prokupac wine fractions investigated in the present study. The pattern of the anthocyanins showed that the sugar substi-

tuents were hexose (about 70 % of the total) followed by acetyl hexose (3.6 % of the total) and coumaroyl hexose (1.2 % of the total). The presence of pyrano-anthocyanins vitisin A, pinotin A and (*p*-hydroxyphenyl)pyranomalvidin 3-*O*--glucoside were also evidenced. These compounds are produced during alcoholic fermentation and wine aging. Although cyanidin derivatives have been detected in red wines of different grape varieties,[18,23,24] cyanidin compounds were not found in the present fractions of Prokupac wine.

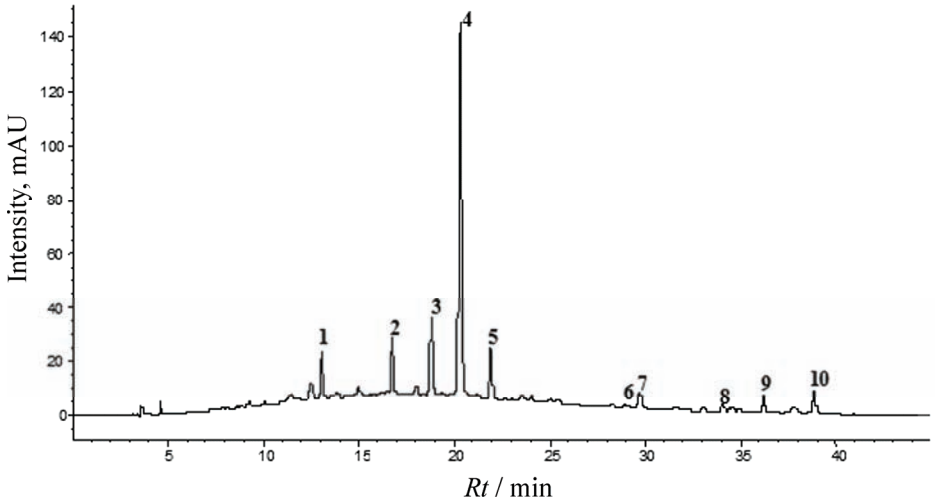

Fig. 1. HPLC profile of the aqueous fraction of Prokupac wine: delphinidin 3-*O*-glucoside (1), petunidin hexoside (2), peonidin hexoside (3), malvidin 3-*O*-glucoside (4), vitisin A (5), peonidin acetyl-hexoside (6), malvidin acetyl-hexoside (**7**), malvidin coumaroyl-hexoside (8), pinotin A (9), and (*p*-hydroxyphenyl)pyranomalvidin glucoside (10).

Non-anthocyanin phenolic compounds, including 4 benzoic acids, 7 cinnamic acids, 5 flavan-3-ols and 2 flavonols were detected in the organic fractions (Tables III and IV). Benzoic acids are minor components in wines, whereas hydroxycinnamates are the most important class of non-flavonoid phenolics.[25] The EtOAc fraction obtained at pH 2.0 was found to be rich in hydroxycinnamic acids and their derivatives. The main phenolic acids found in this fraction were *trans*-caftaric acid (13.2 % of the total) and gentisic acid (11.8 % of the total). *cis*-Caftaric acid, coutaric acid, fertaric acid (*cis* and *trans* isomers), ellagic acid and caffeic acid (the hydrolysis product of caftaric acid) were also detected.

Phenolic acids and their derivatives identified in the EtOAc fraction at pH 7.0 were protocatechuic acid, ethyl gallate and ethyl caffeate. Flavan-3-ols, which are mainly responsible for the astringency, bitterness, and structure of the wines, were also detected and among them catechin, epicatechin and three proanthocyanidin dimers were identified in the EtOAc fraction at pH 7.0.

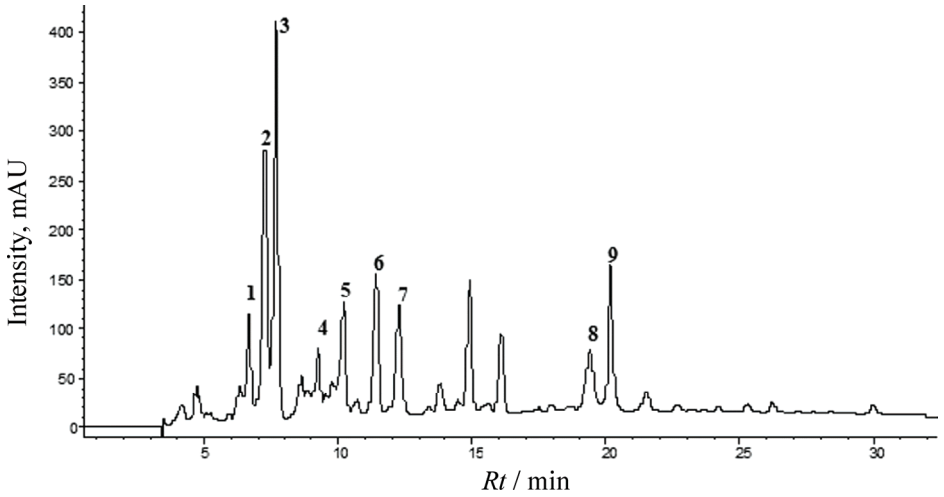

Fig. 2. HPLC profile of the EtOAc fraction at pH 2.0: *cis*-caftaric acid (1), gentisic acid (2), *trans*-caftaric acid (3), *trans*-fertaric acid (4), coutaric acid (5), caffeic acid (6), *cis*-fertaric acid (**7**), quercetin 3-*O*-glucuronide (8) and ellagic acid (9).

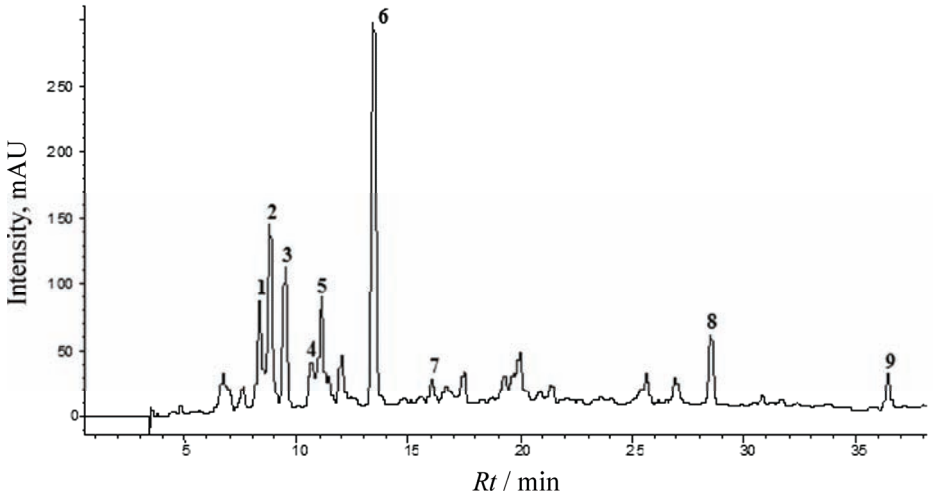

Fig. 3. HPLC profile of the EtOAc fraction at pH 7.0: catechin (1), proanthocyanidin dimer (2), epicatechin (3), proanthocyanidin dimer (4), protocatechuic acid (5), ethyl gallate (6), proanthocyanidin dimer (7), ethyl caffeate (8) and quercetin (9).

Radical scavenging activity of the Prokupac wine fractions

Although wines contain various phenolics, and their antioxidant activities could be connected with a synergy of these compounds, it is important to determine which group of phenolic compounds has most influence on the radical scavenging properties of wines. The fractions obtained in the present study were

subjected to a radical scavenging activity assay employing the stable DPPH radical widely used to characterize the radical scavenging activity of a variety of natural polyphenols. The measurement of the consumption of the DPPH radical allowed the exclusive determination of the intrinsic ability of a substance to donate hydrogen atoms or electrons to this reactive species in a homogeneous system. The method is based on the reduction of an alcoholic DPPH· solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH–H.[26] In this study, both organic fractions demonstrated effective scavenging activity against DPPH radicals (Table I). The order of scavenging activity was: EtOAc fraction at pH 7.0 > EtOAc fraction at pH 2.0 > aqueous fraction. The fractions that contained higher levels of total phenolics showed better radical scavenging activity. A negative, but insignificant, correlation between the total polyphenol content in the examined fractions and the DPPH IC_{50} values was found. Radovanović *et al.*[27] analyzed wines produced from three autochthonous grape cultivars, *i.e.*, Vranac, Kratošija and Prokupac, and showed that all samples possessed antioxidant activity. Atanacković *et al.*[17] also showed that the Prokupac wine they analyzed exhibited antioxidant potential. The present results and those previously published showed that Serbian red wines produced from the indigenous variety Prokupac may serve as a good source of potential antioxidant agents.

Several authors have described significant and positive correlations between the total polyphenol levels of wines and their antioxidant activities evaluated by DPPH.[5,28,29] In contrast, other studies have shown a lack of the aforementioned correlation and even some negative correlation was found, thus indicating that wines having the highest contents of total polyphenols did not always show the highest values for antioxidant activity.[30] It was suggested that the antioxidant activity of wine is more related to the type of the phenolic compounds present than to their total content.[31] There are disagreements regarding the main compounds that act as antioxidants. Di Majo *et al.*[32] showed high correlation between antioxidant activity and the flavonoid fraction as did Radovanović *et al.*,[1] who confirmed high correlation between the total anthocyanin content and DPPH scavenging activity of the 5 vines they tested. On the other hand, Sánchez-Moreno *et al.*[33] found poor correlations between the ability of wines to block free radicals and their anthocyanin levels (whether total or monomeric). Regarding flavonols, Brenna and Pagliarini[34] obtained good correlations between antioxidant activity and the quercetin and myricetin contents. On the contrary, Arnous *et al.*[35] found quercetin levels to be negatively correlated with antioxidant activity and suggested that quercetin might be a pro-oxidant. Fernández-Pachón *et al.*[29] studied the anti-radical ability of various polyphenol fractions in wines (phenolic acids, flavanols, anthocyanins and flavonols) and concluded that flavo-

nols play no prominent role as antioxidants. Other authors found positive correlations between the anti-radical ability of wines and their flavanol levels.[29,33–36]

The antioxidant activity of hydroxybenzoic acids basically depends on the number of hydroxyl groups in the molecule, whereas for hydroxycinnamic acids, the presence of methoxy groups seemed to positively influence their antioxidant activity.[37] Ethyl gallate as the major compound (19.7 % of total) of the most active fraction (EtOAc at pH 7.0) presents three available hydroxyl groups that could donate a hydrogen to stabilize free radicals. In contrast to anthocyanins, the content of which decreased during time, the concentration of the hydroxybenzoic acids and their derivatives increased with time.[3]

The antioxidant activity of proanthocyanidins is, in part, dictated by the oligomer chain length. Flavan-3-ol monomers and dimers were found to inhibit more efficiently LDL oxidation than trimers and tetramers.[38] In the EtOAc fraction at pH 7.0, the amounts of detected dimers and monomers were almost equal (14 and 14.1 % of the total, respectively). Several structures appear to be important for these antioxidant activities, including an 3′4′-dihydroxyl (catechol) group or 3′4′5′-trihydroxyl (gallate) group in the B ring, a gallate group esterified at the 3 position of the C ring, and hydroxyl groups at the 5 and 7 positions of the A ring.[31]

From the flavonol group, quercetin glucuronide and its corresponding aglycon, released by hydrolysis in wine, were detected in the EtOAc fraction at pH 2 and the EtOAc fraction at pH 7.0, respectively. Besides five hydroxyl groups, quercetin also contains a 2,3-double bond in its C ring and a 4-oxo function. This structure enhances the total antioxidant activity of quercetin towards free radicals by allowing electron delocalization across the molecule.[37]

According to the obtained results, non-anthocyanin polyphenols, mainly flavan-3-ols, ethyl gallate and quercetin that dominates in the most active fraction (EtOAc at pH 7.0), could be considered as the main phenolic compounds responsible for radical scavenging activity of Prokupac wine. This is consistent with a study of Rice-Evans *et al.*,[31] in which anthocyanins, such as malvidin 3-glucoside, were found to be less effective as antioxidants than non-anthocyanin components, such as gallic acid, catechin and quercetin. Moreover, Arnous *et al.*[40] affirmed that catechin, epicatechin and proanthocyanidins are the compounds that mostly contribute to the antioxidant activity of wines.

CONCLUSIONS

The phenolic composition of different fractions obtained from wine produced from the Serbian autochthonous grape variety Prokupac was reported for the first time. Twenty-eight phenolic compounds belonging to five different classes (anthocyanins, flavonols, flavan-3-ols, hydroxycinnamic acids and hydroxybenzoic acids) were characterized. Anthocyanins dominated in the aqueous frac-

tion while other classes of compounds were separated in the organic fractions. Non-anthocyanin phenolics were indicated as the key compounds responsible for the radical scavenging activity of Prokupac wine. It is important to bear in mind that the polyphenols identified in this study represent a proportion of the total polyphenols of Prokupac wine, indicating that other non-identified compounds could contribute in a significant manner to the antioxidant activity. In addition, besides polyphenols, many other bioactive compounds, such as vitamins and minerals.[40] could also be connected with the free radical scavenging capacity of red wines generally.

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И З В О Д

ФЕНОЛНИ САСТАВ И СПОСОБНОСТ ХВАТАЊА СЛОБОДНИХ РАДИКАЛА ФРАКЦИЈА РАЗДВОЈЕНИХ ИЗ ЦРНОГ ВИНА ПРОИЗВЕДЕНОГ ОД СРПСКЕ АУТОХТОНЕ СОРТЕ ГРОЖЂА ПРОКУПАЦ

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Садржај фенолних једињења је веома значајан параметар квалитета вина због утицаја ових једињења на боју, укус и лековита својства. Циљ овог рада је одређивање фенолног састава и способности хватања слободних радикала водене и органских фракција добијених применом течно–течне екстракције из црвеног вина произведеног од српске аутохтоне сорте грожђа Прокупац. Садржај укупних фенола у испитиваним фракцијама износио је од 48,22 до 289,12 mg GAE g^{-1} суве фракције. Фенолне киселине (углавном хидроксициметне киселине) и кверцетин-3-глукуронид главне су компоненте етилацетатне фракције при pH 2,0; катехини, фенолне киселине (углавном хидроксибензоеве) и кверцетин нађени су у етилацетатној фракцији при pH 7,0, док су антоцијани идентификовани у воденом остатку након екстракције етилацетатом. Главни антоцијан водене фракције је малвидин-3-глукозид, док су најзаступљенија неантоцијанска фенолна једињења органских фракција етил-галат и *trans*-кафтарна киселина. Способност хватања слободних радикала значајно се разликовала међу фракцијама па је IC_{50} вредност за водену фракцију износила је 138,58 μg mL^{-1}, док су за етилацетатне фракције при pH 2,0 и 7,0 ове вредности износиле 17,83 и 3,47 μg mL^{-1}, редом. Показано је да су етилацетатне фракције снажнији хватачи слободних радикала, па се може претпоставити да су неантоцијанска фенолна једињења одговорна за ову активност испитиваног вина.

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