Antioxidant activity and polyphenol profile of Vranac red wines from Balkan region

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Abstract

The objective of the present study was to investigate the correlation between the radical--scavenging properties (measured by evaluating the quenching of the stable 2,2-diphenyl--1-picrylhydrazil radical) of Serbian, Macedonian and Montenegrin red wine Vranac of different geographical origins, and their contents of total phenolics, total flavonoids and polyphenol profile. All tested Vranac wines samples showed a high antioxidant activity ranging from 13.00 to 15.02 mmol/L, while the total polyphenolic content was between 3478.70 and 3935.19 mg/L. The predominant anthocyanin was malvidin-3-glucoside (179.04–281.31 mg/L), predominant flavonol was quercetin-3-glucoronide (5.88–11.78 mg/L), predominant flavan-3-ol was catechin (24.43–76.78 mg/L) and predominant hydroxycinnamic acid was *t*-caftaric acid (13.46–38.56 mg/L). Generally, red wines Vranac produced from Balkan regions are rich source of phenolics, which the evident antioxidant capacity showed.

Keywords: red wine, Vranac, phenolic profile, antioxidant activity, HPLC-DAD.

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Phenolic compounds play a very important role in the quality of red wine, owing to their contribution to the wine sensory properties, mainly colour, astringency and bitterness [1].

Wine phenolics can be divided into two groups: non-flavonoid (hydroxybenzoic and hydroxycinnamic acids and stilbenes) and flavonoid compounds (anthocyanins, flavan-3-ols and flavonols). Anthocyanins are the main phenolic compounds involved in the colour of the red wine. Flavan-3-ols are a large family of polyphenolic compounds which are mainly responsible for the astringency, bitterness and structure of wines, and also play an important role in the stabilisation of colour during aging. Flavonols, which also contribute to bitterness, display antioxidant activity and affect red wine colour acting as co-pigments of anthocyanins [2]. The polyphenolic contents of wine depend on the grape variety, vineyard location, cultivation system, climate, soil type, vine cultivation practices, harvesting time, production process and ageing [3].

In recent years an increasing number of studies have demonstrated the role of phenolic compounds in the antioxidant activity of many food products [4]. Wine is an excellent source of various classes of polyphenols, including benzoic and cinnamic acid derivatives, flavan-3-ols, flavonols and anthocyanins. A lot of effort has been put into the analysis of red wine polyphenols, and the relationship between polyphenolic content and antioxidant activity. The antioxidant properties of red wines have been correlated with their content in total polyphenols [5,6], anthocyanins [7] and hydroxicinnametes [8]. Red wines contain significantly higher amounts of total polyphenols compared to white wines.

Vranac is a variety of red grapes cultivated in Serbia, Montenegro and Macedonia used in the production of high quality wines. In this study, we evaluated the antioxidant activity of 9 Vranac wines produced from different agronomical and winemaking regions, and their correlation with the total phenolics and flavonoids.

MATERIALS AND METHODS

Chemicals

Standards of catechin, epicatechin, quercetin, myricetin and the phenolic acid standards, such as gallic, ferulic, *p*-coumaric and caffeic acids, were purchased from Sigma Chemicals Co. (St. Louis, Mo). Malvidin-3--glucoside was purchased from Extrasynthèse (e, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich (Steinheim, Germany). 6-Hydroxy-2,5,7,8-tetramethylchromancarboxylic acid (Trolox) and Folin–Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Other chemicals and solvents were of analytical grade.

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Wine samples

Nine Vranac wines, produced from three different Balkan countries (Serbia, Macedonia and Montenegro) originating from different wine regions were taken after production from the wineries (Table 1).

Determination of total phenolic content (TPC)

The total phenolic content of wines was determined by the Folin–Ciocalteau method [9] using gallic acid as the standard. One milliliter (diluted 1:100 with methanol) of red wines was added to a 25 mL volumetric flask filled with 9 mL deionised water. A reagent blank using deionized water sample was prepared, too. Folin––Ciocalteau phenol reagent (0.5 mL) and 5 mL of 5% sodium carbonate solution was immediately diluted to 25 mL with deionized water and mixed thoroughly. The absorbance was measured at 765 nm by a UV–Vis spectrophotometer (Agilent, Santa Clara, CA USA) after incubation for 1 h in dark at room temperature. The quantification was based on the calibration curve generated by the gallic acid standard solutions, and the content was expressed as mg gallic acid equivalent (GAE)/L of wine. All samples were analyzed in triplicate.

Determination of total flavonoid content (TFC)

The measurement of the total flavonoid content in the investigated wines has been determined spectrometrically [10], using a method based on the formation of complex flavonoid-aluminium. 0.5 mL (diluted 1:50 with methanol) of wine has been placed in 10 mL volumetric flask, and 5 mL of deionised water and 0.3 mL of 5% $NaNO_2$ have been added and mixed. After 5 min, 0.6 mL of 10% $AlCl_3{\cdot}6H_2O$ was added. Two milliliters of 1 mol/L NaOH were added 5 min later, and the volume was then made up to 10 mL with deionised water. The solution was mixed well and the absorbance was measured immediately at 510 nm. The flavonoid contents were calculated using a standard calibration curve, prepared from (+)-catechin.

Free radical scavenging activity

The DPPH radical scavenging method [11] has been based on the reduction of DPPH radicals in the presence of a hydrogen donating antioxidants in methanol solution. DPPH radical solution showed an absorption band at 515 nm and was of intensively violet color. The absorption and color intensity decreased when DPPH was reduced by an antioxidant compound. The remaining DPPH radical corresponded inversely to the radical scavenging activity of the antioxidant. Each wine was diluted 1:10 with methanol immediately before the analysis. In the test tubes, 0.2 mL of samples was added to 4.8 mL of DPPH solution (5.2×10^{-6} mol/L in methanol) and the mixture was well mixed. The absorbance at 515 nm was measured at 30 min against a blank (0.2 mL methanol and 4.8 mL DPPH solution in methanol). All determinations were performed in triplicate.

The radical scavenging capacity (*RSC*) expressed in percentage was calculated by the following equation [12]:

$$RSC\ (\%) = 100(A_{\text{blanc}} - A_{\text{sample}})/A_{\text{blanc}} \quad (1)$$

The chart of the remaining DPPH concentration against the concentration of Trolox in the standard samples was used to calculate the total antioxidant activity (TAA) of the wines.

Performance liquid chromatography (HPLC-DAD) analysis of individual phenolic compounds

The concentration of individual anthocyanins, flavonols, flavan-3-ols and hydroxycinnamic acid was determined by HPLC, employing a direct-injection method [13,14]. The equipment used was an HPLC Agilent-1200 series with UV–Vis DAD for multi wavelength detection and fluorescence detection for acquisition of the emission response. The column was thermostated at 30 °C. After injecting 5 μL of sample, the separation was performed in an Agilent-Eclipse XDB C-18 4.6·150 mm column. Two solvents were used for the gradient elution: A – (H_2O+5%HCOOH) and B – (80% CAN + 5% HCOOH + H_2O). The elution program used was as follows: from 0 to 10 min 0% B, from 10 to 28 min gra-

Table 1. Studied Vranac wine samples

No.	Wine and vintage	Wine producer
1	Vranac, 2009.	Vinoprodukt Čoka, Subotica, Serbia
2	Vranac, 2009.	Rubin Kruševac, Serbia
3	Vranac, 2009.	Vino Župa, Aleksandrovac, Serbia
4	Crnogorski vranac, 2009	Plantaže 13. jul, Montenegro
5	Vranac-Pro corde, 2009.	Plantaže 13. jul, Montenegro
6	Crmnički Vranac-Barrique, 2009.	Vinarija Mašanović-Virpazar, Montenegro
7	Vranac, 2009.	Povardarie, Negotino, Macedonia
8	Tga za jug, 2009.	Tikveš, Macedonia
9	Vranac, 2009.	Skovin, Macedonia

dually increases 0–25% B, from 28 to 30 min 25% B, from 30 to 35 min gradually increases 25-50% B, from 35 to 40 min gradually increases 50–80% B, and finally for the last 5 min gradually decreases 80–0% B. All identifications of individual compounds were based on the retention times of the original standards where available, and spectral data [15–18]. Monitoring of the evaluation was performed at 520 nm for the identification of anthocyanins, 360 nm for the identification of flavonols and 320 nm for the identification of hydroxycinnamic acids. Three flavan-3-ols including catechin, procyanidin B2 (dimer of proanthocyanidins epicatechin–epicatechin), and epicatechin were monitored at 275/322 nm ($\lambda_{ex}/\lambda_{em}$) with fluorescence detector. The results were expressed as mg/L of wine.

Statistical analysis

The experiments were reported at least 3 times, and the data were analyzed statistically. All results were given as mean ±standard deviation (*SD*). Statistical analysis was done by one-way analysis of variance (ANOVA), and significant differences between the results were determined by Duncan's multiple range test. The differences were considered significant at $p < 0.05$. Relationships between phenolic compound contents and antiradical efficiency were established using the Pearson correlation test ($p < 0.05$).

RESULTS AND DISCUSSION

Spectrophotometric analysis the content of polyphenols and flavonoids in Vranac wine samples

The results of the determination of total phenolic content in Vranac wine samples from different Balkan wine-producing regions by Folin–Ciocalteu method are presented in Table 2. The total phenolic content varied from 3478.70 to 3935.19 mg GAE/L (averaging 3729.90 mg GAE/L). There is a significant difference in the total phenolic content between the wines made of Vranac grape from different wine-producing regions of the Balkans. Wines from Montenegro have the highest level of total polyphenols (3830.32 mg GAE/L), as opposed to Macedonian (3808.20 mg GAE/L) and Serbian ones (3551.43 mg GAE/L). The total phenolic content of Vranac (Subotica, Serbia) and Vranac (Kruševac, Serbia) was significantly lower from the others ($p < 0.05$). Significant differences were found in the total phenolic content in comparison between "Crnogorski vranac" and "Crmnički vranac-Barrique" ($p < 0.05$); however, significant differences in total phenolic content were not found between Crnogorski vranac and "Vranac-Pro corde". Also, significant differences were found in the total phenolic content in comparison between "Tga za jug" (Macedonia) and Vranec (Negotino, Macedonia); however, significant differences in the total phenolic content were not found between Vranec (Negotino, Macedonia) and Vranec (Skovin, Macedonia). It is well known that genetic and agronomic or environmental factors play important roles in the phenolic composition and thus nutritional quality of crops. The mean concentration of the phenolic content of wine Vranac from the Balkan region was 3729.9 mg GAE/L. Our values for red wine are in the range of values determined by other authors. Majo *et al*. [19] measured 2360–3730 mg GAE/L for Sicilian red wines. Lucena *et al*. [4] measured 3200–5900 mg GAE/L for Brazilian red wines. But, Anli and Vural [20] measured lower values, 1070–2410 mg GAE/L, for Turkish red wines.

Total flavonoids of the Vranac wines were measured (Table 2). The Vranec (Negotino, Macedonia) showed the highest flavonoid content (2630.22 mg CE/L), followed by Vranac-Pro Corde, Crnogorski vranac and Crmnicki vranac-Barrique. The flavonoid content of wine samples 4, 5 and 6 (Macedonia) was significantly different from samples 1 and 2 (Serbia), $p < 0.05$. However, significant differences in the total flavonoid content were not found in comparison among sample 3 (Serbia) and samples 4–9. Only 1.14-fold difference in the in total flavonoid content was found between the highest and the lowest ranked wine samples, Vranec (Negotino, Macedonia) and Vranac (Kruševac, Serbia).

Table 2. Total phenol content (TP), total flavonoid content (TF), radical scavenging capacity (RSC) and total antioxidant activity (TAA) of wines; data are reported as mean±SD (n = 3); bars with no letters in common are significantly different (p < 0.05) in the same column

No. of sample	pH	Polyphenols, mg GAE/L	Flavonoids, mg CE/L	*RSC* / %	*TAA* / mmol TE L^{-1}
1	3.42	3528.04±28.53$^{fg1)}$	2328.53±8.35^{c}	75.62±0.36^{e}	13.22±0.20^{c}
2	3.45	3478.70±23.23^{g}	2297.32±8.56^{c}	74.40±0.36^{f}	13.00±0.26^{c}
3	3.42	3647.52±9.49^{ef}	2553.27±13.35^{ab}	78.91±0.26^{d}	13.79±0.26^{cb}
4	3.50	3935.19±22.68^{ab}	2597.14±12.50^{a}	86.12±0.26^{a}	15.02±0.35^{a}
5	3.48	3850.50±21.65^{cd}	2618.03±6.50^{a}	84.23±0.26^{b}	14.79±0.20^{a}
6	3.52	3705.22±18.59^{edc}	2593.54±14.76^{a}	79.09±0.20^{d}	13.82±0.20^{cb}
7	3.45	3868.54±19.17^{b}	2630.22±15.51^{a}	82.51±0.26^{c}	14.41±0.26^{ab}
8	3.51	3693.81±15.79^{ed}	2470.24±14.70^{b}	77.89±0,26^{d}	13.63±0.17^{c}
9	3.54	3862.23±17.51^{b}	2548.91±5.18^{a}	83.12±0.36^{c}	14.50±0.26^{ab}

Total antioxidant activity of Vranac wines using the DPPH scavenging assays

The radical scavenging activity was evaluated by measuring the scavenging activity of the examined red wine samples on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. All results of antioxidant analysis are summarised in Table 2. The investigated wines showed antioxidant behavior in the range from 74.40 to 86.12%. The percentage for Cabernet Sauvignon wines from the Balkan region was 71.30–83.53% [21]. The percentage for Croatian red wines was 54.6–82.6% [22]. Significant differences were found in the radical scavenging activity when comparing all samples (except for sample 3 and samples 6 and 8, and between samples 7 and 9)

Total antioxidant activity (*TAA*) of the Vranac wines, expressed in mmol of Trolox equivalent per L of red wine is shown in Table 2. Crnogorski vranac had the highest antioxidant activity (15.02 mmol TE/L), followed by Vranac-Pro Corde (14.79 mmol TE/L) and Vranec, Skovin, Macedonia (14.50 mmol TE/L).

TAA values of the Vranac wines from the Balkan region analyzed in this study were in the range obtained from red wines from other wine-producing countries such as Italy (7.8–19.8 mmol TE/L, [23]), Spain (4.65–17.41 mmol TE/L, [24]) and South Africa (9.51–12.30 mmol TE/L, [25]).

The wines containing high total phenolic contents had higher antioxidant activities. The present study reveals a very strong correlation between the total antioxidant activity and total phenolics (R^2 = 0.97). Also, a very good correlation between the total flavonoid content, and antioxidant activity (R^2 = 0.85) of the tested Vranac wine samples was confirmed.

HPLC Analysis

For a better description of Vranac red wine from the Balkan region, the profile of individual polyphenolic compounds were studied. In order to separate and determine individual phenolic compounds present in 9 wine samples from different geographical regions, HPLC method was applied.

The content and the distribution of anthocyanins in Vranac wine samples

The total of 12 phenolics compounds was identified as anthocyanins due to the information provided by their UV–Vis spectra. This primary information differentiated 5 glucosylated anthocyanins (*λ* maximum at 520 nm): delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside: and 7 acylglucosylated anthocyanins: cyanidin-3-acetylglucoside, petunidin-3-acetylglucoside, peonidin-3-acetylglucoside, malvidin-3-acetylglucoside, petunidin-3-*p*-coumaroylglucoside, peonidin-3-*p*-coumaroylglucoside and malvidin-3-*p*--coumaroylglucoside.

Certain quantitative differences in anthocyanins between wine samples were presented in Table 3. The total content of anthocyanins varied from 298.39 (Vranac, Kruševac, Serbia) to 448.82 mg/L (Crnogorski vranac*,* Montenegro). The wines from Montenegro have the highest level of total anthocyanins (mean 408.38 mg/L), as opposed to Macedonian (mean 381.69 mg/L) and Serbian ones (mean 334.14 mg/L). Malvidin-3-*O*--glucoside, which is responsible for the blue-red color of wines, was the most abundant anthocyanin and its concentration was dependent on winemaking, whereas low concentrations (10 to 20 time lower that malvidin-3-*O*-glucoside) of delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were detected. Malvidin-3-*O*-glucoside content ranged from 179.04 to 281.31 mg/L. Cyanidin-3-*O*-glucoside was the anthocyanin present in the lowest amounts (2.98–9.15 mg/L). The values are within the range reported for red wines by other researchers [26,14,27].

Quantitatively, the content of glucosided anthocyanins was predominant in the investigated Vranac wines (mean amount of content was 310.82 mg/L – from 246.74 to 378.07 mg/L), followed by the content of acetylated ones (mean amount was 45.70 mg/L – from 37.47 to 53.69 mg/L) and the content od *p*-coumarylated ones (mean amount was 18.21 mg/L – from 14.18 to 22.14 mg/L).

The acetylated forms were mainly detected in wines produced through cryo-macetation, delestage, prolonged maceration and traditional technology [27]. Acelylated malvidin-3-glucoside was the main compound. In our case, malvidin-3-acetylglucoside varied from 67.6 to 92.2% with respect to total acetyl derivatives.

The coumarolated anthocyanin fraction in Vranac wines was composed of only three anthocyanin coumarates (malvidin-3-*p*-coumaroylglucoside, peonodin-3-p-coumaroylglucoside and petunidin-3-*p*-coumaroylglucoside). The most predominant p-coumaroyl derivative was malvidin-3-*p*-coumaroylglucoside which accounted for 77.5% (from 53.8 to 84.8%) of total *p*-coumaroyl derivative content. The importance of coumarylated forms is related to sensory analysis.

Another point worth mentioning is that the order of abundances based on average value of distribution for each anthocyanin was the following (Table 3):

Mvgl > Mvgl-ac > Ptgl > Pngl >Mvgl-p-coum >Dpgl, for Vranac wines produced in Serbia;

Mvgl > Mvgl-ac > Ptgl ≈ Pngl > Dpgl > Mvgl-p-coum, for Vranac wines produced in Montenegro;

Mvgl > Mvgl-ac > Pngl > Ptgl >Dpgl >Mvgl-p-coum, for Vranac wines produced in Macedonia.

Table 3. Anthocyanin composition of wine samples; (1) delphinidin-3-O-glucoside; (2) cyanidin-3-O-glucoside; (3) petunidin-3-O-glucoside; (4) peonidin-3-O-glucoside; (5) malvidin-3-O-glucoside; (6) cyanidin-3-acetylglucoside; (7) petunidin-3-acetylglucoside; (8) peonidin-3-acetylglucoside; (9) malvidin-3-acetylglucoside; (10) petunidin-3-p-coumaroylglucoside; (11) peonidin-3-p-coumaroylglucoside; (12) malvidin-3-p-coumaroylglucoside; TA, total anthocyanins; TA-G, total anthocyanin-glucosides, TA-A, total anthocyanin-acetylglucosides, TA-C, total anthocyanin-coumaroylglucosides. Results expressed as mg/L of wines and are presented with mean (n = 3)

Cmpd.	Sample No.								
	1	2	3	4	5	6	7	8	9
1	10.95±0.29[2]	8.56±1.14	12.03±2.36	18.83±2.15	12.32±1.14	14.18±0.09	20.18±2.11	17.94±1.54	19.30±2.09
2	3.25±0.01	2.98±0.01	9.15±1.10	4.88±0.03	6.05±0.01	2.87±0.21	2.85±0.21	5.23±0.29	5.80±0.00
3	29.05±2.01	28.55±2.15	29.32±3.00	36.51±3.14	27.78±1.14	20.08±3.00	25.62±3.01	28.78±0.24	30.72±4.54
4	28.53±2.42	27.61±3.12	27.98±3.14	36.54±2.15	27.69±2.01	19.92±1.14	30.89±5.00	32.12±1.14	36.23±2.54
5	*205.38*±6.38	*179.04*±4.06	*227.93*±8.01	*281.31*±5.14	*278.52*±8.62	*235.32*±6.67	*238.72*±9.01	*197.34*±2.77	*252.63*±6.67
6	1.93±0.29	1.97±0.03	4.05±0.04	2.26±0.01	1.22±0.29	1.79±0.04	2.58±0.01	2.29±0.01	1.93±0.00
7	5.07±0.31	4.47±0.78	6.08±0.11	4.69±0.07	2.00±0.21	3.17±0.01	3.26±0.04	3.08±0.06	2.85±0.04
8	2.85±0.22	1.98±0.21	4.25±0.23	2.38±0.04	0.88±0.45	2.38±0.01	3.02±0.32	2.67±0.11	1.57±0.00
9	32.73±3.04	20.05±2.66	30.02±3.18	44.36±4.12	48.28±3.14	34.58±2.15	36.58±2.96	35.78±4.17	45.28±5.24
10	1.83±0.21	1.59±0.21	1.92±0.11	1.65±0.29	3.90±0.06	2.85±0.01	2.13±0.11	1.44±0.00	2.18±0.11
11	1.12±0.14	1.47±0.14	1.88±0.01	1.38±0.21	4.88±0.07	2.38±0.01	1.49±0.00	1.29±0.00	1.40±0.00
12	13.52±0.36	11.12±0.24	13.22±0.45	14.03±1.14	10.24±1.14	13.05±0.45	18.52±0.45	15.34±1.04	18.05±1.14
TA	**336.21**	**298.39**	**367.83**	**448.82**	**423.76**	**352.57**	**385.84**	**341.30**	**417.94**
TA-G	277.16	246.74	306.41	378.07	352.36	292.37	318.26	281.41	344.68
TA-A	42.58	37.47	44.40	53.69	52.38	41.92	45.44	41.82	51.63
TA-C	16.47	14.18	17.02	17.06	19.02	18.28	22.14	18.07	21.63

According to the wine sample results, it is obvious that the content of anthocyanin constituents of single-cultivar wines coming from different viticulture regions and wine producers were different, which may be related to the thickness of the grape skin, the climate in which the grape was grown, the degree of ripeness of the grape, the application of different vinification techniques and the wines ages [21,28].

The content of flavonols, flavan-3-ols and hydroxycinnamic acids in Vranac wine sample

The concentrations of flavonols, flavan-3-ols and hydroxycinnamic acids determined by HPLC in the investigated wine samples are shown in Table 4.

The main flavonol was quercetin-3-glucoronide, followed quercetin in second and third place, respectively,

Table 4. Amount of flavonols (1–5), hydroxycinnamic acids (6–11) and flavan-3-ols (12–14) in wine samples; (1) myricetin-3-glucoside; (2) quercetin-3-glucoside; (3) quercetin-3-glucoronide; (4) myricetin; (5) quercetin; (6) t-caftaric acid; (7) GRP; (8) t-coutaric acid; (9) caffeic acid; (10) p-coumaric acid; (11) ferrulic acid; (12) catechin; (13) procyanidin dimer B2; (14) epicatechin. Results are presented with mean (n = 3) and expressed in mg/L of wine

Cmpd.	Sample No.								
	1	2	3	4	5	6	7	8	9
1	2.05±0.22[2]	1.85±0.02	2.26±0.01	1.85±0.04	1.22±0.00	0.95±0.00	2.18±0.01	2.03±0.08	1.89±0.00
2	2.95±0.11	3.17±0.37	3.08±0.01	3.15±0.07	2.98±0.02	2.65±0.01	4.79±0.10	4.65±0.14	3.08±0.04
3	*7.23*±1.00	5.*88*±0.07	*8.53*±0.03	*9.18*±1.08	*11.78*±0.96	*10.08*±0.84	*8.28*±1.00	*6.03*±0.23	*7.92*±0.33
4	2.65±0.12	1.99±0.00	1.76±0.00	1.68±0.00	1.00±0.00	1.12±0.03	3.39±0.22	0.89±0.00	1.85±0.02
5	3.23±0.11	2.02±0.09	3.65±0.01	4.25±0.16	5.32±0.21	3.62±0.11	5.96±0.25	2.83±0.01	6.32±0.29
Total	**18.13**	**14.93**	**19.28**	**20.60**	**22.30**	**18.42**	**23.60**	**16.43**	**21.06**
6	27.34±3.01[2]	13.46±0.78	23.58±1.24	15.34±0.77	17.89±1.16	16.93±0.95	32.52±3.54	17.74±1.10	38.56±2.96
7	2.97±0.02	1.86±0.01	1.52±0.01	4.18±1.11	1.22±0.00	2.11±0.01	4.69±0.22	3.97±0.09	1.35±0.00
8	17.56±2.31	12.85±1.67	17.62±2.81	2.45±0.09	12.58±1.47	10.86±0.87	9.52±0.45	4.80±0.04	14.32±2.78
9	3.12±0.14	1.55±0.00	2.96±0.12	6.95±2.01	4.72±0.02	3.28±0.04	1.52±0.01	6.02±0.03	3.05±0.02
10	3.96±0.01	3.08±0.03	3.28±0.17	2.81±0.12	1.92±0.01	1.32±0.00	1.03±0.00	2.39±0.04	1.85±0.01
11	0.98±0.00	2.11±0.01	0.62±0.00	3.62±0.14	1.89±0.00	1.18±0.00	10.43±1.12	4.28±0.32	1.33±0.00
Total	**55.83**	**34.91**	**49.58**	**35.35**	**40.22**	**35.68**	**59.71**	**39.20**	**60.46**

Table 4. Continued

Cmpd.	Sample No.								
	1	2	3	4	5	6	7	8	9
12	*62.05*±9.57[2]	*24.43*±1.74	*58.32*±4.12	*55.38*±6.01	*60.82*±5.67	*50.98*±4.65	*76.78*±6.13	24.50±2.10	*68.30*±5.63
13	23.78±0.44	12.39±1.12	19.90±1.40	30.28±2.63	38.09±2.14	37.80±3.00	14.50±2.61	12.37±1.04	17.82±1.74
14	19.53±1.23	15.98±0.99	18.98±1.22	39.70±3.11	29.31±1.74	17.30±2.51	14.74±0.57	*30.15*±2.94	19.20±2.11
Total	**105.36**	**52.80**	**97.20**	**125.36**	**128.22**	**105.38**	**109.02**	**67.02**	**105.32**

by quercetin-3-glucoside. The content in myricetin and its derivatives accounts for approximately 15.0–38.4% of total flavonols in Vranac variety wines. Kaempferol are not detected in all wine samples.

The levels of quercetin and its derivatives (11.07––20.08 mg/L) and myricetin and its derivative (2.07––5.57 mg/L) are highest compared to Croatian wines (from Central and Southern Dalmatia, 3.6–10.4 mg/L and 0.5–3.3 mg/L, respectively [29]) and are in agreement with values obtained from Italian red wines (2.8––28.5 mg/L and 0.6–9.6 mg/L, respectively [23]). Variations in flavonol content of individual wines may be explained by several factors. It has long been known that the increased biosynthesis of polyphenols, especially flavonols, is greatly influenced by sunlight exposure and temperature, so it would be normally expected that the wines made from grapes, which are grown in warmer, sunnier areas, have a higher level of flavonols. Wines from Montenegro and Macedonia have the highest average level of total flavonols (20.44 and 20.36 mg/L, respectively) as opposed to Serbian ones (17.45 mg/L).

Hydroxycinnamic acids and their tartaric acid derivatives have been monitored at 320 nm, since is their characteristic wavelength, together with GRP (grape reaction product or 2-5-glutathionyl-*t*-caftaric acid). GRP is the product of the reaction between caftaric acid and glutation, which, as can be observed, is only detectable in the wine and not in the grape skins [14].

The predominant hydroxycinnamic acid was *t*-caftaric (13.46–38.56 mg/L), followed by *t*-coutaric (2.45––17.62 mg/L), caffeic (1.55–6.95 mg/L) and *p*-coumaric (1.32–3.96 mg/L). Ferulic acid was the hydroxycinnamic acid present in the lowest amounts (0.62–3.62 mg/L) in the Vranac wines from Serbia and Montenegro. *p*-Coumaric acid was the hydroxycinnamic acid present in the lowest amount (1.03–2.39 mg/L) in Vranac wines from Macedonia. There is a significant difference in the total hydroxycinnamic acid content between wines made of Vranac grape from different wine-producing regions of the Balkans. Wines from Macedonia have the highest average level of total hydroxycinnamic acids (53.12 mg/L), as opposed to Serbian (46.77 mg/L) and Montenegran ones (37.08 mg/L).

Many studies on the health benefits studies of red wine have been linked to the catechin content. Catechins possess antioxidant properties [20] and exert a more potent antioxidant effect than flavonols and polymeric anthocyanidins [30]. During vinification, only a portion of catechins and procyanidins is extracted from seeds and is diffused to wine [31]. The use of fluorescence detector has allowed increasing selectivity and sensitivity for the determination of the concentration of catechin and epicatechin in addition to other phenolic compounds [32]. Catechin, epicatechin, and procyanidin dimer (B2) were identified by fluorescence detector in wine samples (Fig. 4). The relative amounts of catechin, epicatechin and procyanidin B2 (three flavan-3-ols compounds) were the highest in Vranac wines from Montenegro (means, 55.73, 35.39 and 28.77 mg/L, respectively). Similar values for the determination of catechin, epicatechin and procyanidin B2 have been reported by others (55.2, 48.5 and 10.8 mg/L, [26]; 31.01, 12.78 and 8.96 mg/L [14]).

PCA Analysis

The large number of the data which are the results of anthocyanin, flavanols, hydroxycinnamic and flavan-3-ols analysis of vranac wine samples, was reduced using PCA analysis regarding to make easily interpretation of the experimental data. According to such a plot, it is possible to classify Vranac wine samples by their geographical origin.

One of the main objectives of PCA is to identify factors that are substantially meaningful. Experimental data were set in matrix with dimensions 9×26. Using latent root (eigenvalue) criteria [32], as a result of PCA calculations, 6 new variables were obtained which were characterized by consecutive eigenvalues 7.71 (29.6%), 6.16 (23.69), 4.34 (16.68%), 3.38 (13.01%), 1.74 (6.71%), 1.37 (5.28%). Two first PCs account 53.30% of the total variance. Taking into account that the first two components show high percentage of the total variance (50%), the distribution of wine samples is illustrated in two-dimensional plot of PC1 versus PC2.

As illustrated in Fig. 1, it is possible to separate three groups of samples what is according to their geographical origin. They are differenced because of their content of individual phenolic compounds. Vranac wine samples with low concentrated of the analyzed phenolic compounds are located on the left side of the

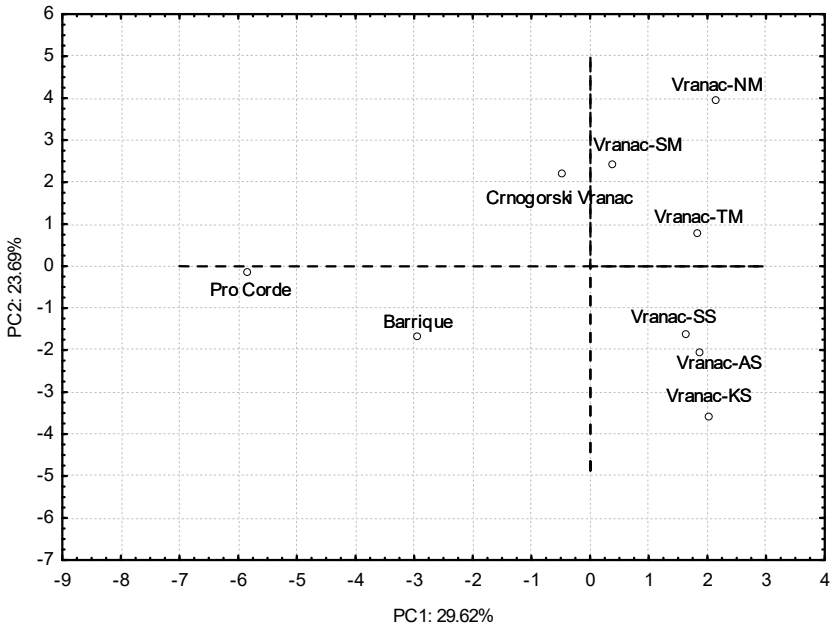

Figure 1. Scatterplots of the first two principal components (PC1 vs. PC2) for Vranac wine samples.

plot, but samples rich in phenolic compounds, can be found on the opposite side.

The behavior of variables on the PC1 and PC2 is shown in Figure 2. Also, the loadings and communality of each phenolic compound are given in Tables 5 and 6. The loading were large for cyanidin-3-acetylglucoside (6), petunidin-3-acetylglucoside (7), peonidin-3-acetylglucoside (8), myricetin-3-glucoside (13) and myricetin (16) on the first component, for delphinidin-3-*O*-glucoside (1), peonidin-3-*O*-glucoside (4), malvidin-3-*O*-glucoside (5), malvidin-3-acetylglucoside (9), malvidin-3-*p*-coumaroylglucoside (12), quercetin-3-glucoside (14), quercetin (17), *t*-caftaric acid (18), GRP (19), ferrulic acid (23) and catechin (24) on the second component, for myricetin (16), t-caftaric acid (18), t-coutaric acid (20) and catechin (24) on the thrd component, for cyanidin-3-*O*-glucoside (2), *t*-coutaric acid (20) and catechin (24) on the forth component, for cyanidin-3-*O*--glucoside (2) on the sixth component. Table 6 gives the score values for each principal component for each vranac wine sample. From the scores on the first principal component it can be interpreted that the concentrations of cyanidin-3-acetylglucoside (6), petunidin-3--acetylglucoside (7), peonidin-3-acetylglucoside (8), myr-

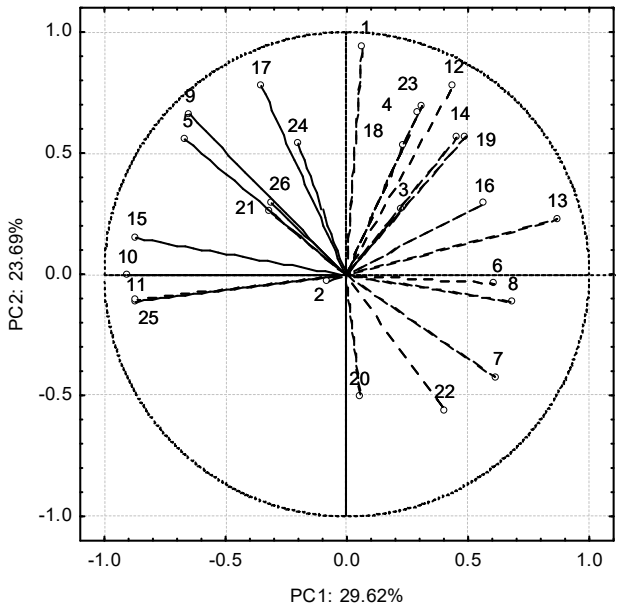

Figure 2. The score plot of the phenolic compounds in the space of the two first PCs (PC1 vs. PC2).

Table 5. The loadings of the first six principal components; (1) myricetin-3-glucoside; (2) quercetin-3-glucoside; (3) quercetin-3-glucoronide; (4) myricetin; (5) quercetin; (6) t-caftaric acid; (7) GRP; (8) t-coutaric acid; (9) caffeic acid; (10) p-coumaric acid; (11) ferrulic acid; (12) catechin; (13) procyanidin dimer B2; (14) epicatechin. Results are presented with mean (n = 3) and expressed in mg/L of wine

Phenolic compound	PC1	PC2	PC3	PC4	PC5	PC6
1	0.065	0.939	–0.091	–0.072	–0.078	0.127
2	–0.076	–0.033	–0.247	0.758	–0.188	0.557
3	0.222	0.271	–0.679	0.477	0.312	–0.177
4	0.289	0.666	–0.433	0.305	0.412	0.021
5	–0.669	0.557	–0.113	0.385	–0.153	–0.168
6	0.609	–0.041	0.003	0.494	–0.546	0.257
7	0.612	–0.424	–0.175	0.491	–0.264	–0.302
8	0.686	–0.115	0.056	0.293	–0.627	0.020
9	–0.646	0.660	–0.186	0.259	0.109	0.018
10	–0.905	–0.003	0.351	0.040	–0.091	0.057
11	–0.869	–0.118	0.098	0.043	–0.132	0.163
12	0.440	0.781	0.276	0.013	0.075	0.127
13	0.868	0.228	–0.074	0.343	0.041	0.0599
14	0.492	0.568	–0.014	–0.429	–0.185	0.295
15	–0.867	0.152	0.128	0.246	–0.354	–0.134
16	0.567	0.290	0.530	0.099	0.0297	–0.501
17	–0.354	0.776	0.397	0.311	0.096	0.046
18	0.235	0.533	0.595	0.394	0.301	0.079
19	0.453	0.565	–0.267	–0.401	–0.315	–0.339
20	0.052	–0.506	0.634	0.510	0.195	0.037
21	–0.315	0.257	–0.872	0.034	–0.103	0.021
22	0.403	–0.562	–0.412	0.421	0.191	–0.268
23	0.311	0.694	0.181	–0.426	–0.279	–0.087
24	–0.202	0.537	0.530	0.518	–0.118	–0.317
25	–0.869	–0.110	–0.059	0.112	–0.264	–0.333
26	–0.310	0.291	–0.890	0.075	–0.0518	–0.090

Table 6. The scores of the first six principal components; Vranac-SS (Vinoprodukt Čoka, Subotica, Serbia); Vranac-KS (Rubin Kruševac, Serbia); Crnogorski Vranac (Plantaže 13. jul, Montenegro); Pro Corde (Plantaže 13. jul, Montenegro); Barrique (Vinarija Mašanović-Virpazar, Montenegro); Vranac NM (Povardarie, Negotino, Macedonia); Vranac-TM (Tikveš, Macedonia); Vranac-SM (Skovin, Macedonia)

Wine sample	PC1	PC2	PC3	PC4	PC5	PC6
Vranac-SS	0.596	–0.772	–0.902	0.455	0.642	–1.520
Vranac-KS	0.729	–1.457	–0.851	–0.930	0.961	–0.110
Vranac-AS	0.681	–0.828	–0.825	1.808	–1.325	0.916
Crnogorski Vranac	–0.160	0.867	–1.349	0.399	–0.349	–1.312
Pro Corde	–2.102	–0.057	–0.015	0.181	0.147	0.287
Barrique	–1.058	–0.696	0.706	–0.976	–0.960	–0.164
Vranac-NM	0.785	1.577	1.401	–0.665	–0.827	–0.386
Vranac-TM	0.670	0.308	–0.827	–1.146	–0.066	1.490
Vranac-SM	–0.141	0.958	0.610	0.872	1.776	0.798

icetin-3-glucoside (13) and myricetin (16) on the first principal component loadings are higher for Vranac-SS, Vranac-KS, Vranac-As, Vranac-NM and Vranac-TM than the other wine samples and are lower for Pro Corde, Barrique and Crnogorski Vranac. When the second principal component is interpreted delphinidin-3-*O*--glucoside (1), peonidin-3-*O*-glucoside (4), malvidin--3-*O*-glucoside (5), malvidin-3-acetylglucoside (9), malvidin-3-*p*-coumaroylglucoside (12), quercetin-3-glucoside (14), quercetin (17), t-caftaric acid (18), GRP (19),

ferrulic acid (23) and catechin (24) are higher for Crnogorski Vranac, Vranac-NM and Vranac-SM and are lower for Vranac-KS and Vranac-AS than the other wine samples investigated. On the third principal component myricetin (16), *t*-caftaric acid (18), *t*-coutaric acid (20) and catechin (24) are higher for Vanac-NM, Barrique and Vranac-SM and are lower for Crnogorski Vranac, Vranac-KS and Vranac-TM than for the other samples. Cyanidin-3-*O*-glucoside (2), *t*-coutaric acid (20) and catechin (24) concentrations in the fourth principal component are higher for Vranac-AS and Vranac-SM and lower for Vranac-TM, Barrique and Vranac-KS. Finally, cyanidin-3-*O*-glucoside (2) concentration on the sixth principal component are higher for Vranac-TM, Vranac-AS and Vranac-SM and lower for Vranac-SS and Crnogorski Vranac.

The classification of the Vranac wine samples from the view point of phenolic compounds was also made using two (Fig. 3) and three way PC score graphs (Fig. 4). The PC 1-2 and PC 1-2-3 graph shows the highest percentage of total variance of 53.30 and 69.99%, respectively. It can be seen from the PC 1-2 and PC 1-2-3 graph that the vranac wine sample can be classified into three groups. These groups include: Vranac-SS, Vranac-KS and Vranac-AS (group 1), Pro Corde and Barrique (group 2) and Vranac-NM, Vranac-TM, Vranac-SM and Crnogorski Vranac (group 3).

CONCLUSIONS

In recent years, phytochemicals, especially phenolics, have attracted increasing attention for their anti-

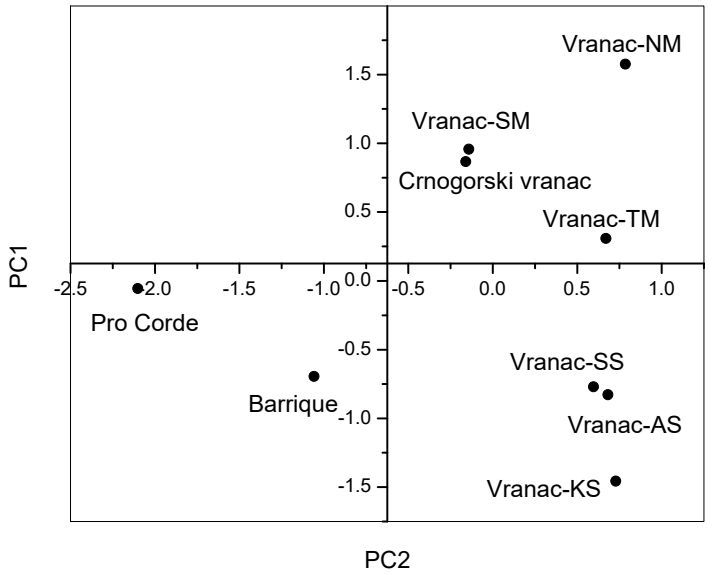

Figure 3. Two way PCA score plot (PC1 vs. PC2).

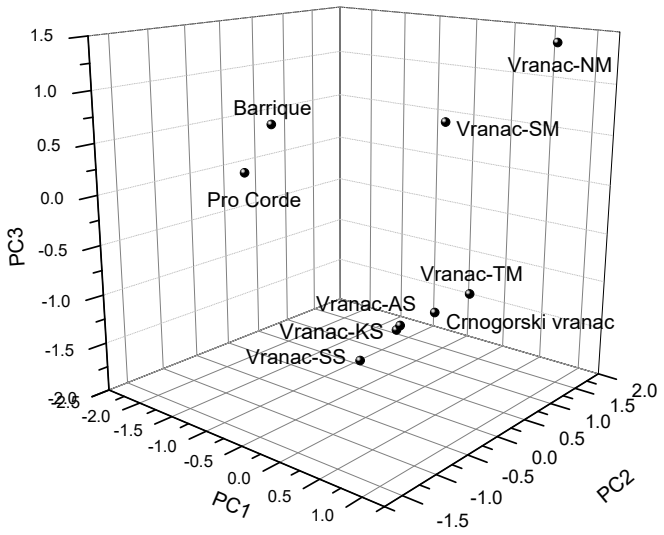

Figure 4. Three way PCA score plot (PC 1-2-3).

oxidant activities. Wines provide phenolic antioxidant, which contribute to their potential health benefits. This work has shown that the phytochemicals present in wines produced in the Balkan region have potent antioxidant activities and that the antioxidant activity in wines is positively correlated with total phenolic content. Red wines produced in Montenegro were found high in anthocyanins and flavonols. Also, red wines from Serbia and Macedonia were good source of hydroxycinnamic acids. From the obtained results, we can conclude that the content of phenolic compounds in the single-cultivar investigated wines (Vranac) depends most of all on agroclimatic factors and genological practices of the particular Balkan vineyard producing wine.

Acknowledgments

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IZVOD

ANTIOKSIDATIVNA AKTIVNOST I POLIFENOLNI SASTAV CRVENOG VINA VRANAC SA PODRUČJA BALKANA

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Poslednjih godina mnoga istraživanja su dokazala da fenolna jedinjenja imaju presudnu ulogu u antioksidativnoj aktivnosti mnogih prehrambenih proizvoda. Vina su odličan izvor polifenola, uključujući derivate benzoeve i cimetne kiseline, flavan-3-ole, flavonole i antocijane. Glavni cilj našeg istraživanja je bio da se ispita korelacija između radikal-hvatačkog kapaciteta srpskih, makedonskih i crnogorskih vina Vranac sa različitih geografskih područja u odnosu na sadržaj fenola, flavonoida i individualnih polifenolnih jedinjenja. Sva analizirana vina su pokazala visoku antioksidativnu aktivnost od 13.00 do 15.02 mmol/L, dok je sadržaj fenolnih jedinjenja bio između 3478.70 i 3935.19 mg/L. U cilju razdvajanja i određivanja individualnih fenolnih jedinjenja (flavan-3-ola, flavonola, hidroksicimetnih kiselina i antocijana) upotrbljena je HPLC metoda, metoda direktnog injektiranja. Identifikacija je vršena na osnovu retencionih vremena i spektralnih osobina određenih komercijalnih standarda. Nakon analize je utvrđeno da je najzastupljeniji antocijanin malvidin-3-glukozid (179,04–281,31 mg/L), najzastupljeniji flavonol je kvercetin-3-glukoronid (5,88–11,78 mg/L), najzastupljeniji flavan-3-ol je katehin (24,43–76,78 mg/L), dok *t*-kaftarna kiselina (13,46–38,56 mg/L) spada u red najzastupljenijih hidroksicimetnih kiselina. Na osnovu dobijenih rezultata, spektrofotometrijskom i HPLC metodom, mozemo pouzdano tvrditi da vina sa područja Balkana pokazuju visoku antioksidativnu aktivnost i da je ona u pozitivnoj korelaciji sa fenolnim sadržajem. Takođe se može zaključiti da sadržaj fenolnih jedinjenja u vinima proizvedenim od iste sorte grožđa pre svega zavisi od agroklimatskih faktora, uslova gajenja grožđa i procesa proizvodnje vina.

Ključne reči: Crveno vino • Vranac • Fenolni profil • Antioksidativna aktivnost • HPLC-DAD