Polyphenol content and antioxidant activity of commercial blackberry wines from Croatia: Application of multivariate analysis for geographic origin differentiation

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Summary

Since polyphenolic compounds have been proposed to have beneficial effects on human health as antioxidants, the objectives of this study were to evaluate the polyphenol content and antioxidant activity in 17 commercial blackberry wines (BW) from three different Croatian subregions, and to investigate the relationship between these compounds and geographic origin of BW. A statistically significant difference was found between the concentration of monomeric anthocyanins present in the samples from three subregions. Furthermore, the concentrations obtained for gallic acid were significantly higher than for chlorogenic, caffeic and *p*-coumaric acids. The half-maximal inhibitory concentrations (IC_{50}) were slightly higher for ABTS (2,2'-azino-bis-[3-ethylbenthiazoline-6-sulfonic acid]) test than for DPPH (2,2-diphenyl-1-picrylhydrazyl) test. Based on the results obtained by multivariate data analysis (principal component analysis and linear discriminant analysis) three models were created that gave a satisfactory grouping of the investigated BW based on their geographic origin. The best discriminating model showed not only extreme separation concerning subregions, but also a very narrow area of dispersion of individual wine samples intraregionally.

Keywords

blackberry wine; *Rubus fruticosus;* polyphenols; antioxidant activity; HPLC; multivariate analysis

Blackberry (*Rubus fruticosus, Rosaceae*) is an edible berry which has been used in Europe for over 2000 years for eating and medicinal purposes. Nowadays, a significant proportion of fresh fruits is directly processed into blackberry products – jam, juice or wine. Blackberry wine (BW) is a product of yeast fermentation of natural saccharides present in blackberry juice. This popular fruit wine is usually served as a dessert wine, being enjoyed with meals in moderate quantities. Daily consumption of blackberry wine in recommended quantities (about 250 ml) is a well-known natural source of many bioactive phytochemicals, which may play an important role in health promotion and disease prevention. Polyphenolic compounds constitute one of the most important quality parameters of wines since they contribute to their organoleptic characteristics, particularly colour, taste, astringency and bitterness. Moreover, these compounds have received much attention in prevention of human neurodegenerative diseases, as cardiovascular disorders and cancer, as they are known to have a high antioxidant activity [1]. Polyphenols have also been reported to possess antibacterial, antimutagenic, anti-inflammatory and vasodilatory properties [2–5]. The concentration of total polyphenols in blackberry wine may be strongly influenced not only by blackberry varieties and cultivation, but also by the way of their extraction from berries during the winemaking process, transport and storage. Furthermore, during wine aging process, the amount of total polyphenols present in wine, particularly anthocyanins, may be directly affected by several factors including pH, light, temperature, oxygen, enzymes, ascorbic acid, saccharides, sulfur dioxide or sulfite

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salts, metal ions and copigments [6, 7].

In the continental part of Croatia, blackberry is grown on small farms using traditional techniques of cultivation and harvesting in summer (July-August) by hand. Thornless Logan, Thornfree, Black Satin and Tayberry are the most common blackberry cultivars used for winemaking. In Croatia, blackberry wine is traditionally called "željezno vino" (ferrous wine), due to an elevated iron content and content of dietary compounds such as ascorbic, citric and malic acids, carotenoids, fructose and alcohol that enhance iron absorption [8–10]. Therefore it has been used as a popular medicine for anaemia and iron deficiency. This fruit wine is one of the most popular alcoholic beverages in Croatia, being mainly produced in the continental part of the country. This is divided into a number of subregions: from the Danube Basin, through Slavonia, Moslavina, Pokuplje, Plešivica, to Prigorje-Bilogora and Zagorje-Međimurje. During the last decade, the classification of wines and other food products according to their geographic origin have received considerable attention in the scientific literature. Principal component analysis (PCA), linear discriminant analysis (LDA) and other multivariate statistical methods have been widely applied for this kind of wine classification using different variables such as contents of minerals, trace elements, amino acids, phenolic, volatile and aroma compounds, colour and sensorial parameters [11–14].

It should be pointed out that the production of blackberry wine has considerably increased in all Croatian subregions for the last few years. Although blackberry has a long history and there have been numerous studies on its chemical composition and pharmacological activity [15–17], the research studies related to this fruit wine are in the initial stages and there is no information or data available on polyphenol content and on their antioxidative activity. For this reason, the aims of this work were: (i) to obtain data on the concentration of polyphenols in Croatian blackberry wines and to investigate this kind of wines as a source of polyphenols, (ii) to determine and compare the antioxidant activity of Croatian blackberry wines using three methods: ABTS (2,2'-azino-bis-[3-ethylbenthiazoline-6-sulfonic acid]), DPPH (2,2-diphenyl-1-picrylhydrazyl) and reducing power assay (RPA), (iii) to evaluate the contribution of polyphenolic compounds to the antioxidant properties of blackberry wines and (iv) to decide which of these compounds could be used in the differentiation of blackberry wines according to the subregion of production.

MATERIALS AND METHODS

Samples

Seventeen commercially available samples of Croatian blackberry wines were collected in a local drugstore during 2000–2004, being categorized into three classes according to the subregion of their cultivation: Slavonia (SL, five samples: BW 1 – BW 5), Prigorje-Bilogora (PB, six samples: BW 6 – BW 11) and Zagorje-Međimurje (ZM, six samples: BW 12 – BW 17). Wine samples were stored at 2–8 °C in the dark until analysed. The alcoholic content declared on the bottle for all samples and on the label ranged from 11.6% to 15.6% ethanol (v/v). In order to get a representative sample of each wine sample, three different bottles of particular wine type were collected and contents of all three bottles were mixed before analysis.

Reagents and standards

All reagents used in this work were of analytical reagent grade or better. Standards of phenolic acids were purchased from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's phenol reagent was obtained from Fluka (Buchs, Switzerland). $K_3[Fe(CN)_6]$ and butylated hydroxyanisole(BHA) were from HiMedia (Mumbai, India). As a source of free radicals, DPPH (2,2-diphenyl-1-picrylhydrazyl, 95%) and ABTS (2,2'-azino-bis-[3-ethylbenthiazoline-6-sulfonic acid]) in the crystallized diammonium salt form were obtained from Sigma-Aldrich. Double-deionized water (DDW) was used in all experiments.

Analytical methods

pH determination

A digital pH meter MP 225 with a pH electrode INLAB 413 (Mettler Toledo, Greifensee, Switzerland) was used to determine the pH values of blackberry wines [18].

Total nitrogen

Total nitrogen concentration of blackberry wines was measured by the Kjeldahl method. A Kjeltec TM 2300 equipped with Digestor 2006, Scrubber 2001 and Controller 2000 (Foss Tecator, Höganäs, Sweden) was used for the determination of total nitrogen in samples [18].

HPLC analysis of phenolic acids

The concentrations of phenolic acids (gallic acid, chlorogenic acid, caffeic acid and *p*-coumaric acid) were determined by high performance liquid chromatography (HPLC). The chromatographic analyses were performed in a Dionex chromato-

graphic system (Sunnyvale, California, USA), with a P680 pumping system, an ASI 100 automatic sample injector, a TCC-100 oven for columns, a UVD170S detector and Chromeleon 6.8 software. All samples were filtered through Minisart RC4, 0.45 μm filters (Sartorius, Göttingen, Germany), which did not retain any of the analytes. After direct injection of 20 μl of sample, phenolic acids were separated on a Lichrospher 100-RP18 column (250 × 4 mm, 5 μm; Agilent Technologies, Santa Clara, California, USA) with a suitable guard column. The chromatographic conditions were a modification of those by GAMBELLI and SANTARONI [19]. The solvents used for gradient elution were (A) acetic acid : water (5 : 95) and (B) acetonitrile. The concentration of acetonitrile was increased from 0% to 80% during 30 min and during the next 3 min was constantly 80% (total run time, 33 min). Elution was carried out at a flow rate of 1 ml·min^{-1} and the column was thermostatically controlled to maintain a temperature of 30 °C. During each run, absorbance was recorded at 280 nm (gallic acid), 313 nm (*p*-coumaric acid) and 323 nm (chlorogenic and caffeic acid). Pure standards of phenolic acids in methanol were used to construct the standard curve and to determine retention times. The linear calibration curves were obtained by injecting different concentration of standards. Phenolic acids were identified by the comparison of retention times and UV spectra with the corresponding pure commercial standards (as external standards). Quantitative analysis was performed in triplicate using external calibration curves. Compounds detected in HPLC were quantified on the basis of peak area obtained by integration.

Determination of total polyphenols – Folin-Ciocalteu Index (TPH)

The total polyphenol concentration in blackberry wines was estimated by Folin-Ciocalteu colorimetric assay based on the procedure described by Organisation Internationale de la Vigne et du Vin (O.I.V.) [18]. Gallic acid was used as a standard. After incubation for 2 h at room temperature, the absorbance was measured at 760 nm using a spectrophotometer (model Lambda 25 UV-VIS; Perkin-Elmer, Waltham, Massachusetts, USA). Results were expressed as gallic acid equivalents (milligrams of gallic acid per litre) determined from a standard calibration curve.

Determination of total monomeric anthocyanins (ACY)

The total concentration of monomeric anthocyanins in blackberry wines was determined using the pH differential method [20]. Absorbance was measured at 510 nm and 700 nm. ACY values were expressed as milligram of malvidin-3-glucoside per litre (molar extinction coefficient of 28 000 l·cm^{-1}·mol^{-1} and molecular weight of 463.3 g·mol^{-1}).

Antioxidant activity

DPPH method

The capacity of blackberry wines to scavenge the 'stable' free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored by a slightly modified method of HATANO et al. [21]. Blackberry wines were diluted with methanol and an aliquot of 4 ml of various concentrations of sample added to a methanolic solution of DPPH (1 mmol·l^{-1}, 0.5 ml). The mixture was vortexed for 15 s and then left to stand at room temperature for 30 min. The absorbance of the resulting solution was read at 517 nm. Instead of pure methanol, a methanolic solution of 2 mg of BHA dissolved in 4 ml of methanol with 0.5 ml of the DPPH solution was used for background correction.

ABTS method

The spectophotometric analysis of ABTS radical-scavenging activity was determined according to RE et al. [22]. The solution of ABTS monocation radical was prepared by mixing equal volumes of a 7 mmol·l^{-1} ABTS solution and a 2.45 mmol·l^{-1} potassium persulfate solution, both in DDW. The mixture was stored in the dark at room temperature for 12 h before use and was diluted to get an absorbance of 0.700 ± 0.025 at 730 nm with DDW. Then, 0.5 ml of ABTS solution was added to an aliquot of 3 ml of blackberry wine diluted in DDW at different concentrations. After 30 min, the absorbance of mixture was measured at 730 nm. The percentage of inhibition was calculated for each concentration relative to the blank absorbance (DDW).

For DPPH and ABTS methods, the radical-scavenging activity (*RSA*) was calculated as a percentage of DPPH or ABTS radical discoloration using the following Eq.(1):

$$RSA = \left(1 - \frac{A_{BW}}{A_0}\right) \times 100 \quad (1)$$

where A_{BW} is the absorbance of the solution when the blackberry wine was added at a particular level, and A_0 is the absorbance of the DPPH or the ABTS solution.

Further, the half-maximal inhibitory concentrations (IC_{50}) were calculated from regression equations, where the abscissa represented

Tab. 1. Total nitrogen, pH, total polyphenolic compounds, monomeric anthocyanins, individual phenolic acids and antioxidant activity in Croatian blackberry wines.

Sample	TN [mg·l^{-1}]	pH	TPH [mg·l^{-1}]	ACY [mg·l^{-1}]	Gallic acid [mg·l^{-1}]	Chlorogenic acid [mg·l^{-1}]	Caffeic acid [mg·l^{-1}]	*p*-Coumaric acid [mg·l^{-1}]	DPPH [mg·l^{-1}]	ABTS [mg·l^{-1}]
BW 1	279.95 ± 0.64	3.15 ± 0.01	1 513 ± 110	6.21 ± 0.55	111.52 ± 0.48	1.028 ± 0.017	2.864 ± 0.015	1.319 ± 0.002	5.52 ± 0.19	6.12 ± 0.09
BW 2	71.65 ± 1.11	3.15 ± 0.01	829 ± 1	10.74 ± 0.22	28.14 ± 0.39	1.797 ± 0.046	2.602 ± 0.052	1.501 ± 0.042	7.21 ± 0.26	5.90 ± 0.16
BW 3	119.55 ± 0.64	3.39 ± 0.01	1 907 ± 44	14.20 ± 1.19	60.91 ± 3.02	1.249 ± 0.027	4.010 ± 0.054	3.005 ± 0.052	4.36 ± 0.18	5.76 ± 0.07
BW 4	200.48 ± 0.64	3.43 ± 0.01	1 695 ± 13	5.95 ± 0.18	99.12 ± 0.24	1.370 ± 0.009	3.972 ± 0.022	4.354 ± 0.004	5.29 ± 0.07	5.75 ± 0.12
BW 5	455.06 ± 3.55	3.24 ± 0.10	1 526 ± 122	4.98 ± 0.33	58.65 ± 0.75	1.442 ± 0.021	3.574 ± 0.013	2.515 ± 0.013	6.05 ± 0.13	7.74 ± 0.23
BW 6	65.36 ± 0.30	3.63 ± 0.01	733 ± 18	2.00 ± 0.02	122.41 ± 1.42	1.061 ± 0.023	3.882 ± 0.016	3.319 ± 0.036	6.29 ± 0.34	5.77 ± 0.14
BW 7	193.13 ± 3.31	3.22 ± 0.01	1 574 ± 12	28.45 ± 1.98	49.09 ± 0.29	3.115 ± 0.012	3.849 ± 0.006	1.616 ± 0.012	5.11 ± 0.18	5.77 ± 0.09
BW 8	128.01 ± 5.06	3.32 ± 0.01	1 433 ± 38	8.93 ± 0.34	58.97 ± 0.45	3.942 ± 0.031	3.540 ± 0.036	2.636 ± 0.074	4.43 ± 0.26	5.05 ± 0.04
BW 9	137.14 ± 6.77	3.25 ± 0.01	1 126 ± 71	6.63 ± 0.12	43.23 ± 1.40	2.997 ± 0.010	2.615 ± 0.057	1.997 ± 0.038	6.21 ± 0.12	6.03 ± 0.10
BW 10	73.57 ± 0.64	3.41 ± 0.01	1 994 ± 4	23.17 ± 0.37	113.95 ± 0.93	1.935 ± 0.063	4.721 ± 0.028	1.749 ± 0.014	4.32 ± 0.25	4.56 ± 0.05
BW 11	217.78 ± 5.10	3.41 ± 0.01	1 225 ± 46	1.30 ± 0.12	115.32 ± 0.75	1.910 ± 0.055	3.032 ± 0.018	2.385 ± 0.035	4.38 ± 0.12	9.27 ± 0.17
BW 12	235.43 ± 2.55	3.36 ± 0.01	1 256 ± 14	30.64 ± 0.38	60.91 ± 3.02	2.246 ± 0.034	3.018 ± 0.061	1.033 ± 0.021	5.40 ± 0.11	5.62 ± 0.12
BW 13	85.36 ± 0.68	3.20 ± 0.01	1 433 ± 20	9.50 ± 1.19	75.65 ± 0.34	2.561 ± 0.026	3.467 ± 0.010	2.424 ± 0.032	4.63 ± 0.13	5.74 ± 0.14
BW 14	213.73 ± 1.27	3.29 ± 0.01	2 698 ± 113	74.09 ± 0.61	92.31 ± 0.96	1.594 ± 0.044	4.351 ± 0.036	2.231 ± 0.021	4.53 ± 0.14	5.30 ± 0.06
BW 15	96.38 ± 3.37	3.23 ± 0.01	1 816 ± 48	18.55 ± 0.26	88.86 ± 0.56	3.552 ± 0.011	3.543 ± 0.004	2.997 ± 0.012	5.54 ± 0.35	4.88 ± 0.27
BW 16	105.94 ± 2.92	3.17 ± 0.01	2 284 ± 8	125.31 ± 0.74	70.33 ± 0.85	2.431 ± 0.063	2.031 ± 0.028	1.246 ± 0.024	6.04 ± 0.03	5.82 ± 0.02
BW 17	159.29 ± 6.74	3.33 ± 0.01	1 275 ± 21	25.84 ± 0.39	61.41 ± 0.54	2.304 ± 0.038	4.845 ± 0.012	2.369 ± 0.037	6.89 ± 0.06	4.52 ± 0.01

Values represent mean concentration ± standard deviation.
TN – total nitrogen, TPH – total polyphenolic compounds (values are expressed as milligrams of gallic acid per litre of sample), ACY – monomeric anthocyanins (values are expressed as milligrams malvidin-3-glucoside per litre of sample).

the total polyphenol concentration in blackberry wines (expressed as gallic acid equivalents) and the ordinate the percent of scavenging capacity from the test. IC_{50} is the concentration of blackberry wine required for 50% inhibition.

Reducing power assay (RPA)

The reducing power of the blackberry wines was determined by the method of Oyaizu [23] and Yen and Chen [24] with some slight modifications. Briefly, 0.1–1.0 ml of each investigated blackberry wine was diluted with DDW. In 1.0 ml of the prepared aliquot, 2.5 ml of a 0.2 mol·l^{-1} phosphate buffer (pH 6.6) and 2.5 ml of a 1% (w/v) solution of potassium ferricyanide were added in the test tube. The mixture was incubated in a water bath at 50 °C for 20 min. Following this, 2.5 ml of a 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 1 750 ×*g* for 10 min. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of DDW and 0.5 ml of a 0.1% (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read at 700 nm. An increased absorbance of the reaction mixture indicated a greater reducing power.

Statistical methods

All determinations were conducted in triplicate. Descriptive statistical analysis, correlation analyses and the univariate characterization of blackberry wines by their geographic origin were done as described by Amidžić Klarić et al. [25].

Principal component analysis (PCA) was used to provide a new set of variables (the least possible number) that are calculated in a way to keep most of the information present in the original data set. Principal components (PC) derived from the original data set with the cumulative percentage of total explained variance of >99.0 were used for further analysis with linear discriminant analysis (LDA). Principal components were separately produced for the model of polyphenol content and antioxidant activity, and for the model

with the addition of metal content. The mineral and heavy metal contents (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Co, Cr and Cd) in the investigated samples of blackberry wines were determined by FAAS/FAES and GFAAS and the model using only metal content was published by Amidžić Klarić et al. [25].

LDA was conducted using the original data set and compared with the same analysis done with PC for the model of polyphenol content and antioxidant activity, as well as for the model with the addition of metal content [25] using backward stepwise approach to produce the discriminant model with the least number of variables, but still enough statistical power to discriminate significantly between subregions. A classification model for subregional separation was provided for all LDA models.

The statistical package Statistica ver. 7.1 from StatSoft (Tulsa, Oklahoma, USA) was used for analyses of all data.

RESULTS AND DISCUSSION

Wines, like many other natural food products, contain varying amounts of different nitrogenous substances including proteins, polypeptides, peptides, amino acids, amides, ammonia, nitrates and nitrites. Total nitrogen values were obtained from the sum of all nitrogenous compounds present in investigated blackberry wines (Tab. 1). The total nitrogen concentrations in the analysed blackberry wines were found to fall in a rather wide range (65.36–455.06 mg·l^{-1}), the median value being 137.14 mg·l^{-1} (standard deviation (*SD*): 0.30–6.77 mg·l^{-1}; relative standard deviation (*RSD*): 0.2–4.9%). It was observed that blackberry wines had a relatively low total nitrogen concentration compared to the average concentration of 226.8 mg·l^{-1}, which was found in Spanish red wines by Alcaide-Hidalgo et al. [26].

Generally, table wines have pH between 3.3 and 3.7, while the range of pH values of the investigated blackberry wines were from 3.15 (BW 1 and BW 2) to 3.63 (BW 6), the average value being 3.31. The pH values of sample wines are listed in Tab. 1.

Polyphenol content of blackberry wine

Because the composition of polyphenolic compounds in blackberry wines may be strongly affected, not only quantitatively but also qualitatively, by berry cultivar, maturity degree, environmental factors, winemaking techniques and technological treatments, a major part of our research efforts was to apply the spectrophotometric and chromatographic methods to investigate the content of total polyphenolic compounds, monomeric anthocyanins and phenolic acids.

The results on total polyphenol concentration in blackberry wines (Tab. 1), reported as milligram of gallic acid equivalents per litre of sample, demonstrate that the investigated blackberry wines differed significantly in the amount of total polyphenols (from 733 mg·l^{-1} for BW 6 to 2698 mg·l^{-1} for BW 14; mean value: 1548 mg·l^{-1}).

In order to investigate whether the region has an influence on the polyphenol contents of Croatian blackberry wines, the polyphenol content of wines from different subregions were compared. Our results showed that two wines from Zagorje-Međimurje had the highest contents of polyphenols (above 2000 mg·l^{-1}), while the only samples with the content below 1000 mg·l^{-1} belonged to Slavonia region (829 mg·l^{-1}) and Prigorje-Bilogora (733 mg·l^{-1}). Comparing our results to the literature data it was found that polyphenol content of investigated Croatian BW was mostly similar to the content of Portuguese, Brazilian, Chilean [27, 28] and Greek [29] red wines. It should be also pointed out that Croatian BW had a significantly higher content of polyphenols compared to white wines from the above mentioned regions as well as Croatian white wines [27, 29, 30].

As it can also be seen from Tab. 1, the concentration of monomeric anthocyanins, expressed as malvidin-3-glucoside, differed quite markedly, ranging from 1.30 mg·l^{-1} for BW 11 to 125.31 mg·l^{-1} for BW 16; the median value: 10.74 mg·l^{-1}. A statistically significant difference ($p = 0.032$) was found

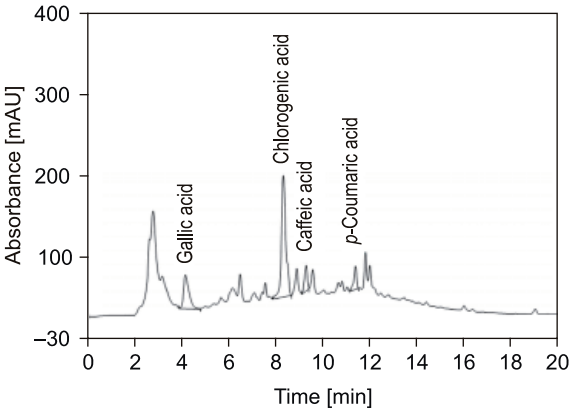

Fig. 1. Chromatogram of blackberry wine (sample No. 13) monitored at 313 nm.

Conditions: Lichrospher 100-RP18 column (250 × 4 mm, 5 μm); eluents: (A) acetic acid : water (5 : 95) and (B) acetonitrile; total run time: 33 min; flow rate: 1 ml·min^{-1}; column temperature: 30 °C; volume of injection: 20 μl of sample.

between the concentration of monomeric anthocyanins in the samples from different Croatian subregions (median values for wines from individual subregions were ZM – 28.24 mg·l^{-1}, SL – 6.29 mg·l^{-1} and PB – 7.78 mg·l^{-1}). In addition, the obtained results demonstrate that the wines with the highest contents of total polyphenols (BW 14 and BW 16) were also the richest source of monomeric anthocyanins. Similar data, not only for total polyphenols but also for monomeric anthocyanins, were found in Greek red wines [29].

Since phenolic acids in blackberry fruit are represented by benzoic acid and cinnamic acid derivatives [31, 32], liquid chromatography was used to investigate the presence of four phenolic acids (gallic, chlorogenic, caffeic and *p*-coumarinic acid) in BW. A satisfactory separation was achieved using the above described method. A representative chromatogram is presented in Fig. 1. The performance characteristics of the proposed method were evaluated on the basis of linearity, detection limit (*LOD*), quantification limit (*LOQ*), precision and accuracy. The linearity was examined by analysing solutions in a range of concentrations between 6 mg·l^{-1} and 125 mg·l^{-1} for gallic acid and from 1 mg·l^{-1} to 15 mg·l^{-1} for chlorogenic, caffeic and *p*-coumarinic acids. The correlation coefficient of the linear regression of the standard curves was greater than 0.999 for all investigated phenolic acids. Detection and quantification limits were determined using progressively lower concentrations for a signal-to-noise ratio of 3:1 and 10:1, respectively. The highest detection and quantification limits were determined for gallic acid (*LOD* = 0.529 mg·l^{-1} and *LOQ* = 5.292 mg·l^{-1}), while for other investigated acids were significantly lower (*LOD* = 0.079 mg·l^{-1} and *LOQ* = 0.790 mg·l^{-1} for chlorogenic acid; *LOD* = 0.074 mg·l^{-1} and *LOQ* = 0.740 mg·l^{-1} for caffeic acid; and *LOD* = 0.074 mg·l^{-1} and *LOQ* = 0.736 mg·l^{-1} for *p*-coumarinic acid). The accuracy of the method was investigated by multiple injections (n = 3) of three different standard solutions. The recovery rate for gallic acid was 99.8% ± 0.5%, for chlorogenic acid was 97.8% ± 1.6%, for caffeic acid was 99.9% ± 1.2% and for *p*-coumarinic acid was 99.5% ± 0.8%. The repeatability of the method was tested by repeated injections of the standard solutions mixture (n = 3) over three consecutive days. The relative standard deviations (*RSD*) were in the range from 0.2% to 1.2% for intra-day repeatability and from 0.5% to 1.6% for inter-day repeatability. These results clearly show that the chromatographic method was suitable for determination of the investigated phenolic acids in samples of BW. All samples were analysed in triplicate and the concentration of phenolic acids is given in Tab. 1. The obtained results demonstrate the presence of these four acids in all wines included in the study. It is also noteworthy that the concentrations of gallic acid in Croatian BW showed a relatively high variability (28.14–122.41 mg·l^{-1}). The concentrations of gallic acid were significantly higher than those of chlorogenic acid (1.028–3.942 mg·l^{-1}), caffeic acid (2.031–4.845 mg·l^{-1}) and *p*-coumaric acid (1.033–4.354 mg·l^{-1}). Our results agree in general with results of earlier studies of wines [27, 33, 34]

Antioxidant activity of blackberry wine

Since blackberry wine contains a wide variety of free radical-scavenging molecules, such as polyphenols, the aim of this work was also to investigate the antioxidative activity of Croatian blackberry wines. Chromogen radicals, such as DPPH and ABTS, are useful reagents for investigating the free-radical-scavenging activities of compounds and they are often used as substrates to evaluate the antioxidative activity of phytochemicals. These free radicals, on interaction with antioxidants, accept an electron or hydrogen atom to become a stable molecule. The degree of discoloration indicates the radical-scavenging potential of the antioxidant. Arnao [35] pointed out some methodological problems in the determination of antioxidant activity of these chromogenic radicals. Firstly, DPPH is acquired directly without preparation, while ABTS has to be generated by chemical or enzymatic reactions. Another important difference is that DPPH can only be dissolved in organic media, which is an important limitation in analysing antioxidative activity of hydrophilic compounds. In contrast, ABTS may be solubilized in organic or aqueous media, and can be easily used for interpreting the role of lipophilic and hy-

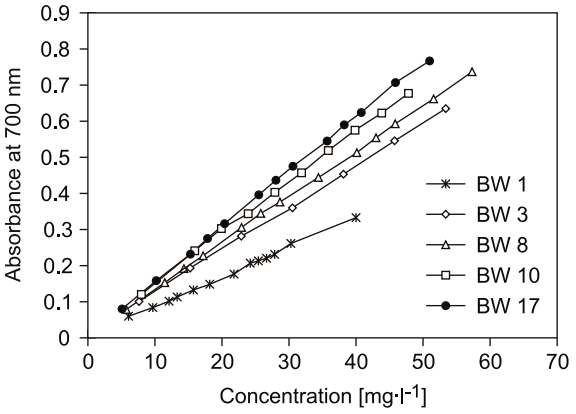

Fig. 2. Antioxidant activity of investigated blackberry wines expressed as reducing power.

drophilic antioxidants. Thus, free-radical-scavenging activities of blackberry wines at different concentrations were tested by both methods, DPPH and ABTS. The results, expressed as IC_{50} values, are presented in Tab. 1 and it should be pointed out that a lower IC_{50} value indicates a greater antioxidant activity. The IC_{50} values obtained by the DPPH method ranged from 4.32 mg·l^{-1} (BW 10) to 7.21 mg·l^{-1} (BW 2), the average value being 5.42 mg·l^{-1} (*SD*: 0.03–0.35 mg·l^{-1}; *RSD*: 0.6–6.3%). The IC_{50} values obtained by the ABTS method did not significantly differ from values obtained by the DPPH method and ranged from 4.52 mg·l^{-1} (BW 17) to 9.27 mg·l^{-1} (BW 11), the average being 5.86 mg·l^{-1} (*SD*: 0.01–0.27 mg·l^{-1}; *RSD*: 0.2–5.6%).

As mentioned above, the antioxidative activity of Croatian blackberry wines was also investigated by the reducing power assay. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of each sample. The presence of reductants (i.e. antioxidants) in the samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm [36]. In other words, the $FeCl_3/K_3Fe(CN)_6$ system offers a sensitive method for the "semi-quantitative" determination of concentrations of dilute polyphenolics, which participate in the redox reaction. RPA of the samples was evaluated at a concentration of 40 mg·l^{-1} and, as it can be seen from Fig. 2, the lowest absorbance was found for BW 1 (0.330) and the highest for BW 17 (0.609).

The investigation, whether subregion has influence on the antioxidant activity of blackberry wines, showed no significant difference ($p > 0.05$) between the antioxidant activities measured by all above mentioned tests. Comparing our results with those obtained by other authors using DPPH, ABTS and RPA assays was rather difficult because of differences in the sample preparation and in the expressions used. For example, Lucena et al. [37] investigated the antioxidant activity of monovarietal northeastern Brazilian wines after extraction using ethyl acetate at different pH values, yielding three fractions for each sample assayed, while Anastasiadi et al. [38] evaluated the antioxidant activity of Greek wine using a XAD-4 resin column for sample preparation. Furthermore, some authors [39-43] used millimoles of Trolox equivalent per litre to express the antioxidant activity, whereas others [44, 45] employed IC_{50} values, which is the amount of wine needed to reduce the initial DPPH or ABTS value by 50%. However, our results for DPPH test are quite consistent with those of Woraratphoka et al. [46] obtained for red wines from Thailand (2.7–6.7 mg·l^{-1}).

Furthermore, a significant correlation between results obtained by ABTS and RPA methods was observed ($r = –0.58$; $p = 0.016$). Also, the antioxidant activity of the blackberry wines slightly yet significantly correlated with their total polyphenols composition (DPPH and TPH: $r = –0.50$; $p = 0.04$) and monomeric anthocyanins (ABTS and ACY: $r = –0.57$; $p = 0.02$). Monomeric anthocyanins showed weak and non-significant correlations with results obtained by the DPPH method, similar to the results previously published by Giovanelli [44] and Granato et al. [28]. These authors also found that there was no correlation between the contents of anthocyanins and the antioxidant activity measured by the DPPH assay. Furthermore, non-significant correlations were found between the antioxidant activity and contents of individual phenolic compounds, which is in agreement with earlier reports for wines [44]. The antioxidant activity depends on many factors other than the phenolic composition. BW is a complex matrix that contains large quantities of phenolic and non-phenolic compounds and thus antioxidant activity cannot be predicted by the content of a specific class of substances or a substance alone.

According to this study, a considerable amount of polyphenols can be found in Croatian BW and the extent of antioxidant activity of this popular fruit wine is in conformity with the content of these bioactive phytochemicals.

Multivariate analysis

Applied to our data set, PCA for the model of polyphenol content and antioxidant activity revealed the first two and three eigenvectors representing 46.3% and 64.3%, respectively, of the total variability of the data (Tab. 2) by reducing the number of features from 11 original variables (manifest variables) to two or three principal components (latent variables). Also, for this model four principal components that accounted for 74.9% of the variance were chosen on the basis of Kaiser's criterion (eigenvalues higher than 1.0).

Furthermore, PCA for the model with the addition of metal content [25] revealed the first three and four eigenvectors representing 52.4% and 63.5%, respectively, of the total variability of the data by reducing the number of features from 22 original variables to three or four principal components. In addition for this model, seven principal components that accounted for 83.5% of the variance were chosen on the basis of Kaiser's criterion, and the first fourteen principal compo-

Tab. 2. Variance explained by 11 principal components derived by PCA for the model of polyphenol content and antioxidant activity, and for the model with the addition of metal content.

PC	Model of polyphenol content and antioxidant activity			Model of polyphenol content and antioxidant activity with the addition of metal content		
	Eigenvalue	Variance explained [%]	Cumulative variance explained [%]	Eigenvalue	Variance explained [%]	Cumulative variance explained [%]
1	2.915	26.5	26.5	4.505	20.5	20.5
2	2.179	19.8	46.3	3.914	17.8	38.3
3	1.973	17.9	64.3	3.117	14.2	52.4
4	1.171	10.6	74.9	2.423	11.0	63.5
5	0.909	8.3	83.2	1.888	8.6	72.0
6	0.563	5.1	88.3	1.493	6.8	78.8
7	0.552	5.0	93.3	1.033	4.7	83.5
8	0.335	3.0	96.3	0.948	4.3	87.8
9	0.241	2.2	98.5	0.659	3.0	90.8
10	0.133	1.2	99.7	0.584	2.7	93.5
11	0.029	0.3	100.0	0.439	2.0	95.5
12				0.362	1.6	97.1
13				0.267	1.2	98.3
14				0.160	0.7	99.1
15				0.120	0.5	99.6
16				0.089	0.4	100.0

nents (out of 16) explained 99.1% of total variance (Tab. 2). These 10 principal components for the model of polyphenol content and antioxidant activity, as well as 14 principal components for the model with the addition of metal content, were used for further analysis.

As one of the aims of this study was to investigate the relationship between polyphenol content, antioxidant activity with/without metal content and the geographic origin of the blackberry wine, LDA was applied to determine the discriminant power of both original data sets and principal components determined previously, using backward stepwise procedure. The non error rate (*NER%*) of the LD model of polyphenol content and antioxidant activity for the original data set was 100% and for principal components model 94.1%, giving a slightly lesser discriminate power. The model based on the original data set of polyphenol content and antioxidant activity of the wines finally included 9 original variables: pH, total nitrogen, ACY, ABTS, RPA, gallic acid, chlorogenic acid, caffeic acid and *p*-coumaric acid. It facilitated a satisfactory separation of three subregions of the wine origin. The data for sensitivity and specificity associated with this LDA model showed good results with sensitivity of 100%and specificity of 100%. Canonical scores for both roots for each sample from three subregions are depicted in Fig. 3A.

The non error rate (*NER%*) of the LD model of polyphenol content and antioxidant activity with the addition of metal content for the original data set was 100% as well as for PCs model. Although the model based on principal components produced a comparable discriminant power with less variables included into the model (7 principal components vs. 9 original variables), these 7 principal components actually included data from all original dataset variables. The model based on the original data set of antioxidant activity, polyphenol and metal content of investigated wines (finally including 9 original variables: chlorogenic acid, Ca, Zn, ABTS, ACY, RPA, pH, Cu, *p*-coumaric acid) gave a satisfactory separation of three production areas. The data for sensitivity and specificity associated with this LDA model showed good results with sensitivity of 100% and specificity of 100%. Canonical scores for both roots for each sample from three subregions are presented in Fig. 3B.

The interpretation of the results of the multivariate analyses is mainly based on the comparison of the *F* values and of the squared Mahalanobis distances. The *F* value gives us information about the strength of the entire discrimination, while the squared Mahalanobis distances are calculated between groups, two by two, and facilitate comparison of the distances between the groups of samples (subregions). In our study, the best discriminating model was produced using 12 (out

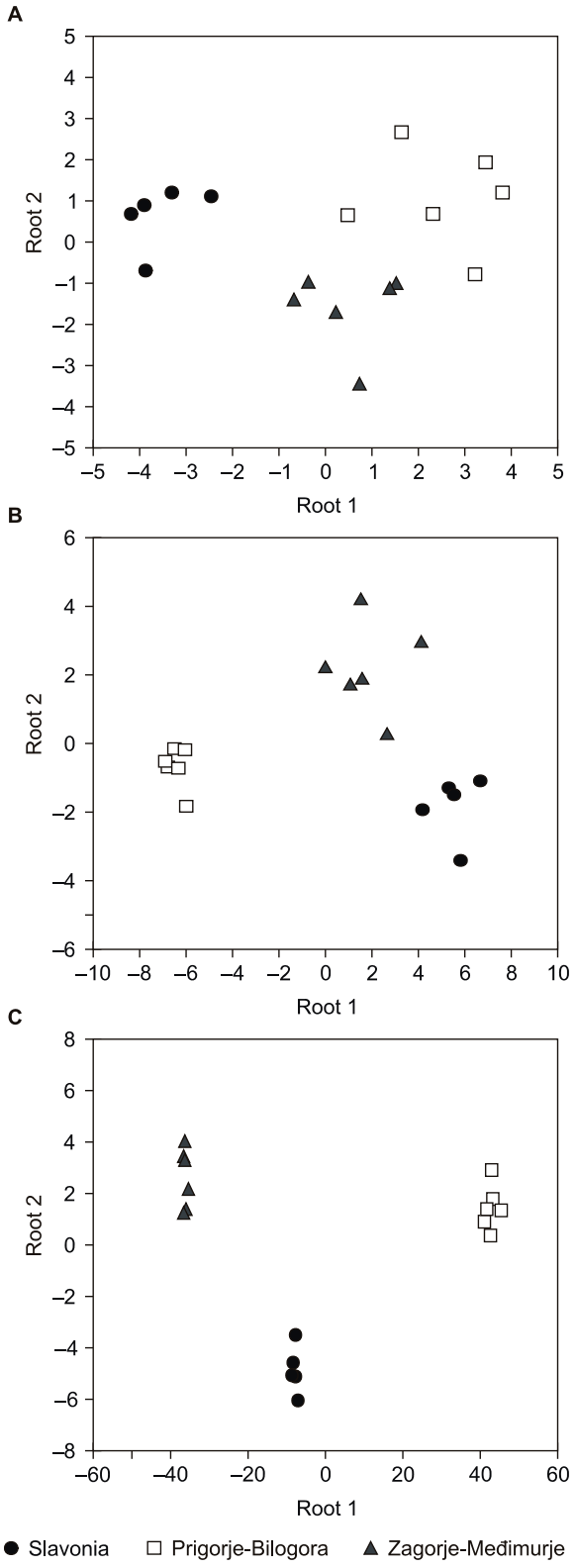

Fig. 3. Scatter plot of canonical scores for two roots for each sample according to the subregion.

A – polyphenol content and antioxidant activity using 9 original dataset variables; B – polyphenol content, antioxidant activity and metal content using 9 original dataset variables; C – polyphenol content, antioxidant activity and metal content using 12 principal components.

of 14) principal components (principal components 1–10, 12 and 13) used for LDA. It is evident from squared Mahalanobis distances that *F*- and *P*- values for discrimination between subregions are: SL vs PB (2612.55; $F = 127.23$; $P = 0.0001$), SL vs ZM (857.07; $F = 41.74$; $P = 0.0013$), PB vs ZM (6247.40; $F = 334.68$; $P < 0.0001$). This model showed not only extreme separation concerning subregions but also a very narrow area of dispersion of individual wine samples intraregionally (Fig. 3C).

CONCLUSION

The results of our study show a significant polyphenol content and antioxidant activity of Croatian blackberry wines. Generally, the results indicate that the extent of antioxidant activities of the investigated blackberry wines is in accordance with the amount of polyphenols and monomeric anthocyanins present in this popular fruit wine. Furthermore, the results of this study clearly demonstrate that it is possible to obtain excellent classification of Croatian blackberry wines according to their geographic origin.

REFERENCES

1. Granato, D. – Katayama, F. C. U. – de Castro, I. A.: Phenolic composition of South American red wines classified according to their antioxidant activity, retail price and sensory quality. Food Chemistry, *129*, 2011, pp. 366–373.
2. Renaud, S. – de Lorgeril, M.: Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet, *339*, 1992, pp. 1523–1526.
3. Mazza, G.: Health aspects of natural colors. In: Lauro, G. J. – Francis, F. J. (Ed.): Natural food colorants: science and technology. New York: Marcel Decker, 2000, pp. 290–294.
4. Smith, M. – Marley, K. – Seigler, D. – Singletary, K. – Meline, B: Bioactive properties of wild blueberry fruits. Journal of Food Science, *65*, 2000, pp. 352–356.
5. Katsube, N. – Iwashita, K. – Tsushida, T. – Yamaki, K. – Kobori, M.: Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. Journal of Agricultural and Food Chemistry, *51*, 2003, pp. 68–75.
6. Castañeda-Ovando, A. – Pacheco-Hernández, M. L. – Páez-Hernández, M. E. – Rodríguez, J. A. – Galán-Vidal, C. A.: Chemical studies of anthocyanins: A review. Food Chemistry, *113*, 2009, pp. 859–871.
7. Francis, F. J.: Food colorants: anthocyanins. Critical Reviews in Food Science and Nutrition, *28*, 1989, pp. 273–314.
8. Reilly, C.: The nutritional trace metals. Oxford:

Blackwell Publishing, 2004. 238 pp. ISBN 1-4051-1040-6.
9. García-Casal, M. N.: Carotenoids increase iron absorption from cereal-based food in the human. Nutrition Research, *26*, 2006, pp. 340–344.
10. Péneau, S. – Dauchet, L. – Vergnaud, A. – Estaquio, C. – Kesse-Guyot, E. – Bertrais, S. – Latino-Martel, P. – Hercberg, S. – Galan, P.: Relationship between iron status and dietary fruit and vegetables based on their vitamin C and fiber content. The American Journal of Clinical Nutrition, *87*, 2008, pp. 1298–1305.
11. García-Rodríguez, G. – Hernández-Moreno, D. – Soler, F. – Pérez-López, M.: Characterization of "ribera del Guadiana" and "méntrida" Spanish red wines by chemometric techniques based on their mineral contents. Journal of Food and Nutrition Research, *50*, 2011, pp. 41–49.
12. Arvanitoyannis, I. S. – Katsota, M. N. – Psarra, E. P. – Soufleros, E. H. – Kallithrakay, S.: Application of quality control methods for assessing wine authenticity: Use of multivariate analysis (chemometrics). Trends in Food Science and Technology, *10*, 1999, pp. 321–336.
13. Kadegowda, A. K. G. – Piperova, L. S. – Erdman, R. A.: Principal component and multivariate analysis of milk long-chain fatty acid composition during diet-induced milk fat depression. Journal of Dairy Science, *91*, 2008, pp. 749–759.
14. Bentabol Manzanares, A. – Hernández García, Z. – Rodríguez Galdón, B. – Rodríguez Rodríguez, E. – Díaz Romero, C.: Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition. Food Chemistry, *126*, 2011, pp. 664–672.
15. Elisia, I. – Hua, C. – Popovich, D. G. – Kittsa, D. D.: Antioxidant assessment of an anthocyanin – enriched blackberry extract. Food Chemistry, *101*, 2007, pp. 1052–1058.
16. Marinova, D. – Ribarova, F.: HPLC determination of carotenoids in Bulgarian berries. Journal of Food Composition and Analysis, *20*, 2007, pp. 370–374.
17. Wang, W. D. – Xu, S. Y.: Degradation kinetics of anthocyanins in blackberry juice and concentrate. Journal of Food Engineering, *82*, 2007, pp. 271–275.
18. Compendium of international methods of wine and must analysis [online]. Paris: Organisation Internationale de la Vigne et du Vin, 2008 [cit. 22 March 2010]. Available at <http://news.reseau-concept.net/images/oiv_uk/Client/PNO04b_EN_Recueil_2006_Vol1.pdf>
19. Gambelli, L. – Santaroni, G. P.: Polyphenols content in some Italian red wines of different geographical origins. Journal of Food Composition and Analysis, *17*, 2004, pp. 613–618.
20. Lee, J. – Durst, R. W. – Wrolstad, R. E.: Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. Journal of AOAC International, *88*, 2005, pp. 1269–1278.
21. Hatano, T. – Kagawa, H. – Yasuhara, T. – Okuda, T.: Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chemical and Pharmaceutical Bulletin, *36*, 1988, pp. 2090–2097.
22. Re, R. – Pellegrini, N. – Proteggente, A. – Pannala, A. – Yang, M. – Rice-Evans, C.: Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, *26*, 1999, pp. 1231–1237.
23. Oyaizu, M.: Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition, *44*, 1986, pp. 307–315.
24. Yen, G. C. – Chen, H. Y.: Antioxidant activity of various tea extracts in relation to their antimutagenicity. Journal of Agricultural and Food Chemistry, *43*, 1995, pp. 27–37.
25. Amidžić Klarić, D. – Klarić, I. – Velić, D. – Vedrina Dragojević, I.: Evaluation of mineral and heavy metal content in Croatian blackberry wines. Czech Journal of Food Sciences, *29*, 2011, pp. 260–267.
26. Alcaide-Hidalgo, J. M. – Moreno-Arribas, M. V. – Martín-Álvarez, P. J. – Polo, M. C.: Influence of malolactic fermentation, postfermentative treatments and ageing with lees on nitrogen compounds of red wines. Food Chemistry, *103*, 2007, pp. 572–581.
27. Minussi, R. C. – Rossi, M. – Bologna, L. – Cordi, L. – Rotilio, D. – Pastore, G. M. – Durán, N.: Phenolic compounds and total antioxidant potential of commercial wines. Food Chemistry, *82*, 2003, pp. 409–416.
28. Granato, D. – Katayama, F. C. U. – Castro, I. A.: Assessing the association between phenolic compounds and the antioxidant activity of Brazilian red wines using chemometrics. Lebensmittel-Wissenschaft und -Technologie - Food Science and Technology, *43*, 2010, pp. 1542–1549.
29. Roussis, I. G. – Lambropoulos, I. – Soulti, K.: Scavenging capacities of some wines and wine phenolic extracts. Food Technology and Biotechnology, *43*, 2005, pp. 351–358.
30. Piljac, J. – Martinez, S. – Valek, L. – Stipčević, T. – Kovačević Ganić, K.: A comparison of methods used to define the phenolic content and antioxidant activity of Croatian wines. Food Technology and Biotechnology, *43*, 2005, pp. 271–276.
31. Siriwoharn, T. S. – Wrolstad, R. E.: Polyphenolic composition of marion and evergreen blackberries. Journal of Food Science, *69*, 2004, pp. 233–240.
32. Szajdek, A. – Borowska, E. J.: Bioactive compounds and health-promoting properties of berry fruits: a review. Plant Foods for Human Nutrition, *63*, 2008, pp. 147–156.
33. Villańo, D. – Fernández-Pachón, M. S. – Troncoso, A. M. – García-Parrilla, M. C.: Influence of enological practices on the antioxidant activity of wines. Food Chemistry, *95*, 2006, pp. 394–404.
34. Staško, A. – Brezová, V. – Mazúr, M. – Čertík, M. – Kaliňák, M. – Gescheidt, G.: A comparative study on the antioxidant properties of Slovakian and Austrian wines. LWT - Food Science and Technology, *41*, 2008, pp. 2126–2135.
35. Arnao, M. B.: Some methodological problems in

the determination of antioxidant activity using chromogen radicals: a practical case. Trends in Food Science and Technology, *11*, 2000, pp. 419–421.
36. Chung, Y. C. – Chang, C. T. – Chao, W. W. – Lin, C. F. – Chou, S. T.: Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. Journal of Agricultural and Food Chemistry, *50*, 2002, pp. 2454–2458.
37. Lucena, A. P. S. – Nascimento, R. J. B. – Maciel, J. A. C. – Tavares, J. X. – Barbosa-Filho, J. M. – Oliveira, E. J.: Antioxidant activity and phenolics content of selected Brazilian wines. Journal of Food Composition and Analysis, *23*, 2010, pp. 30–36.
38. Anastasiadi, M. – Pratsinis, H. – Kletsas, D. – Skaltsounis, A. L. – Haroutounian, S. A.: Bioactive non-coloured polyphenols content of grapes, wines and vinification by-products: Evaluation of the antioxidant activities of their extracts. Food Research International, *43*, 2010, pp. 805–813.
39. Arnous, A. – Makris, D. P. – Kefalas, P.: Effect of principal polyphenolic components in relation to antioxidant characteristics of aged red wines. Journal of Agricultural and Food Chemistry, *49*, 2001, pp. 5736–5742.
40. Arnous, A. – Makris, D. P. – Kefalas, P.: Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. Journal of Food Composition and Analysis, *15*, 2002, pp. 655–665.
41. Zafrilla, P. – Morillas, J. – Mulero, J. – Cayuela, J. M. – Martínez-Cachá, A. – Pardo, F. – López Nicolás, J. M.: Changes during storage in conventional and ecological wine: Phenolic content and antioxidant activity. Journal of Agricultural and Food Chemistry, *51*, 2003, pp. 4694–4700.
42. Fernández-Pachón, M. S. – Villano, D. – García-Parrilla, M. C. – Troncoso, A. M.: Antioxidant activity of wines and relation with their polyphenolic composition. Analytica Chimica Acta, *513*, 2004, pp. 113–118.
43. Makris, D. P. – Kefalas, P.: Association between in vitro antiradical activity and ferric reducing power in aged red wines: A mechanistic approach. Food Science and Technology International, *11*, 2005, pp. 11–18.
44. Giovanelli, G.: Evaluation of the antioxidant activity of red wines in relationship to their phenolic content. Italian Journal of Food Science, *17*, 2005, pp. 381–393.
45. Cimino, F. – Sulfaro, V. – Trombetta, D. – Saija, A. – Tomaino, A.: Radical-scavenging capacity of several Italian red wines. Food Chemistry, *103*, 2007, pp. 75–81.
46. Woraratphoka, J. – Intarapichet, K. O. – Indrapichate, K.: Phenolic compounds and antioxidative properties of selected wines from the northeast of Thailand. Food Chemistry, *104*, 2007, pp. 1485–1490.

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