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A S T R A C T

Phenolic composition of twenty-two Macedonian red wines, including ten autochthonous monovarietal Vranec wines produced with different yeasts for fermentation, and twelve wines from international varieties (Syrah, Merlot and Cabernet Sauvignon) from different wine regions was studied. All wines presented relatively high value of total phenols and antioxidant activity. A total of 19 phenolic compounds were identified and quantified using HPLC-DAD and among them, malvidin-3-glucoside and its derivatives were the major compounds, followed by the petunidin derivatives, while caftaric acid was the predominant cinnamic acid derivative in all wines. The anthocyanin content was mainly affected by the grape variety and to a less extent by the yeast used in fermentation. In particular, the use of locally isolated yeasts affected higher amount of anthocyanins and phenolic acids compared to the wines fermented with commercial yeasts. Principal Component Analysis showed a satisfactory grouping of red wines according to the grape variety.

Keywords:
Anthocyanins
Autochthonous variety
HPLC
Phenolic acids
Vinalco yeasts
Vranec wine

1. Introduction

Wine production has a long tradition in Republic of Macedonia since the ancient Roman times and now it is the second most important export agro-food product after the tobacco, thus representing an economic opportunities for the new generation. In 2010 there were 86 registered wineries in Macedonia with a total capacity of ca. 2 million hectolitres per year and the total capacity of bottling is around 0.65 million hL per year, which is insufficient to cover the entire wine production in the country although a significant wine share (51% in 2006) is in bulk (MAFWE 2010). In 2008, 70.3 million litres were exported with value of around 39 million euros i.e. 10.4% of the total value of agri-food exports (SSO, 2009).

To increase the competitiveness of Macedonian wines on the global market there is a need to achieve distinctiveness and recognition of the products with specific quality characteristics, that origin from a particular geographical region or area (Nacka, Georgiev, & Dabovic Anastasovska, 2012). According to the climate characteristics and classification of the EU, Republic of Macedonia is classified as one geographic area for vine growing, i.e. zone III-C-b, that includes three viticultural regions divided into sixteen sub regions (districts) with specific favourable natural condition for production of quality wine. The wineries are mainly located in the region of the river Vardar valley, in particular in Skopje, Tikveš, and Gevgelija-Valandovo. Red wine represents approx. 60% of the national production and includes both autochthonous and international grape varieties such as Cabernet Sauvignon, Syrah, Merlot and Vranec. Vranec is probably the Macedonia's best known grape with dark and ruby-coloured on the vine, it produces dry, full-bodied red wines with high tannins. It is well known that polyphenolic compounds of red wine, including anthocyanins and tannins, are natural dietary antioxidant with potential health benefit and affect the quality of red wines, in terms of astringency, bitterness and colour (Mazza & Francis, 1995; Versari, du Toit, & Parpinello 2013).

Preliminary studies on the phenolic composition of wines from Macedonia have been preformed applying HPLC-DAD-MS, MALDI-TOF-MS and spectrophotometry techniques. Information is available on identification and semi-quantification of phenolic compounds of Vranec wines produced by different vinification conditions (Ivanova et al. 2011a) as well as stilbene levels and antioxidant activity of Vranec and Merlot wines, produced under different vinifications (Kostadinovic et al., 2012) applying HPLC methods. Moreover, MALDI-TOF-MS was applied for pigments

fingerprinting of Macedonian red wines (Ivanova et al., 2011b, Ivanova Petropulos et al. 2014) and spectrophotometry was used for determination of total phenolics, anthocyanins, flavan-3-ols, flavonoids and colour on commercial wines and wines prepared under different maceration time, yeasts and different doses of SO_2 (Ivanova, Stefova, & Chinnici 2010; Ivanova, Stefova, & Vojnoski 2009; Ivanova, Vojnoski, & Stefova 2012).

However, to the best of our knowledge, there has been no report so far on the quantification of individual phenolic compounds in Macedonian red wines from different grape varieties and viticulture areas. Considering this, the objectives of the present work were (1) to characterize the flavonoid and non-flavonoid composition and to determine the antioxidant activity in red wines made from Vranec, Syrah, Merlot and Cabernet Sauvignon varieties, and (2) to assess the influence of different yeast preparations, Vinalco (Macedonian autochthonous yeast) and yeasts from Lallemand, on the phenolic composition and antioxidant activity of Vranec wines.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and reagents were from commercial source: methanol, acetonitrile, perchloric acid, formic acid (Merck, Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, (+)-catechin, (−)-epicatechin, caffeic acid, syringic acid (Sigma–Aldrich, Milano, Italy), protocatechuic acid, vanillic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid (Extrasynthese, Genay, France). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox©) used for DPPH assay calibration was from Fluka *Analytical* (Sigma–Aldrich, Milano, Italy).

2.2. Wine samples

Twelve red wines from different *Vitis vinifera* L. grape international varieties, (Merlot, Cabernet Sauvignon and Syrah) from vintage 2103, were collected directly from the commercial wineries located in different wine regions of Macedonia, including Skopje, Gradsko, Negotino and Kavadarci.

The Vranec wines (10 samples), also collected from wineries, were produced from Vranec grapes (*Vitis vinifera* L.) grown in Tikveš region using similar winemaking protocol, as follows. Grapes were harvested at optimal technological maturity (23–24°Brix) and processed using electrical inox crusher/destem-mer, then added with SO_2 (ca. 65 mg/L total concentration) before inoculation of four wines with one of the following *Saccharomyces cerevisiae* yeast strains: Clos, RC212, D254, BDX (Lallemand, Bordeaux, France), whereas other six wines were inoculated with Vinalco yeast (Bitola, Republic of Macedonia) that was isolated from the Tikveš wine region. In all trials the grape mash was macerated for 7–10 days at 23 ± 2 °C, with pumping over and delastage performed once per day during the first three days of maceration, followed by pumping over two times a day.

2.3. Proximate chemical composition of wines

The following parameters: total acidity, volatile acidity, total and free SO_2, alcohol, dry extract and specific density, were analyzed according to the official methods of analysis of wines (OIV. 2014).

2.4. Spectrophotometric analyses

Spectrophotometric analyses were performed by direct measurements of wines or using appropriate dilution of wine in distilled water when necessary, at the following wavelength: 280 nm (total phenols), 420 nm (browning degree), 520 nm and 620 nm (anthocyanins) nm with a UV–VIS spectrophotometer (Shimadzu, UVmini 1240, Milan, Italy) using a cuvette with 1 cm optical path against the blank, i.e. water (Harbertson & Spayd, 2006). Based on the Vis measurements the following parameters were calculated: colour intensity (CI = Σ 420 + 520 + 620 nm) and colour tonality (H = 420/520 nm) (OIV 2014).

Total phenols content, expressed as mg/L gallic acid equivalent (GAE/L), was determined by reading of the absorbance of the diluted samples (1:100 dilutions in distilled water) at 280 nm, and using a calibration curve of gallic acid standard solution in the range of 1.95 to 31.25 mg/L.

2.5. Determination of antioxidant activity of wines

Antioxidant activity of wines was determined as a radical scavenging ability following the procedure described by Brand-Williams, Cuvelier, and Berset (1995). Briefly, 200 μL of wine was added to 3 mL of a methanol solution of the radical DPPH with concentration of 0.025 mol/L, and measured at 515 nm after 1 h storage at dark. Antioxidant activity was calculated from a calibration curve constructed using methanol solutions of Trolox with concentrations ranged between 93–0.19 mg/L, and expressed as mg Trolox equivalent/L (TE/L).

2.6. HPLC analysis

An High Performance Liquid Chromatography (HPLC) system equipped with temperature control oven, photodiode array detector (DAD) and a Chromeleon chromatography manager software v. 6.60 SP2 (Dionex DX500, Milano, Italy) was used for identification and quantification of anthocyanins, phenolic acids and flavan-3-ols in wines. The samples were always filtered using 0.20 μm cellulose acetate membrane (Millipore, Milano, Italy) before direct injection into the HPLC system, kept at 30 °C.

Anthocyanins and related pigments were analyzed with the Gemini RP-C18 column (250 × 4.6 mm; 5 μm particle size; 110 Å porosity; Phenomenex, Bologna, Italy) using the following mobile phases: water/methanol (70/30, *v/v*) containing 6 mL/L of 70% perchloric acid (solvent A) and water/methanol (25/75, *v/v*) containing 6 mL/L of 70% perchloric acid (solvent B), at flow rate of 0.9 mL/min. The proportions of solvent B were as follows: 0 min, 0%; 23 min, 25%; 51 min, 70%; 60 min, 100%; 65 min, 0%. Anthocyanins were recorded at 530 nm.

Hydroxycinnamic acid derivatives and flavan-3-ols were analyzed with the Aquapore ODS-300 RP-C18 column (250 × 4.6 mm; 7 μm particle size; 300 Å porosity; Applied Biosystems, San Jose, CA, USA) using the following mobile phases: solvent A (water/formic acid, 98/2, *v/v*) and solvent B (acetonitrile/water/formic acid, 80:12:2, *v/v/v*), at flow rate of 0.5 mL/min. The propor-tions of solvent B were: 0–50 min, 9%; 65–70 min, 10%; 77 min, 30%; 80–97 min, 0%. Protocatechuic, *p*-hydroxybenzoic and vanillic acids were quantified at 256 nm, (+)-catechin, (−)-epicatechin, gallic and syringic acids at 280 nm, whereas *p*-coumaric acid at 308 nm, and caftaric, caffeic, coutaric and fertaric acids at 324 nm.

2.7. Statistical analysis

Statistical treatment, including Principal Component Analysis (PCA) was performed using the XLSTAT Software, Version 7.5.2, Addinsoft (Paris, France). PCA was carried out to evaluate relationships among the groups of variables, e.g. concentration of anthocyanins, flavan-3-ols, hydroxybenzoic acids and hydroxycinnamic acids and their derivatives.

3. Results and discussion

3.1. Proximate chemical composition of wines

Table 1 shows the chemical composition of all wines according to the different grape varieties (Merlot, Syrah, Cabernet Sauvignon and Vranec), the wine regions of origin (Skopje, Gradsko, Negotino and Kavadarci) and the use of different yeasts (the autochthonous yeast, Vinalco and four commercial yeasts, Clos, RC212, D254, BDX, from Lallemand) for the vinification of Vranec wines. In general, all wines showed high amount of total acidity (ranged from 5.5 to 7.9 g/L) that peaked for Vranec wine (V-Vi2) fermented with Vinalco, autochthonous yeast. These results are consistent with previous findings that found the Vranec wines characterized by high value of total acidity, typical for this variety (Košmerl et al. 2013; Rajković & Sredović, 2009). Moreover, a high value of total acids helps the microbial stabilization and the freshness of these wines. The alcohol level is always within the regulatory values of 20% for wines of area C III b (Reg. CE 606/2009), and two Vranec wines (V-V1 and V-L1) showed the highest content of alcohol (16.44% and 16.15%, *v/v*, respectively) and highest content of dry extract (36.0 and 35.3 g/L, respectively) as well. The volatile acidity showed an overall average value of 0.55 ± 0.1 g/L with no influence on the quality of wines that was protected from further oxidation and microbial contamination by the free SO_2 present in a sufficient level in the wines (14 to 28 mg/L).

3.2. Total phenols, colour, hue and antioxidant activity determined by spectrophotometry

The results for total phenols content, colour intensity, hue (colour tonality) and antioxidant activity are presented in Table 1. For all wines, total phenols ranged from 1394 to 3097 mg/L GAE (mean 2037 mg/L GAE). On average, Syrah wines contained slightly lower phenolic levels compared to Vranec, Merlot and Cabernet Sauvignon. These results are comparable to those reported for Macedonian Vranec and Merlot wines produced under different vinification conditions (Ivanova et al. 2009; Ivanova et al. 2012). Regarding the effect of yeast, it was observed that the different (yeast) strains influenced the phenolic content of wines produced under the same operative conditions, observing a higher average phenolic content in wines fermented with Vinalco yeasts, probably because this strain absorbs less phenolic compounds compared to the Lallemand ones.

All wines presented relatively high values for the antioxidant activity, ranged between 82–117 mg/L TE, regardless the variety and yeast strain used for fermentation. Plotting total phenols concentration against antioxidant activity for the Merlot, Cabernet Sauvignon and Syrah wines from different wine regions (Fig. 1a), as well as Vranec wines produces with different yeast strains (Fig. 1b), the corresponding correlation coefficients (r^2) that obtained were 0.8251 and 0.6836, respectively, suggesting that total phenols are related to the antioxidant properties of the wines.

Regarding colour intensity (CI) and hue (H), Vranec wines presented higher average values (21.04) for the CI than the other varieties (14.83), principally due to the higher content of the red compounds. Hue values were ranged between 0.52 to 0.75, characteristic for young red wines (Glories, 1984a; Glories, 1984b), as our wines were, that is consistent with values from literature (Tsanova-Savova, Dimov, & Ribarova, 2002; Kontkanen, Reynolds, Cliff, & King 2005). Hue values, which increase throughout aging, were slightly lower for Vranec wines (average 0.61) compared to the other varieties (average 0.65). It is well know that as the red wine age the absorption at 520 nm decreases whereas increases that one at 420 nm, and this explains the colour shift of wine with aging from red to orange, and finally to red brick.

3.3. Anthocyanins composition of wines

Table 2 summarizes the concentration of individual anthocyanins in the Vranec wines produced by different fermentation yeasts and red wines from different varieties (Syrah, Merlot and Cabernet

Table 1
Proximate chemical composition, total phenolics content (mg/L, GAE) colour intensity, hue and antioxidant activity (AA) of varietal red wines (Vranec, Syrah, Merlot and Cabernet Sauvignon).

Wines*	Total SO_2 (mg/L)	Free SO_2 (mg/L)	Total acidity (g/L)	Volatile acidity (g/L)	Alcohol (%,*v/v*)	Dry extract (g/L)	Specific density	TPC (mg/L, GAE)	CI	H	AA (mg/L, TE)
V–L1	55.0	28.2	7.0	0.55	16.15	35.2	0.9932	3097	23.27	0.66	116
V–L2	58.9	24.3	6.8	0.65	13.61	30.1	0.9939	1868	17.95	0.64	106
V–L3	64.0	25.6	6.3	0.54	13.13	28.6	0.9939	1394	15.43	0.58	99
V–L4	53.8	26.9	6.3	0.60	13.64	29.2	0.9935	1577	15.42	0.58	103
V–Vi1	51.2	33.3	6.9	0.63	16.44	36.0	0.9930	2257	27.47	0.60	105
V–Vi2	56.3	24.3	7.9	0.67	15.83	35.3	0.9934	2333	27.51	0.52	117
V–Vi3	55.0	24.3	5.6	0.62	13.11	28.6	0.9939	2053	24.18	0.60	111
V–Vi4	69.1	26.9	5.9	0.53	12.56	29.6	0.9966	1909	17.19	0.62	108
V–Vi5	57.6	26.9	5.8	0.45	12.78	27.6	0.9963	2224	21.71	0.62	114
V–Vi6	48.6	24.3	6.4	0.51	14.29	32.3	0.9940	2059	20.30	0.65	109
S–S	51.2	26.9	5.9	0.47	15.95	34.6	0.9933	1991	18.04	0.65	104
CS–S	52.5	24.3	6.1	0.55	15.89	36.6	0.9942	2131	17.98	0.61	111
M–S	51.2	29.4	5.8	0.43	14.35	32.0	0.9936	2564	24.20	0.57	116
S–G	55.0	26.9	5.6	0.48	13.40	35.6	0.9965	1733	11.31	0.63	91
CS–G	57.6	30.7	6.0	0.42	13.21	36.7	0.9969	1975	9.42	0.70	99
M–G	62.7	33.3	5.6	0.44	13.20	35.6	0.9966	1992	11.41	0.72	109
S–N	25.6	14.1	7.1	0.76	14.68	32.3	0.9935	1701	13.15	0.58	89
CS–N	80.6	34.6	7.1	0.64	15.30	34.9	0.9939	1791	11.03	0.66	95
M–N	34.6	20.5	6.0	0.72	14.65	31.7	0.9934	1918	12.35	0.68	100
S–K	49.9	24.3	5.8	0.51	14.38	30.7	0.9933	1612	11.37	0.75	82
CS–K	55.0	32.0	5.5	0.43	14.04	35.9	0.9952	2126	16.63	0.65	105
M–K	57.6	28.2	5.6	0.45	14.51	32.3	0.9939	2507	21.08	0.58	115

Abbreviation of wine regions: S – Skopje, G – Gradsko, N – Negotino, K – Kavadarci. Abbreviation of *Saccharomyces cerevisiae* yeasts: Vi – Vinalco; L – Lallemand yeasts. TPC – total phenols content, in Gallic acid equivalents (GAE), CI – colour intensity, H – hue, AA – antioxidant activity in Trolox equivalents (TE).

* Abbreviation of wines: V – Vranec, S – Syrah, CS – Cabernet Sauvignon, M – Merlot.

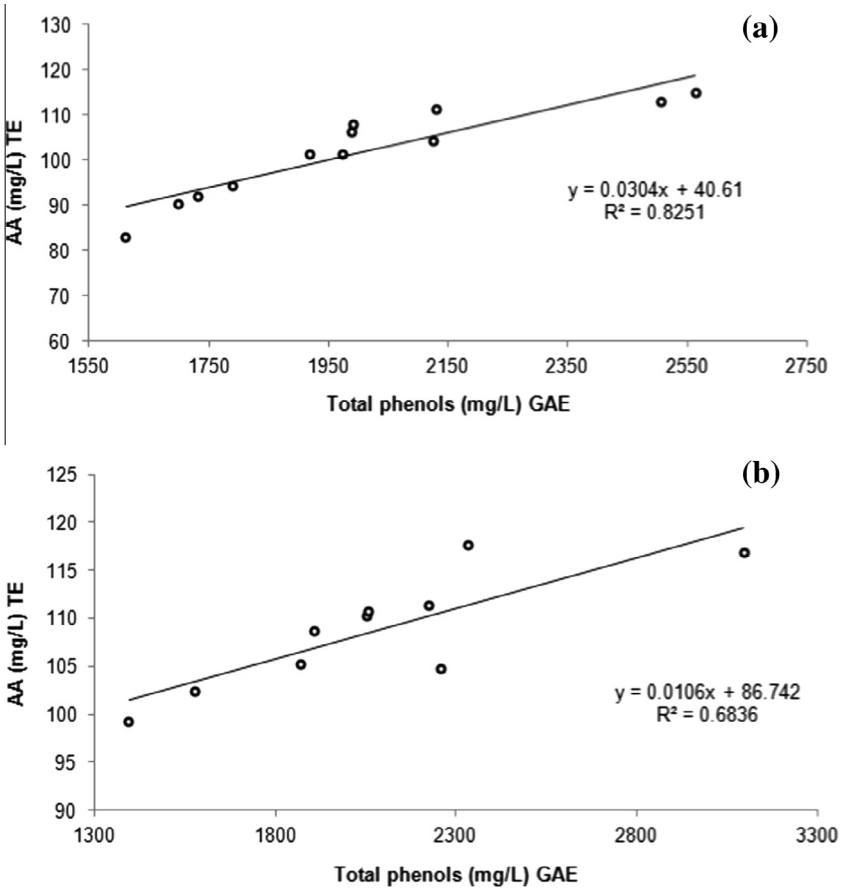

Fig. 1. Correlation between total phenols and antioxidant activity of (a) Merlot, Cabernet Sauvignon and Syrah wines from different wine regions and (b) Vranec wines produced with different yeast strains for fermentation.

Sauvignon) produced in different wine regions (Skopje, Gradsko, Negotino and Kavadarci) determined by HPLC. The chromatogram presenting the separation of anthocyanins at 530 nm is shown at Fig. 2a.

In total, 10 anthocyanins were identified and quantified in wines including five monoglucosides, three acetylglucosides and two *p*-coumaroylglucosides. The malvidin-3-glucoside was the dominant anthocyanin in all analyzed wines as expected for most of the *V. Vinifera* cultivars, followed by petunidin-3-glucoside, whereas the cyanidin-3-glucoside showed the lowest amount to below detection in some wines (Table 2). The group of anthocyanin monoglycosides represented the highest proportion of all anthocyanins in all wines, ranging from 60.3% (CS-G) to 88.5% (V-L1), followed by the acetyl derivatives ranged between 6% (V-L1) to 32.5% (CS-G) and *p*-coumaroylglucosides ranged from 5.3% (M–S) to 8.5 (V-V1). The ratio of acetylglucosides and *p*-coumaroylglucosides (Σ acetylated/Σ coumaroylated), proposed as a verification factor for varietal authenticity of red wines, was calculated. The values obtained ranged as follows: for the Vranec wines 1–1.7, for Syrah 2.6–4.8 (an highest ratio was noticed for Syrah wines from Skopje region, 4.8), for Cabernet Sauvignon 3.2–4.6 and for Merlot wine 2.3–3.8, with the highest ratio (4.6 and 3.8) for both wine varieties (Cabernet Sauvignon and Merlot, respectively) produced in the Gradsko region.

In terms of variety, the malvidin-3-glucoside was always higher in Vranec wines (range: 235–887 mg/L) compared to the international varieties Merlot, Cabernet Sauvignon and Syrah (overall range: 173–541 mg/L). In particular, the Vranec wines (V-Vi2, V-Vi3 and V-Vi1) fermented with the local Vinalco yeast strain showed the highest amount of monoglucoside, acetylglucoside and *p*-coumaroylglucoside derivatives, followed by Syrah wine from Skopje region and Cabernet Sauvignon from Gradsko and Negotino (data not shown). On average, Vranec wines contained higher amount of anthocyanins (738 mg/L) than other varieties (651 mg/L). The analyzed wines presented higher concentration of malvidin-3-glucoside as well as of anthocyanin monoglucosides compared to Cabernet Sauvignon and Merlot wines produced in Spain (Ortega et al. 2008), but similar values with Cabernet Sauvignon, Cencibel and Syrah wines produced in region of La Mancha in Spain (Hermosín Gutiérrez, Sánchez-Palomo Lorenzo, & Vicario Espinosa 2005). The results for the anthocyanins content correlate with the colour intensity values (Table 1).

It is well known that the anthocyanins content of wine can be affected by the yeast during fermentation. In this view we observed that Vranec wines, fermented with the Vinalco yeast had higher amounts of anthocyanins (on average 853 mg/L), compared to the wines fermented with the four Lallemand yeasts (on average 567 mg/L) and among them, wine V-Vi2 presented highest value (1530 mg/L). These results are in agreement with our previous findings (Ivanova et al., 2011a; Ivanova et al., 2012) and can be explained by the reversible adsorption of anthocyanins onto the yeast cell walls during the fermentation (Vasserot, Caillet, & Maujean, 1997). Moreover, it is well known that the metabolic pathways of the yeast can differ among the strains which affect the content of phenolic (e.g. tyrosol, pyruvic acid, vinylphenol) and other compounds (e.g. acetaldehyde) during maceration, and some of these compounds can react with anthocyanins modifying their adsorption properties.

Table 2
Concentration of anthocyanins (mg/L) of varietal red wines (Merlot, Cabernet Sauvignon and Syrah) from different Macedonian wine regions and Vranec wines fermented with different yeasts.

Wines*	Dp-Glc	Cy-Glc	Pt-Glc	Pn-Glc	Mv-Glc	Total Glc	Pt-AcGlc	Pn-AcGlc	Mv-AcGlc	Total AcGlc	Pn-coumGlc	Mv-coumGlc	Total coumGlc	Vitisin B	Total anthocyanins	ΣGlc/ ΣAcG	ΣGlc/ ΣcoumGlc	ΣAcGlc/ ΣcoumGlc
V–L1	2.1	27.2	0.9	143.0	344	388	4.1	3.6	27.5	35.2	4.3	28.2	32.5	n.d.	456	11.0	11.9	1.1
V–L2	19.8	1.1	43.3	24.3	347	435	4.0	5.1	45.2	54.3	2.9	29.0	31.9	3.7	525	8.0	13.7	1.7
V–L3	63.2	5.0	99.1	40.2	335	543	9.7	5.0	27.8	42.4	6.1	29.0	35.1	0.9	622	12.8	15.5	1.2
V–L4	16.5	n.d.	41.5	19.5	355	433	4.2	3.5	44.9	52.6	4.2	30.8	34.9	4.5	525	8.2	12.4	1.5
V–Vi1	38.8	8.1	82.3	73.0	544	746	7.9	12.8	68.8	89.5	8.1	69.9	78.0	n.d.	914	8.3	9.6	1.1
V–Vi2	99.6	20.1	154	134	887	1296	14.7	15.1	96.4	126	15.3	93.5	108	n.d.	1530	10.3	11.9	1.2
V–Vi3	76.6	7.5	114	68.1	524	790	12.8	11.9	61.5	86.3	9.5	54.9	64.4	n.d.	941	9.2	12.3	1.3
V–Vi4	35.7	1.0	63.1	33.6	378	512	7.1	8.1	46.1	61.3	5.1	36.2	41.3	n.d.	614	8.3	12.4	1.5
V–Vi5	59.7	5.5	89.4	50.6	411	617	8.7	6.9	45.0	60.6	5.9	36.2	42.1	2.7	722	10.2	14.6	1.4
V–Vi6	28.9	4.8	47.4	27.9	235	345	3.2	2.6	19.6	25.4	2.8	22.6	25.4	n.d.	395	13.5	13.5	1.0
S–S	18.9	n.d.	41.3	12.8	541	614	10.2	8.4	215	234	5.2	43.4	48.6	3.1	899	2.6	12.6	4.8
CS–S	31.6	0.1	28.8	10.3	258	329	6.1	3.2	85.9	95.2	1.1	24.9	26.0	n.d.	450	3.5	12.6	3.7
M–S	50.8	2.7	51.4	23.1	251	379	9.4	5.4	52.3	67.1	3.2	21.8	25.1	2.6	474	5.6	15.1	2.7
S–G	14.9	n.d.	32.7	16.9	408	472	6.8	12.3	178	197	4.7	48.3	52.9	n.d.	723	2.4	8.9	3.7
CS–G	13.6	n.d.	17.1	4.7	472	507	4.8	3.5	265	273	0.7	59.2	59.9	n.d.	840	1.9	8.5	4.6
M–G	15.7	n.d.	27.7	17.5	397	458	5.2	9.0	168	182	2.4	45.9	48.3	n.d.	688	2.5	9.5	3.8
S–N	14.7	n.d.	29.6	15.2	316	375	6.6	11.4	136	154	5.8	54.6	60.4	n.d.	590	2.4	6.2	2.6
CS–N	26.6	n.d.	32.4	11.9	497	568	6.4	7.6	212	226	1.8	69.4	71.2	n.d.	866	2.5	8.0	3.2
M–N	26.4	0.7	41.8	24.4	408	501	7.8	10.7	141	159	4.6	65.2	69.8	n.d.	730	3.2	7.2	2.3
S–K	15.1	n.d.	34.7	13.4	489	552	9.7	11.7	185	206	4.6	63.7	68.3	n.d.	827	2.7	8.1	3.0
CS–K	27.8	n.d.	26.9	9.5	229	294	5.9	2.8	74.1	82.7	1.2	23.6	24.9	0.2	402	3.5	11.8	3.3
M–K	34.7	1.3	35.1	16.0	173	260	4.9	3.1	34.3	42.4	2.2	15.3	17.4	1.2	321	6.1	14.9	2.4

Abbreviation of wine regions: S – Skopje, G – Gradsko, N – Negotino, K – Kavadarci.
Abbreviation of yeasts: Vi-Vinalco yeast, *Saccharomyces serevisiae*; L – Lallemand yeasts. Dp – delphinidin, Cy – cyanidin, Pt – petunidin, Pn – peonidin, Mv – malvidin, Glc – glucoside, AsGlc – acetylglucoside, coumGlc – coumaroylglucoside.
* Abbreviation of wines: V – Vranec, S – Syrah, CS – Cabernet Sauvignon, M – Merlot.

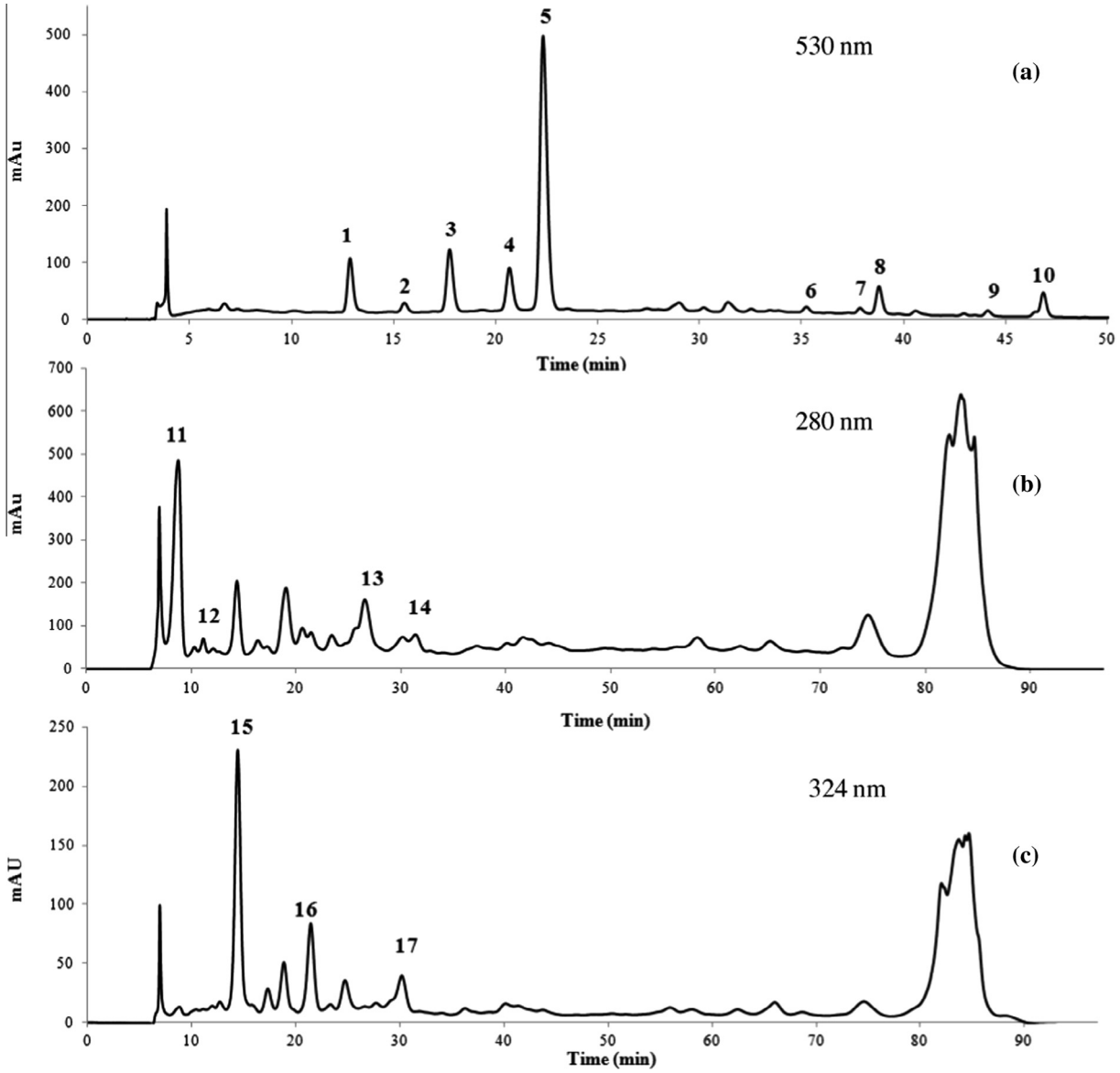

Fig. 2. UV–Vis chromatogram of V-L1 Vranec wine sample recorded at 530 nm (a), 280 nm (b) and 324 nm (c) for separation and quantification of anthocyanins, flavan-3-ols/hydroxybenozic acids and hydroxycinnamic acids/derivatives, respectively. Peak identification: delphinidin-3-glucoside, (1); cyanidin-3-glucoside, (2); petunidin-3-glucoside, (3); peonidin-3-glucoside, (4); malvidin-3-glucoside, (5); petunidin-(6 acetyl)-3-glucoside, (6); peonidin-(6 acetyl)-3-glucoside, (7); malvidin-(6 acetyl)-3-glucoside, (8); peonidin-coumaroyl-3-glucoside, (9); malvidin-coumaroyl-3-glucoside, (10); gallic acid, (11); protocatecuic acid, (12); syringic acid, (13); (+)-catechin, (14); caftaric acid, (15); caffeic acid, (16); *p*-coumaric acid, (17).

3.4. Non-anthocyanin composition of wines

Separation of phenolic acids in one Vranec wine sample (V-L1), recorded at 280 nm and 324 nm, is presented at Fig. 2b and c, respectively.

Three benzoic acids, including gallic acid, protocatechuic acid and syringic acid, were identified and quantified in the wines. Gallic acid, that originates from the grapes and also from the breakdown of both hydrolyzable and condensed tannins, i.e. the gallic acid esters of flavan-3-ols, was the dominant benzoic acid in all wines, presenting an average value of 478 mg/L in Vranec wines, mainly depending on the high contribution of wine V-L1 (Table 3). The average values of total hydroxybenzoic acids obtained for the international varieties were different, i.e. 491 mg/L for Merlot, 372 mg/L for Cabernet Sauvignon and 325 for Syrah, in accordance with other studies (Ertan Anli & Nilüfer, 2009; Mendoza et al., 2011;). The differences observed in the levels of gallic acid among the samples probably are due to the differences in techniques applied for winemaking, including the addition of enological hydrolizable tannins, whereas the contribution due to the geographical origin or the cultivar seems to play a minor effect (Nicoletti, Bello, De Rossi, & Corradini 2008; Pajovic et al. 2014; Vinković Vrček, Bojić, Žuntar, Mendaš, & Medić-Šarić, 2011). Considerable amounts were also found for protocatechuic acid and syringic acid, higher in Vranec wines (on average 48.2 mg/L and 76.3 mg/L, respectively) compared to the international varieties (on average 39 mg/L and 10.8 mg/L, respectively). Similarly as for the gallic acid, protocatechuic and syringic acids were present in highest concentration in Merlot (on average 57.2 and 14.4 mg/L, respectively), followed by Cabernet Sauvignon (on average 34.7 and 6.62 mg/L, respectively) and Syrah (on average 25.1 and 1.3 mg/L, respectively) probably as a varietal characteristic (Garía-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara 2007; Monagas, Suarez, Gómez-Cordovés, & Bartolomé 2005).

Table 3
Concentration of phenolic acids (mg/L) and catechin of varietal red wines (Merlot, Cabernet Sauvignon and Syrah) from different Macedonian wine regions and Vranec wines fermented with different yeasts.

Wines*	Protocatecuic acid	Gallic acid	Syringic acid	*p*-Coumaric acid	Caftaric acid	Coutaric acid	Caffeic acid	Fertaric acid	Total HBA	Total HCA and HCAD	Catechin
V–L1	98.1	1352	419	60.8	237	13.7	94.5	19.4	1869	425	567
V–L2	44.9	460	161	10.9	243	49.1	31.2	21.4	666	355	93
V–L3	23.7	252	0.5	8.1	176	32.2	10.7	10.7	276	237	482
V–L4	31.1	317	30.0	13.2	226	45.2	14.0	36.5	378	335	348
V–Vi1	42.1	265	66.3	3.2	206	35.1	8.3	29.7	373	282	150
V–Vi2	62.6	311	55.3	18.6	275	55.6	14.6	49.4	429	413	n.d.
V–Vi3	62.6	472	13.5	13.3	507	89.2	11.5	39.0	548	660	347
V–Vi4	29.3	291	17.4	11.0	365	68.4	10.2	38.4	338	493	271
V–Vi5	37.2	391	n.d.	11.5	384	72.9	7.54	30.6	428	507	331
V–Vi6	50.8	671	n.d.	16.3	362	67.1	31.9	43.6	722	521	284
S–S	30.8	310	n.d.	18.3	243	61.1	40.4	35.4	340	398	407
CS–S	39.1	337	n.d.	11.4	412	67.5	16.1	40.7	376	548	203
M–S	104	303	5.4	2.7	257	33.8	16.7	42.9	412	353	298
S–G	34.1	523	19.8	n.d.	147	37.2	20.5	37.2	577	242	238
CS–G	44.4	631	14.1	n.d.	244	53.9	16.4	28.6	689	343	227
M–G	26.2	847	21.2	21.9	182	25.8	19.3	34.2	894	284	649
S–N	30.3	281	25.4	14.1	243	71.2	15.6	38.4	337	382	109
CS–N	5.6	177	n.d.	n.d.	387	74.6	31.5	46.3	183	539	136
M–N	18.5	369	21.5	13.3	266	47.7	19.3	35.8	409	382	n.d.
S–K	5.1	185	n.d.	26.7	243	65.3	25.9	43.6	190	405	411
CS–K	49.7	344	12.4	3.8	387	65.3	11.1	36.7	406	504	114
M–K	80.1	445	9.6	1.1	219	31.4	11.5	42.9	535	306	209

Abbreviation of wine regions: S – Skopje, G – Gradsko, N – Negotino, K – Kavadarci.
Abbreviation of yeasts: Vi – Vinalco yeast, *Saccharomyces servisie*; L – Lallemand yeasts, HBA – hydroxybenzoic acids, HCA – hydroxycinnamic acids, HCAD – hydroxycinnamic acid derivatives.
* Abbreviation of wines: V – Vranec, S – Syrah, CS – Cabernet Sauvignon, M – Merlot.

Regarding the influence of yeast, higher proportions of gallic acid and syringic acid were present in Vranec fermented with the four Lallemand yeasts, while in two wines fermented with Vinalco yeasts (V-Vi5 and V-Vi6) syringic acid was not detected. In addition, similar contents of protocatechuic acid were found in all wines fermented with different yeast strains.

With respect to hydroxycinnamates, five compounds were detected, including caftaric, coutaric, fertaric, caffeic and *p*-coumaric acids. Caftaric acid presented highest average concentration (282 mg/L) representing 69.1% (on average) of all hydroxycinnamates, present in similar concentrations in all wines analyzed (Table 3). Cabernet Sauvignon presented highest proportion of caftaric acid (358 mg/L, on average) and Syrah lowest (219 mg/L, on average). Coutaric and fertaric acids also had an important contribution (mean content 52.9 mg/L [13.02%] and 35.5 mg/L [9.13%], respectively). Differences between varieties were observed for caffeic and *p*-coumaric acids too.

In terms of the influence of yeast, Vranec wines fermented with Vinalco contained higher content of caftaric acid, coutaric acid and fertaric acid (mean value 350, 64.7, and 38.5 mg/L, respectively) compared to wines fermented with Lallemand yeasts (mean value 221, 35.1, and 22 mg/L, respectively). According to Balik et al.(2008) the *Vitis vinifera L.* varieties contain the highest level of caftaric acid in the shoots (474–2257 mg/kg) and the leaves of grape (278–914 mg/kg), which extract's represent a valuable source of bioactive compounds with further application in the food or pharmaceutical industries (Fernandes et al., 2013).

Concerning flavan-3-ols, only (+)-catechin was identified and quantified in wines. Its content was relatively high and different among varieties and wine regions, ranged from 92.9 to 567 mg/L. In particular, the highest average levels of catechin were found in Vranec wines (319 mg/L) similarly to Merlot wines (316 mg/L), followed with Syrah (291 mg/L) and Cabernet Sauvignon (170 mg/L). With regard to the yeast influence on catechin content, wines fermented with Vinalco yeasts contained lower levels (mean content 277 mg/L) then other wines fermented with Lallemand yeasts, Clos, RC212, D254, BDX (mean content 373 mg/L), probably because of a lower adsorption of catechin on the yeast cell walls.

3.5. Principal Component Analysis

Principal Component Analysis was used to explore the contribution of each parameter on the clustering among the wines. Projection of the wines on the first two principal components (explained variability: 59.95%) showed a separation mainly according to the variety (Fig. 3a). Thus, wines from Cabernet Sauvignon variety were clearly separated from the other wines, located in the negative part of PC2, and Syrah wines formed a group in the positive part of PC1. Vranec wines were grouped and further divided in subgroups according to the yeast used for fermentation. Thus, wines fermented with Vinalco yeast were located in the upper positive part of PC1 while wines fermented with Lallemand yeasts were not clearly separated and were located near the centre, with exception of V-L1 which was placed in the upper negative part of PC1, separated from all other wines.

PCA results of the variables used for characterization of the wine samples displayed into the first two principal components are presented in Fig. 3b. It could be noticed that anthocyanins and hydroxycinnamic acid derivatives prevail in the positive part of the first principal component, while catechin, caffeic acid, *p*-coumaric acid and hydroxybenzoic acids prevail in the negative part of PC1. Wines located in the upper right quarter are richer in anthocyanins, which can be related to varietal character.

4. Conclusions

Chemical analysis on Macedonian quality wines are important to support the viticulture and wine strategy aimed at brand development particularly from local grape variety. In this view, comparing Vranec wines with other varieties from Republic of Macedonia, such as Merlot, Cabernet Sauvignon and Syrah, it was found as

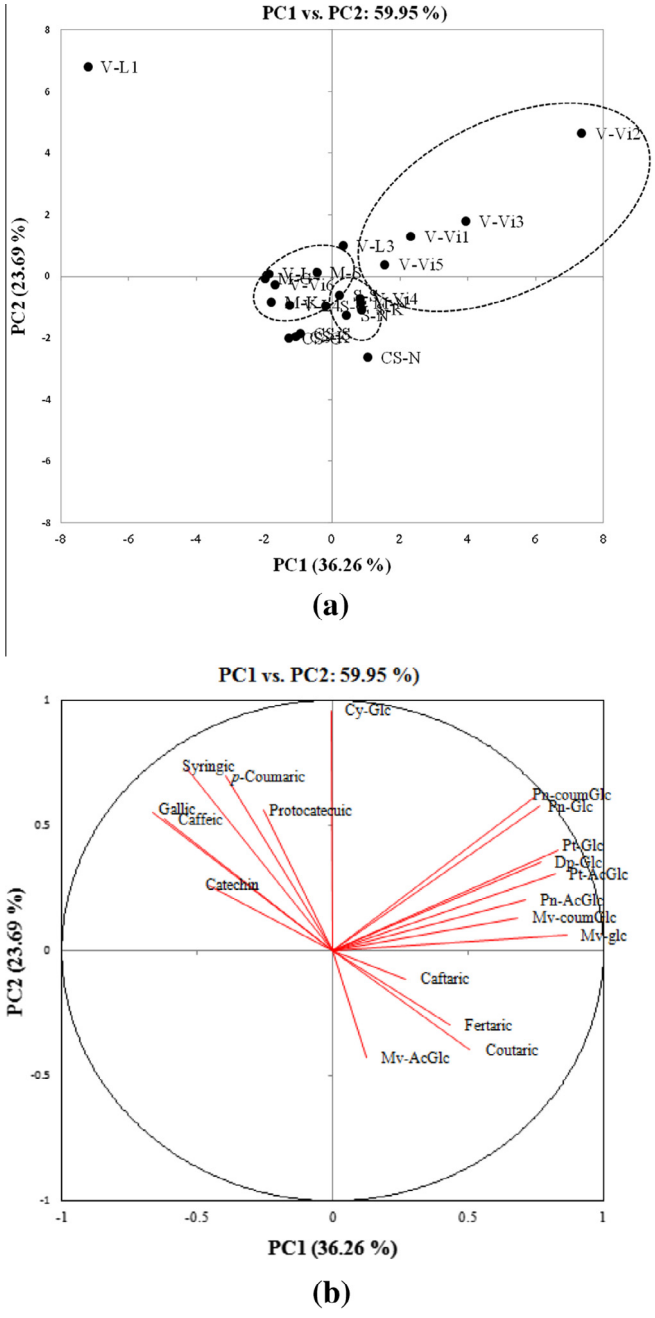

Fig. 3. Principal component score plot (a) and correlation scatterplots (b) of the variables with PC1 and PC2 based on anthocyanins, phenolic acids and catechin for the Vranec, Syrah, Merlot and Cabernet Sauvignon wines.

richer in total acids and polyphenols, especially anthocyanins that make this variety deeply coloured, fresh and stable, appropriate for long term aging and production of high quality wines. Investigating the effect of yeast, the locally isolated yeast strains seem to improve several polyphenolic components, such as higher levels of total phenols, anthocyanins, colour, phenolic acids and high value of antioxidant activity in Vranec wines.

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References

Balik, J., Kyselakova, M., Vrchotova, N., Triska, J., Kumsta, M., Veverka, J., et al. (2008). Relations between polyphenols content and antioxidant activity in vine grapes and leaves. *Czech Journal of Food Sciences, 26*, S25–S32.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology, 28*, 25–30.

Ertan Anli, R., & Nilüfer, V. (2009). Antioxidant phenolic substances of Turkish red wines from different wine regions. *Molecules, 14*, 289–297.

Fernandes, F., Ramalhosa, E., Pires, P., Verdial, J., Valentão, P., Andrade, P., et al. (2013). *Vitis vinifera* leaves towards bioactivity. *Industrial Crops and Products, 43*, 434–440.

Garía-Falcón, M. S., Pérez-Lamela, C., Martínez-Carballo, E., & Simal-Gándara, J. (2007). Determination of phenolic compounds in wines: influence of bottle storage of young red wines on their evolution. *Food Chemistry, 105*, 248–259.

Glories, Y. (1984a). La couleur des vins rouges. Mesure, origine et interprétation. Partie I. *Connaissance de la Vigne et du Vin, 18*, 195–217.

Glories, Y. (1984b). La couleur des vins rouges. Mesure, origine et interprétation. Partie II. *Connaissance de la Vigne et du Vin, 18*, 253–271.

Harbertson, J. F., & Spayd, S. (2006). Measuring phenolics in the winery. *American Journal of Enology and Viticulture, 57*(3), 280–288.

Hermosín Gutiérrez, I., Sánchez-Palomo Lorenzo, E., & Vicario Espinosa, A. (2005). Phenolic composition and magnitude of copigmentation in young and shortly aged red wines made from the cultivars, Cabernet Sauvignon, Cencibel, and Syrah. *Food Chemistry, 92*, 269–283.

Ivanova, V., Dörnyei, Á., Márk, L., Vojnoski, B., Stafilov, T., Stefova, M., et al. (2011a). Polyphenolic content of Vranec wines produced by different vinification conditions. *Food Chemistry, 124*, 316–325.

Ivanova, V., Dörnyei, Á., Stefova, M., Stafilov, T., Vojnoski, B., Kilár, B., et al. (2011b). Rapid MALDI-TOF-MS detection of anthocyanins in wine and grape using different matrices. *Food Analytical Methods, 4*, 108–115.

Ivanova Petropulos, V., Dörnyei, Á., Stefova, M., Stafilov, T., Vojnoski, B., Márk, L., et al. (2014). Application of a novel small-scale sample cleanup procedure prior to MALDI-TOF-MS for rapid pigment fingerprinting of red wines. *Food Analytical Methods, 7*(4), 820–827.

Ivanova, V., Stefova, M., & Chinnici, F. (2010). Determination of polyphenol contents in Macedonian grapes and wines assessed by standardized spectrophotometric methods. *Journal of the Serbian Chemical Society, 75*, 45–59.

Ivanova, V., Stefova, M., & Vojnoski, B. (2009). Assay of the phenolic profile of Merlot wines from Macedonia: effect of maceration time, storage, SO_2 and temperature of storage. *Macedonian Journal of Chemistry and Chemical Engineering, 28*, 141–149.

Ivanova, V., Vojnoski, B., & Stefova, M. (2012). Effect of winemaking treatment and wine aging on phenolic content in Vranec wines. *Journal of Food Science and Technology, 49*, 161–172.

Kontkanen, D., Reynolds, A. G., Cliff, M. A., & King, M. (2005). Canadian terroir: sensory characterization of Bordeaux-style red wine varieties in the Niagara Peninsula. *Food Research International, 38*, 417–425.

Košmerl, T., Bertalanič, L., Maraš, V., Kodžulović, V., Šućur, S., & Abramovič, H. (2013). Impact of yield on total polyphenols, anthocyanins, reducing sugars and antioxidant potential in white and red wines produced from Montenegrin autochthonous grape varieties. *Food Science and Technology, 1*(1), 7–15.

Kostadinović, S., Wilkens, A., Stefova, M., Ivanova, V., Vojnoski, B., Mirhosseini, H., et al. (2012). Stilbene levels and antioxidant activity of Vranec and Merlot wines from Macedonia: Effect of variety and enological practices. *Food Chemistry, 135*, 3003–3009.

MAFWE (2010). Ministry of Agriculture, Forestry and Water Economy of Republic of Macedonia. National Strategy for Viticulture and Winemaking for the period of 2010–2015, Macedonia.

Mazza, G., & Francis, F. J. (1995). Anthocyanins in grapes and grape products. *Critical Reviews in Food Science and Nutrition, 35*(4), 341–371.

Mendoza, L., Matsuhiro, B., Aguirre, M. J., Isaacs, M., Sotés, G., Cotoras, M., et al. (2011). Characterization of phenolics acids profile from Chilean red wines by high performance liquid chromatography. *Journal of Chilean Chemical Society, 56*(2), 688–691.

Monagas, M., Suarez, R., Gómez-Cordovés, C., & Bartolomé, B. (2005). Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC-DAD/ESI-MS. *American Journal of Enology and Viticulture, 56*, 139–147.

Nacka, M., Georgiev, N., Dabovic Anastasovska, J. D. (2012). The economics of industrial property rights: Use of geographical indications as a marketing strategy for competitiveness of wine sector in Republic of Macedonia. In: Proceedings of the 132nd seminar of the european association of agricultural economists (EAAE), "is transition in European agriculture really over?" New dimensions and challenges of transition and post-transition processes in agriculture and food sectors in the European Union and EU acceding and neighboring countries. Skopje, October 25–27, 2012, pp. 1–14.

Nicoletti, I., Bello, C., De Rossi, A., & Corradini, D. (2008). Identification and quantification of phenolic compounds in grapes by HPLC-PDA-ESI-MS on a semimicro separation scale. *Journal of Agricultural and Food Chemistry, 56*, 8801–8808.

OIV. (2014). International organization of vine and wine. Compendium of International Methods of Analysis of Wines and Musts 1.
Ortega, T., De La Hera, E., Carretero, M. E., Gómez-Serranillos, P., Naval, M. V., Villar, A. M., et al. (2008). Influence of grape variety and their phenolic composition on vasorelaxing activity of young red wines. *European Food and Research Technology, 227*, 1641–1650.
Pajovic, R., Raicevic, D., Popovic, T., Sivilotti, P., Lisjak, K., & Vanzo, A. (2014). Polyphenolic characterisation of Vranac, Kratosija and Cabernet Sauvignon (Vitis vinifera L. cv.) grapes and wines from different vineyard locations in Montenegro. *South African Journal of Enology and Viticulture, 35*(1), 139–148.
Rajković, M. B., & Sredović, I. D. (2009). The determination of titratable acidity and total tannins in red wine. *Journal of Agricultural Sciences, 54*, 223–346.
SSO. (2009). State statistical office of the republic of Macedonia (2004–2009). Statistical Yearbooks 2004–2009. Skopje.
Tsanova-Savova, S., Dimov, S., & Ribarova, F. (2002). Anthocyanins and color variables of Bulgarian aged red wines. *Journal of Food Composition and Analysis, 15*, 647–654.
Vasserot, Y., Caillet, S., & Maujean, A. (1997). Study of anthocyanin adsorption by yeast lees. Effect of some physicochemical parameters. *American Journal of Enology and Viticulture, 48*, 433–437.
Versari, A., du Toit, W., & Parpinello, G. P. (2013). Oenological tannins: a review. *Australian Journal of Grape and Wine Research, 19*, 1–10.
Vinković Vrček, I., Bojić, M., Žuntar, I., Mendaš, G., & Medić-Šarić, M. (2011). Phenol content, antioxidant activity and metal composition of Croatian wines deriving from organically and conventionally grown grapes. *Food Chemistry, 124*, 354–361.