CHANGES IN ANTHOCYANINS AND BERRY COLOR OF 'PLAVAC MALI' GRAPE DURING RIPENING

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This study investigated harvest potential of the berries through changes in the anthocyanin composition and berry skin color profile of the grape variety 'Plavac mali' grown in the Mediterranean climatic region of Croatia at four different dates of ripening. Four berry samplings were conducted at 17-days-intervals starting with 20th August and until 10th October. The berries were weighed and the skin color parameters were determined using the CIELab technique. The separation and quantitative determination of anthocyanins was done by high performance liquid chromatography analysis, whereas total anthocyanins and total phenols were determined by applying ultraviolet-visible spectrophotometry. The mono-glucosylated derivatives of five main anthocyanidins were detected, some of which were stable such as cyanidin and peonidin whereas others, delphinidin, petunidin and malvidin concentrations, varied during the ripening. Malvidin and its derivatives, malvidin-3-(6"-O-acetyl) glucoside and malvidin-3-coumaroylglucoside, were most abundant (over 70 %) in the skin of 'Plavac mali'. All chromatic parameters except L* values correlated significantly with malvidin. Grape berry color profile showed plateau of synthesis anthocyanins in the third sampling date when the highest total anthocyanin concentration and chromatic values of L*, a* and b* were obtained. This work advances our understanding of the anthocyanin dynamics during ripening of 'Plavac mali' grape and aids characterization of clones during individual clonal selection.
Keywords: malvidin, skin color, maturation, CIELab, 'Plavac mali', HPLC

Veränderungen bei Anthocyanen und Beerenausfärbung bei 'Plavac mali'-Trauben während der Reifung. Diese Studie untersuchte das Erntepotenzial der Beeren durch Veränderungen in der Zusammensetzung der Anthocyane und des Farbprofils der Beerenhaut bei der Rebsorte 'Plavac mali' aus der mediterranen Klimaregion Kroatiens an vier verschiedenen Tagen der Reifephase. Vier Probenahmen wurden in 17-Tage-Intervallen beginnend mit 20. August bis 10. Oktober durchgeführt. Die Beeren wurden gewogen und die Parameter der Beerenfarbe mittels CIELab-Technik bestimmt. Die Trennung und die quantitative Bestimmung von Anthocyanen wurde mittels HPLC durchgeführt, während die Gesamtgehalte der Anthocyane und Phenole durch UV/VIS-Spektrophotometrie bestimmt wurden. Die monoglucosylierten Derivate von fünf Hauptanthocyanen wurden nachgewiesen, von denen einige, wie Cyanidin

und Peonidin, stabil waren, während andere, Delphinidin, Petunidin und Malvidin, während der Reifung schwankten. Malvidin und seine Derivate, Malvidin-3- (6"-O-Acetyl)-Glucosid und Malvidin-3-Coumaroylglucosid waren am häufigsten (über 70 %) in der Beerenhaut von 'Plavac mali'. Alle chromatischen Parameter mit Ausnahme der L*-Werte korrelierten signifikant mit Malvidin. Die Farbprofile der Beeren zeigten ein Plateau der synthetisierten Anthocyane zum dritten Probenahmetermin, zu dem die höchsten Werte für die Gesamtkonzentration der Anthocyane und die Farbwerte L*, a* und b* festgestellt wurden. Diese Arbeit fördert unser Verständnis der Anthocyandynamik während der Reifung von 'Plavac mali'-Trauben und unterstützt die Identifizierung bei der Selektion einzelner Klone.
Schlagwörter: Malvidin, Beerenhautfarbe, Reifung, CIELab, 'Plavac mali', HPLC

The concentration and composition of anthocyanins at the moment of harvest are quality indicators for wine grapes and significantly affect the enological potential. The composition and total concentration of anthocyanins in grapes combined with their degree of extractability determine the chromatic characteristics of wine. The greatest changes in the concentration and structure of anthocyanins occur during ripening, starting at veraison. They are primarily located in several cell layers of the berry skin or very rarely in the pulp of 'teinturier' varieties. The anthocyanin profile is characteristic for grapevine species and variety (Mazza and Miniati, 1993; Ortega-Regules et al., 2006). Varieties of Vitis vinifera L. commonly contain 3-monoglucoside, acetyl-glucoside and 3-p-coumaroylglucoside derivatives of delphinidin, cyanidin, peonidin, petunidin and malvidin (Eder et al., 1994). There are no reports of pelargonidin derivatives in grape berry skin. Glycosylation, methylation and acylation of anthocyanins occur in the cytosol, increasing the stability of otherwise unstable anthocyanins, which is essential for their accumulation in vacuoles (Springob et al., 2003). The anthocyanin composition of grapes is very distinctive for the variety. 'Pinot Noir' and some wild grape accessions do not contain acylated anthocyanin forms but only 3-monoglucoside anthocyanins (Revilla et al., 2012). Unlike 'Pinot Noir', 'Vranec' contains a very high concentration of coumaroyl derivatives, whereas acetyl derivatives are predominant in 'Cabernet Sauvignon' (Dimitrovska et al., 2011). Anthocyanin composition largely depends on environmental factors, ecological conditions of the region and viticultural practices (Jackson and Lombard, 1993; Eder et al., 2004; Cortell et al., 2007). In 'Cabernet Sauvignon', the concentration of some individual anthocyanins depends on nitrogen availability in the soil (Keller and Hrazdina, 1998). The anthocyanin profile can also be influenced by soil composition (Yokotsuka et al., 1999), water availability (Matthews and Kriedemann, 2006; Castellarin et al., 2007) and temperature conditions (Yamane et al., 2006).

'Plavac mali' is the primary red grape variety (*Vitis vinifera L.*) grown in Croatia, on islands and coastal regions of central and south Dalmatia. Recent genetic studies have confirmed that 'Plavac mali' is a native Croatian variety created by crossing 'Crljenak Kaštelanski' and 'Dobričić' (Maletić et al., 2004). Currently, there is no registered and commercially available clone of 'Plavac mali' in Croatia, but a program to select clone and sanitary selections has found high phenotypic variability in important wine-growing properties (Zdunić et al., 2007). A particularly prominent feature was found to be color variability in the bunch. It was found, that on many mother vines at full maturity there was a significant proportion of green berries. Asynchronous ripening of grapes can lead to inconsistent sensory characteristics in the resulting wine, and may be associated with several viticultural factors in addition to the genetic structure of the variety (Jackson and Lombard, 1993; Gray and Coombe, 2009; Barbagallo et al., 2011; Wu Dai et al., 2011). Clones of one variety can vary significantly in production characteristics and produce wines with different sensory characteristics, making individual evaluation necessary.

There are scarce data regarding the color and anthocyanin profile of 'Plavac mali' grape, which makes evaluating clonal candidates difficult. The anthocyanin profile of 'Plavac mali' grape berries is typical for *Vitis vinifera* L.; the most common anthocyanin found was malvidin-3-monoglucoside (Budić-Leto et al., 2009). The

concentration of individual anthocyanins was significantly higher than that of another native Croatian variety, 'Babić', which is grown in the same region of Dalmatia (Ćurko et al., 2014). To our knowledge, there is no published study on the dynamics of accumulation of individual anthocyanins during the ripening of 'Plavac mali' grapes.

In this study, we focused on the berry skin color and anthocyanin profile of 'Plavac mali' accession OB225 (commercially unavailable) during ripening. During selection, accession OB225 showed excellent production characteristics (high yield, large bunches and berries, vigorous growth), but a significant color variation among the berries in a bunch that can serve as a case study for color evaluation of 'Plavac mali'. The results of this study will contribute to understanding anthocyanin dynamics during ripening of 'Plavac mali' grape and aid characterization of clones during individual clonal selection.

MATERIAL AND METHODS

PLANT MATERIAL

Samples of berries *Vitis vinifera* L. cv. 'Plavac mali' (accession OB225) were collected from the grape germplasm repository of the Institute for Adriatic Crops and Karst Reclamation, which is located in Split (latitude, 43°30.335N; longitude, 016º29.855E; 14 m asl) in Croatia. The vines were grafted on the rootstock 'Börner' (*Vitis riparia* x *Vitis cinerea*). The germplasm repository includes 59 accessions of 'Plavac mali' that were selected throughout the growing area on the basis of phenotypic variability. Each accession in the collection is represented by six vines that were propagated from a single mother plant in 2005. The accessions were previously evaluated using ampelographic description and the cultivar identity was confirmed through analysis of nuclear microsatellites (Zdunić et al., 2012). Spacing between plants was 1.0 m and the distance between rows was 2.0 m, representing a density of 5000 plants/ha. Four spurs with two buds were left at winter pruning and other cultivation practices were the same for all vines. The soil in the vineyard is clay-sand. Just before veraison, we observed that the studied vines had approximately the same number of bunches and yield. Four berry samplings were conducted at 17-days-intervals between 20th August and 10th October 2012, starting eighty-one days after anthesis, when 90 % of the berries were colored. Samples of 100 berries were collected from each of the six individual vines representing the accession early in the morning. A representative sampling was performed with care to take berries from all positions on the vine and cluster (exposed to direct sunlight and shaded). Whole, undamaged berries with attached petioles were immediately transferred to the laboratory. The sample was divided into two, 50-berry sub-samples. The berries from the first sub-sample were weighed, the skin color was determined using the CIELab technique, and the same sample was used for spectrophotometric measurement of total anthocyanins and total phenols. Another sub-sample of grape berries was separated and stored at -80 °C until HPLC analysis.

COLOR MEASUREMENTS (CIELAB)

To avoid irregularities caused by remnants of dust and/or pesticides, the berries were gently cleaned with a cotton cloth. The color was always measured on the same place on the berry with a colorimeter (CR-400, Konica Minolta, Tokyo, Japan) and the computer program SpectraMagic NX Lite, ver. 2.0. The colorimeter was calibrated using a standard calibration plate before use. The transmission values of the berry skin were measured with an illuminant D65 with a 2° observer. CIELab is the most complete color system in which all colors are visible to the human eye as the value of three coordinates: L* measures brightness (0 - 100), chromatic component a* ranges from -a = green to +a = red, and chromatic component b* from -b = blue to +b = yellow, equally distant along the axis. Color saturation C was calculated as $C = [(a^{*2} + b^{*2})]^{0.5}$ whereas h, the value of the hue, was expressed in degrees arctang h = b*/a*. The color index for red grapes (CIRG) was calculated according to a previously published equation (Carreno et al., 1995).

SPECTROPHOTOMETRIC DETERMINATION OF TOTAL ANTHOCYANINS AND TOTAL PHENOLS

Analysis of total anthocyanins and total phenols was carried out by the method of Iland et al. (1996). Briefly, the petioles were removed and the berries were homogenized in a blender (Multiquic Braun, Kronberg, Germany) for 2 min. One g of the resulting homogenate was added to 10 ml ethanol (50 % v/v) and stirred at intervals for 1 h prior to centrifugation at 1,800 g for 10 min. A portion of the supernatant (0.2 ml) was added to 3.8 ml 1.0 M HCl, mixed and incubated at room temperature for 3 h. Subsequently, sample absorbance at 520 and 280 nm was measured with a spectrophotometer (Cary 50 Scan, Agilent, Middelburg, Netherlands). Total anthocyanins and total phenols were expressed as mg per berry and mg per g of fresh berry weight.

HPLC-PDA / ELECTROSPRAY IONIZATION (ESI)-MS OF ANTHOCYANINS

Skins, seeds and pulp were manually separated from the frozen berries. Immediately afterwards, skin and pulp were freeze-dried (Freeze Dry System free zones 2.5l, Labconco, Kansas City, USA). The freeze-dried samples were weighed into a centrifuge vial (500 mg) and extracted twice with 10 ml acidified methanol (methanol/water/formic acid 80/15/5, v/v/v) in a cooled ultrasonic bath. The supernatants after centrifugation (4000 g/15 min) were pooled and made up to 50 ml in a volumetric flask with the extraction solution. Chromatographic separation was achieved on a Surveyor HPLC system (Thermo Fisher, Dreieich, Germany) equipped with a 125 x 2 mm i.d., 3 µm RP-Reprosil-Pur 120 ODS-3 column (Dr. Maisch, Ammerbuch, Germany) protected with a guard column of the same material as described elsewhere (Will and Dietrich, 2013). Injection volume was 4 µl after 0.45 µm filtration of the extracts, the flow rate was 200 µl/min at 40 °C. Elution conditions: solvent A was 5 % formic acid (v/v); solvent B was methanol; 1 min isocratic conditions with 10 % B, linear gradient from 10 to 40 % B in 19 min, followed by washing with 100 % B and re-equilibrating the column. Quantitation was carried out using peak areas a 520 nm using external calibration with the reference substance cyanidin-3-glucoside. Mass detection was performed on a coupled LCQ Advantage Max mass spectrometer (Thermo Fisher, Dreieich, Germany) equipped with an ESI source and an ion trap mass analyzer. For anthocyanins, the mass spectrometer was operated in the positive mode with the following conditions: source voltage, 4.5 kV; capillary voltage, 32 V; capillary temperature, 275 °C; collision energy, 30 (MS^2) and 33 % (MS^3).

SANITARY ANALYSIS

Cuttings from the mother vine of accession OB225 were collected in the original vineyard and cuttings from its clonal progeny were collected from the collection vineyard during dormancy. These were examined for the presence of viruses by several enzyme-linked immunosorbent assay (ELISA) protocols: a double antibody sandwich ELISA (DAS-ELISA) for *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), and *Grapevine leafroll-associated virus* types 1 and 3 (GLRaV-1 and GLRaV-3), a double antibody sandwich indirect ELISA (DASI-ELISA) for *Grapevine fleck virus* (GFkV) and a protein A double antibody sandwich ELISA (protein A-DAS-ELISA) for *Grapevine virus* A (GVA). All reagents were provided by Agritest (Valenzano Bari, Italy) and used according to the manufacturer's instructions.

SATISTICAL ANALYSIS

Significant differences among sampling periods and for each variable were determined by one-way analysis of variance (ANOVA). Differences tested by Fisher test were considered significant at the 5 % level. Correlation of chromatic color parameters (CIELab) and anthocyanin concentrations were explored by correlation index. Statistical analysis was done by the statistical software package Statistica 8.0 (StatSoft, Inc, USA; www.statsoft.com).

RESULTS AND DISCUSSION

THE SKIN COLOR OF BERRIES AND CONCENTRATION OF TOTAL ANTHOCYANINS

At the beginning of sampling (August), around veraison, the weather conditions were extremely dry (0 mm of rainfall), very hot with mean temperature 28.6 °C, whereas September and October were rainy, 108.8 mm and 143.6 mm and warm 22.8 °C and 18.3 °C, respectively (Fig. 1).

The appearance of green berries in bunches of the accession OB225, which was quite evident in the mother vine, did not manifest in any of its six clonal progenies during sampling (Fig. 2). Only a small proportion of green berries in bunches were detected in the first sampling, but this was expected given the early stage of maturity. At later samplings, all bunches were fully colored. Other accessions also had green berries on the mother vine during the initial stage of selection, and their progeny vines in the collection did not keep this characteristic. This suggests that the appearance of green berries during maturation of 'Plavac mali' bunches was influenced by environmental factors and cultural practices. ELISA tests of the mother vine and clonal progenies showed that OB225 was infected with GLRV-3 virus, which is known to cause asynchronous berry ripening (Guidoni et al., 1997; Cabaleiro et al., 1999). Vines of clonal progenies in the collection have not manifested green berries and we may assume that in these vines, the virus impact was latent. However, there are numerous ecophysiological and genetic factors that influence the variability and composition of berries in addition to viral infections (Wu Dai et al., 2011). Besides the obvious effect of light exposure, asynchronous berry development can depend on the ratio of leaves to fruit on an individual shoot (Kliewer and Dokoozlian, 2001). According to Gray and Coombe (2009), variability in berry size originated prior to fruit set is probably due to asynchronous cell division in the floral buds at the beginning of budding. It appears that 'Plavac mali' is sensitive to asynchronous development of berries and this phenomenon will not be eliminated only by clonal selection, but this still remains to be checked after completion of sanitary selection and virus elimination.

Total berry weight, fresh skin weight, and the concentrations of total anthocyanins and phenolics in 'Plavac mali' grapes were determined at four sampling dates between veraison and harvest (Table 1). The total weights of berries and fresh skins did not differ ($p < 0.05$) between the four different sampling dates. The total anthocyanins (as concentration or per berry) changed over time. The highest concentration was found at the third sampling (2.19 mg/g), but when the result was expressed per berry, there was no difference between the second and third sampling dates. Total phenolics did not differ in concentration, but did differ per berry. Slightly higher total phenol per berry was found at the first and second samplings than at the third and fourth. More anthocyanins at the later dates were expected due to the advanced ripeness of the grapes. However, the high standard deviation at all dates indicates high berry variability and the significant differences in ripeness among the vines, bunches and even within the bunch itself. At full maturity, 'Syrah' berries separated into four categories by weight also had significant differences in the concentrations of total and individual anthocyanins (Barbagallo et al., 2011).

CIELab parameters of grapes and the calculated color index, CIRG, emphasize the differences between berries at the four sampling dates (Table 2). The effect of sampling time on color was dramatic, although the pattern formation of the color was inconsistent and there was non-linear behavior of various parameters over time. Berry lightness L* varied from darker (24.30) at the second sampling date to lighter (28.10) at the third sampling date, whereas the berries at the last sampling became darker again (24.42) and did not differ from the berries from the second sampling date. The redness of the berries (positive a* values) decreased from the first to the third sampling, when it reached its lowest value. The proportion of blue (negative b* values) was highest at the third sampling. Consequently, the hue angle (h) differed significantly between the initial and the final stages of the ripening. C values at all dates behaved very similarly to the L* values, where the color saturation decreased during ripening of the grapes. The calculated

color index of red grapes (CIRG) showed a weak linear rise from 6.34 to 7.36 as the berries ripened. Significant differences were found among the berries of the first, second and fourth sampling dates, whereas the berries of the third sampling did not differ significantly from the berries of the second or fourth sampling. Rolle and Guidoni (2007) tested the chromatic values of 18 different dark-skinned grape varieties and found a strong correlation between chromatic color index and total anthocyanins. In this study, all parameters except L* and C were correlated significantly with total anthocyanin concentration and certain individual anthocyanins (Table 3). The values of a* and b* correlated negatively with total anthocyanin concentration (r = -0.53 and -0.47, respectively), whereas h and CIRG were positively correlated (r = 0.51 and 0.49, respectively). Among individual anthocyanins, malvidin-3-glucoside and its derivatives had the best linear correlation with chromatic parameters. These results are in accordance with Liang et al. (2011), in which the CIELab parameters and anthocyanin content and composition of 78 different white, purple, red and dark-skinned varieties were tested. The results of this study suggest that it is possible to use chromatic parameters directly in the vineyard to estimate fruit quality and maturity of 'Plavac mali'.

ANALYSIS OF INDIVIDUAL ANTHOCYANINS

The skin and flesh fractions of 'Plavac mali' berries were separated manually and HPLC was used to measure individual anthocyanins (Fig. 3). The skin contains the majority of anthocyanins, whereas negligible concentrations of individual anthocyanins were detected in the flesh. Peonidin-3-glucoside, malvidin-3-glucoside and derivatives of malvidin (malvidin-3-(6"-O-acetyl)-glucoside and malvidin-3-coumaroylglucoside; concentrations not shown) were identified in the flesh. The flesh of 'Plavac mali' is considered colorless and these detected values probably result from physical maceration during the separation of skin and flesh.

Seven anthocyanins were identified and quantified in 'Plavac mali' skin extracts (Table 4). At the four sampling dates, 3-O-monoglucoside-delphinidin, cyanidin, petunidin, peonidin, malvidin and 6"-O-acetyl and 6"-O-cou-

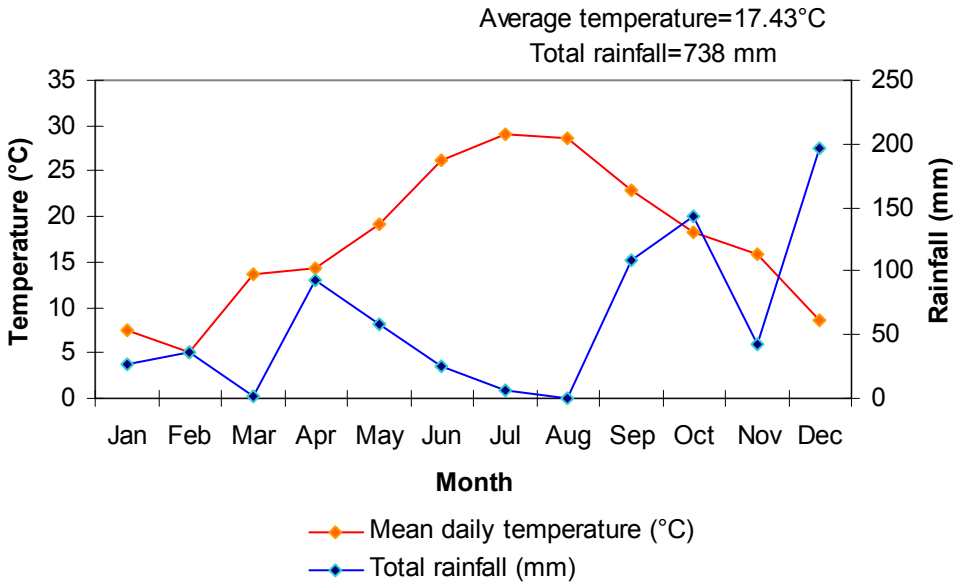

Fig. 1: Average monthly temperature (°C) and rainfall (mm) during 2012 for the Split meteorological station

a) b)

Fig. 2: Bunches of 'Plavac mali' accession OB225 on the mother vine at full maturity with green berries (a) and of its clonal progeny in the germplasm collection, fully painted and without green berries (b)

Table 1: Total berry weight (g), total fresh skin weight (g) and spectrophotometric determination of total anthocyanins and total phenols expressed as mg/g and mg/berry, resp., of 'Plavac mali' accession OB225 grape berries at four sampling dates

	1st sampling 20th Aug	2nd sampling 5th Sep	3rd sampling 23rd Sep	4th sampling 11th Oct
Total weight of 50 berries (g)	72.23 ± 6.51a	78.19 ± 9.24a	72.59 ± 5.68a	74.22 ± 8.72a
Total fresh skin weight (g)	19.05 ± 2.91a	19.76 ± 3.88a	18.92 ± 2.84a	19.34 ± 2.59a
Total anthocyanins (mg/g)	1.54 ± 0.17a	1.72 ± 0.25ab	2.19 ± 0.17c	1.81 ± 0.21b
Total anthocyanins (mg/berry)	2.85 ± 0.49a	3.67 ± 0.53b	3.35 ± 0.58ab	2.84 ± 0.21a
Total phenols (mg/g)	4.25 ± 0.56a	4.28 ± 0.48a	4.55 ± 0.43a	4.06 ± 0.47a
Total phenols (mg/berry)	7.89 ± 1.59bc	9.10 ± 0.76c	6.96 ± 1.18ab	6.38 ± 0.50a

Mean values ± standard deviations followed by different letters among columns indicate statstically significant differences between various sampling dates at $p \leq 0.05$

Table 2: Color parameters of the skin tissue of the 'Plavac mali' accession OB225 during four sampling dates: the lightness coordinate (L*), the green/red coordinate (a*), the blue/yellow coordinate (b*), hue (h), chroma (C) and color index (CIRG)

	1st sampling 20th Aug	2nd sampling 5th Sep	3rd sampling 23rd Sep	4th sampling 11th Oct
L*	25.78 ± 0.55b	24.30 ± 0.34a	28.10 ± 1.44c	24.42 ± 0.37a
a*	2.40 ± 0.39c	1.90 ± 0.22a	1.19 ± 0.38b	1.67 ± 0.32a
b*	0.16 ± 0.20b	0.05 ± 0.12ab	-1.10 ± 0.73c	-0.35 ± 0.12a
C	2.47 ± 0.38b	1.94 ± 0.23a	1.99 ± 0.27a	1.82 ± 0.31a
h	122.57 ± 69.09a	121.16 ± 42.87a	243.65 ± 96.31b	243.23 ± 31.77b
CIRG	6.34 ± 0.25a	6.82 ± 0.10b	7.12 ± 0.52bc	7.36 ± 0.20c

Mean values ± standard deviations followed by different letters among columns indicate statstically significant differences between various sampling dates at $p \leq 0.05$

Table 3: Factors of the Pearson correlation matrix

	Dp	Cy	Pt	Pn	M	M-a-g	M-c-g	TA	L*	a*	b*	C	h	CIRG	TA 1	TA 2	TP 1	TP 2
Dp	1.00																	
Cy	0.80	1.00																
Pt	0.98	0.72	1.00															
Pn	0.77	0.89	0.78	1.00														
M	0.38	-0.01	0.55	0.34	1.00													
M-a-g	0.04	-0.26	0.22	0.09	0.91	1.00												
M-c-g	0.03	-0.22	0.21	0.14	0.86	0.95	1.00											
TA	0.72	0.42	0.84	0.69	0.90	0.69	0.67	1.00										
L*	0.19	-0.04	0.21	0.04	0.26	0.12	0.02	0.23	1.00									
a*	-0.15	0.03	-0.27	-0.24	-0.60	-0.54	-0.56	-0.53	-0.53	1.00								
b*	-0.24	0.02	-0.33	-0.19	-0.52	-0.37	-0.37	-0.47	-0.79	0.82	1.00							
C	-0.01	0.01	-0.12	-0.21	-0.44	-0.49	-0.59	-0.38	0.13	0.72	0.23	1.00						
h	0.28	0.04	0.39	0.25	0.53	0.38	0.48	0.51	0.50	-0.73	-0.86	-0.36	1.00					
CIRG	0.08	-0.06	0.22	0.24	0.58	0.54	0.63	0.49	0.21	-0.80	-0.74	-0.61	0.86	1.00				
TA 1	0.06	-0.11	0.14	0.14	0.45	0.32	0.23	0.35	0.52	-0.64	-0.64	-0.28	0.43	0.45	1.00			
TA 2	-0.25	-0.01	-0.23	0.10	-0.03	0.04	-0.01	-0.07	0.16	-0.35	-0.26	-0.24	0.09	0.26	0.49	1.00		
TP 1	-0.10	-0.10	-0.13	-0.18	-0.11	-0.17	-0.29	-0.16	0.30	-0.09	-0.25	0.15	0.08	0.01	0.58	0.45	1.00	
TP 2	-0.31	0.05	-0.38	-0.13	-0.49	-0.35	-0.38	-0.46	-0.16	0.20	0.21	0.10	-0.27	-0.18	-0.10	0.70	0.49	1.00

Correlation coefficients between the concentration of individual anthocyanins (3-O-monoglucosides of delphinidine (Dp), cyanidine (Cy), petunidine (Pt), peonidine (Pn) and malvidine (M); 3-O-acylated monoglucosides of the malvidine: malvidin-3-(6"-O-acetyl) glucoside (M-a-g), malvidin-3-coumarylglucoside (M-c-g), and total anthocyanins (TA) determined by HPLC), CIELAB color parameters (L*, a*, b*, C, h and CIRG) and total anthocyanins and total phenols determined by spectrometry (total anthocyanins expressed as mg/g (TA 1) or expressed as mg/berry (TA 2) and total phenols expressed as mg/g (TP 1) or expressed as mg/berry (TP 2).
Red numbers: Correlation is significant at the 0.05 level

maroyl derivatives of malvidin-3-glucoside were identified in the skin of 'Plavac mali'. The most abundant group of anthocyanins was 3-O-monoglucosides, and the most common among them was malvidin-3-glucoside. Earlier studies showed that skin extracts of 'Plavac mali' are characterized by high concentrations of malvidin-3-glucoside (Budić-Leto et al., 2009; Ćurko et al., 2014), as are the Italian variety 'Aglianico' (Manfra et al., 2011), several indigenous Andalusian varieties ('Jaen tinto', 'Palomino negro', 'Tintilla de Rota'), 'Cabernet Sauvignon', 'Tempranillo' (Guerrero et al., 2009), 'Vranec' and 'Merlot' (Ivanova et al., 2011). The least abundant anthocyanin in skin extracts was cyanidin-3-glucoside, also consistent with previous results (Budić-Leto et al., 2009; Ćurko et al., 2014). Other forms of acylated anthocyanins (delfinidin, peonidin, cyanidin, and petunidin) were not detected in our extracts, but were detected in 'Plavac mali' in earlier studies (Budić-Leto et al., 2009; Ćurko et al., 2014) and in other varieties (reviewed by Mazza and Miniati (1993)). Also, we detected no malvidin-3- caffeoyl-glucoside, which was previously detected in the skin of 'Plavac mali' (Budić-Leto et al., 2009). However, according to previous studies (Budić-Leto et al., 2009; Ćurko et al., 2014) monoglucosides represent more than 70 % of the total anthocyanins identified in grapes and in our study, the majority of the total anthocyanins in the grapes were identified.

The concentrations of individual anthocyanins in 'Plavac mali' berries were determined at four sampling times (Table 5). The total anthocyanin concentration as mg per kg fresh weight showed a continuous increase and was greater at the third and fourth sampling dates than at the first and second samplings. The accumulation of anthocyanins begins at veraison, increases during ripening, and reaches its greatest concentration at full maturity (Boss et al., 1996; Kennedy et al., 2002; Fournand et al., 2006). The dihydroxylated anthocyanin cyanidin-3-glucoside, precursor of peonidine-3-glucoside, followed by the trihydroxylated form delfinidin-3-glucoside, precursor of the petunidine-3-glucoside and malvidin-3-glucoside occur first (Boss et al., 1996). In this paper, a different behavior of individual anthocyanins was observed during ripening. There was a continuous increase in the malvidin-3-glucoside concentration,

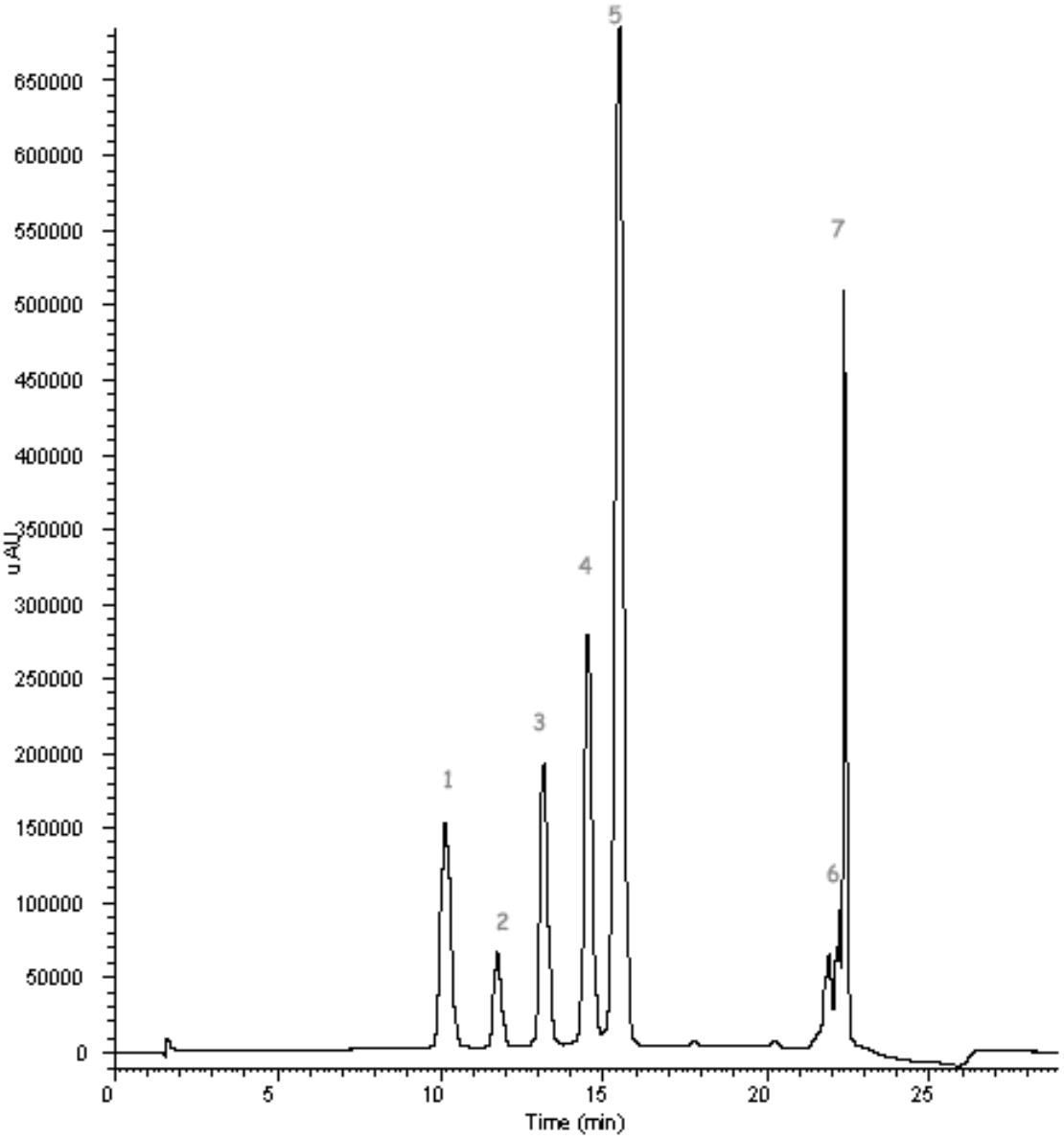

Fig. 3: Separation of anthocyanins in 'Plavac mali' accession OB225 grape skin extract by HPLC, monitored at 520 nm. The proposed identities of compounds associated with the peaks shown are given in Table 4.

Table 4: Chromatography and mass spectrometry identifiers of the anthocyanins found in skins of 'Plavac mali' accession OB225 grape berries by HPLC/MS

Peak no.	t_R (min)	Compound	M^+ (m/z)	Aglycon fragment (m/z)	λ_{max} (nm)
1	10.2	Delphinidin-3-glucoside	464.85	303	523
2	11.8	Cyanidin-3-glucoside	448.84	287	515
3	13.2	Petunidin-3-glucoside	478.86	317	524
4	14.6	Peonidin-3-glucoside	462.88	301	516
5	15.5	Malvidin-3-glucoside	492.85	331	526
6	21.9	Mal-3-(6"-O-acetyl)-glucoside	534.87	331	531
7	22.4	Mal-3-(6"-O-coumaryl)-glucoside	638.90	331	532

Peak numbering as in Fig. 3; HPLC retention time (t_R), protonated molecular ion (M^+), main fragment ions and spectral characteristics of the anthocyanins (λ_{max}), detected in skin

Table 5: Concentrations of anthocyanins (mg/kg fresh berry) in skins of 'Plavac mali' accession OB225 on four sampling dates

Compound	1st sampling 20th Aug	2nd sampling 5th Sep	3rd sampling 23rd Sep	4th sampling 11th Oct
Delphinidin-3-glucoside	312 ± 95ab	299 ± 84a	406 ± 168b	344 ± 109ab
Cyanidin-3-glucoside	98 ± 57a	88 ± 50a	96 ± 48a	95 ± 39a
Petunidin-3-glucoside	313 ± 77a	317 ± 71a	414 ± 118b	367 ± 101ab
Peonidin-3-glucoside	389 ± 145a	387 ± 141a	472 ± 110a	477 ± 116a
Malvidin-3-glucoside	1348 ± 260a	1560 ± 222a	2056 ± 358b	1901 ± 524b
Mal-3-(6"-O-acetyl)-glucoside	138 ± 29a	173 ± 25b	194 ± 43b	192 ± 61b
Mal-3-(6"-O-coumaryl)-glucoside	425 ± 96a	552 ± 94ab	647 ± 225b	673 ± 249b
Total anthocyanin	2713 ± 520a	2967 ± 465a	3789 ± 582b	3541 ± 837b

Mean values of the individual anthocyanins ± standard deviations, followed by different letters among columns indicate statistically significant differences between various sampling dates at $p \leq 0.05$

from 1348 mg/kg fresh berries at the first sampling to 2056 at the third sampling. A similar trend of increasing concentration occurred in other derivatives of malvidin. There was no significant difference in the concentrations of cyanidin-3-glucoside and peonidin-3-glucoside among sampling dates. The delfinidin-3-glucoside concentration varied significantly at the second and third sampling dates. The highest concentration of delfinidin-3-glucoside was found during the third sampling (406 mg/kg fresh berries). The petunidin-3-glucoside concentration increased during ripening and was highest at the third sampling (414 mg/kg fresh berries), after which its concentration decreased. Delfinidin and cyanidin are biosynthetic precursors that undergo methylation during maturation into peonidin, petunidin and malvidin (Roggero et al., 1986). The concentrations of cyanidin-3-glucoside and peonidine-3-glucoside were relatively stable during maturation as reported previously (Boss et al., 1996).

CONCLUSIONS

We examined the color and anthocyanin profile of 'Plavac mali' accession OB225, in which berry maturity visibly affects the concentration and composition of skin anthocyanins. There was high variability of anthocyanin concentrations from vine to vine and also within the vine, although there were no differences in berry weight or skin weight among sampling dates. The total anthocyanins, total phenols, CIELab color coordinates and individual anthocyanins varied significantly at different stages of maturation. The concentration of total anthocyanins increased with maturation and reached its maximum at the third ripening stage, which also coincides with the maximum concentration of total phenols. The most common anthocyanin at all stages was malvidin-3-glucoside, followed by peonidin-3-glucoside and petunidin-3-glucoside. The concentrations of individual anthocyanins increased with maturity and differed signi-

ficantly from the first two samplings. Results suggest that continued clonal selection and virus elimination from this accession is warranted.

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REFERENCES

Barbagallo, M.G., Guidoni, S. and Hunter, J.J. 2011: Berry size and qualitative characteristics of Vitis vinifera L. cv. 'Syrah'. S. Afr. J. Enol. Vitic. 32: 129-136

Boss, P.K., Davies, C. and Robinson, S. 1996: Analysis of the expression of anthocyanin pathway genes in developing Vitis vinifera L. cv. 'Shiraz' grape berries and the implications for pathway regulation. Plant Physiol. 111: 1059-1066

Budić-Leto, I., Vrhovšek, U., Gajdoš-Kljusurić, J. and Lovrić, T. 2009: Anthocyanin pattern of skin extracts from 'Babić' and 'Plavac mali' grapes and anthocyanin pattern of produced wine. Acta Alimenaria 38: 67-75

Cabaleiro, C., Segura, A. and García-Berrios, J.J. 1999: Effects of Grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. 'Albariño' following contamination in the field. Am. J. Enol. Vitic. 50: 40-44

Carreno, J., Martinez, A., Almela, L. and Fernandez-Lopez, J.A. 1995: Proposal of an index for the objective evaluation of the colour of the red table grapes. Food Res. Int. 28: 373-377

Castellarin, S.D., Matthews, A.M., Di Gaspero, G. and Gambetta, A.G. 2007: Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. Planta 227: 101-112

Cortell, J.M., Halbleib, M., Gallagher, A.V., Righetti, L.T. and Kennedy, J.A. 2007: Influence of vine vigor on grape (*Vitis vinifera* L. cv. 'Pinot Noir') anthocyanins. 1. anthocyanin concentration and composition in fruit. J. Agric. Food Chem. 55: 6575-6584

Ćurko, N., Kovačević Ganić, K., Gracin, L., Đapić, M., Jourdes, M. and Teissedre, P.L. 2014: Characterization of seed and skin polyphenolic extracts of two red grape cultivars grown in Croatia and their sensory perception in a wine model medium. Food Chem. 145: 15-22

Dimitrovska, M., Bocevska, M. and Dimitrovski, D. 2011: Anthocyanin composition of 'Vranec', 'Cabernet Sauvignon', 'Merlot' and 'Pinot Noir' grapes as indicator of their varietal differentiation. Eur. Food Res. Technol. 232: 591-600

Eder, R., Wendelin, S. und Barna, S. 1994: Klassifizierung von Rotweinsorten mittels Anthocyananalyse. 1. Mitteilung: Anwendung multivariater statistischer Methoden zur Differenzierung von Traubenproben. Mitt. Klosterneuburg 44: 201-2012

Eder, R., Oswald, B. und Wendelin, S. 2004: Einfluss von pektolytischen Enzympräparaten mit Acetylaseaktivität sowie Botrytisbefall und Maischeerhitzung auf Anthocyanzusammensetzung und Qualität von Rotweinen. Mitt. Klosterneuburg 54: 204-221

Fournand, D., Vicens, A., Sidhoum, L., Souquet, J.M., Moutounet, M. and Cheynier, V. 2006: Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. J. Agric. Food Chem. 54: 7331-7338

Gray, D.J. and Coombe, B.G. 2009: Variation in 'Shiraz' berry size originates before fruitset but harvest is a point of resynchronisation for berry development after flowering. Austr. J. Grape Wine Res. 15: 156-165

Guerrero, F.R., Liazid, A., Palma, M., Puertas, B., González-Barrio, R., Gil-Izquierdo, A., García-Barroso, C. and Cantos-Villar, E. 2009: Phenolic characterisation of red grapes autochthonous to Andalusia. Food Chem. 112: 949-955

Guidoni, S., Mannini, F., Ferrandino, A., Argamante, N. and Di Stefano, R. 1997: The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (Vitis Vinifera L.). Am. J. Enol. Vitic. 48: 438-442

Iland, P.G., Cynakar, W., Francis, I.L., Williams, P.J. and Coombe, B.G. 1996: Optimisation of methods for the determination of total and red-free glycosyl-glucose in black grape berries of Vitis vinifera. Austr. J. Grape Wine Res. 2: 171-178

Ivanova, V., Stefova, M., Vojnoski, B., Dörnyei, A., Márk, L., Dimovska, V., Stafilov, T. and Kilár, F. 2011: Identification of polyphenolic compounds in red and white grape varieties grown in R. Macedonia and changes of their content during ripening. Food Res. Int. 44: 2851-2860

Jackson, D.I. and Lombard, P.B. 1993: Environmental and management practices affecting grape composition and wine quality – a review. Am. J. Enol. Vitic. 44: 409-430

Keller, M. and Hrazdina, G. 1998: Interaction of nitrogen availability during bloom and light intensity during veraison. II. Effect on anthocyanin and phenolic development during grape ripening. Am. J. Enol. Vitic. 49: 341-349

Kennedy, J.A., Matthews, M.A. and Waterhouse, A.L. 2002: Effect of maturity and wine water status on grape skin and wine flavonoids. Am. J. Enol. Vitic. 53: 268-274

Kliewer, W.M. and Dokoozlian, N.K. (2001): Leaf area/crop weight ratios of grapevines: influence on fruit composition and wine quality. In: Rantz, J.M. (ed.): Proc. ASEV 50th Anniversary annual meeting 2000, Seattle, Washington, p. 285-295. – Davis, Ca: ASEV, 2001

Liang, Z., Sang, M., Fan, P., Wu, B., Wang, L., Yang, S. and Li, S. 2011: CIELAB coordinates in response to berry skin anthocyanins and their composition in Vitis. J. Food Sci. 76: 490-497

Maletić, E., Pejić, I., Karoglan Kontić, J., Piljac, J., Dangl, G.S., Vokura, A., Lacombe, T., Mirošević, N. and Meredith, C. 2004: 'Zinfandel', 'Dobričić' and 'Plavac mali': the genetic relationship among three cultivars of the Dalmatian coast of Croatia. Am. J. Enol. Vitic. 55: 174-180

Manfra, M., De Nisco, M., Bolognese, A., Nuzzo, V., Sofo, A., Scopa, A., Santi, L., Tenore, G.C. and Novellino, E. 2011: Anthocyanin composition and extractability in berry skin and wine of Vitis vinifera L. cv. 'Aglianico'. J. Sci. Food Agric. 91: 2749-2755

Matthews, M.A. and Kriedemann, P.E. (2006): Water deficit, yield, and berry size as factors for composition and sensory attributes of red wine. In: Oag, D. et al. (eds.): Proc. Austr. Society of Viticulture and Oenology 'Finishing the Job' - Optimal ripening of Cabernet Sauvignon and Shiraz, Mildura Arts Centre, Mildura, Victoria, 46-54. – Adelaide: ASOV, 2006

Mazza, G. and Miniati, E. (1993): Anthocyanins in fruits, vegetables, and grains. - Boca Raton (Fl.): CRC Press, 1993

Ortega-Regules, A., Romero-Cascales, I., Lopez-Roca, J.M., Ros-Garcia, J.M. and Gomez-Plaza, E. 2006: Anthocyanin fingerprint of grapes: environmental and genetic variations. J. Sci. Food Agric. 86: 1460-1467

Revilla, E., Carrasco, D., Carrasco, V., Benito, A. and Arroyo-García, R. 2012: On the absence of acylated anthocyanins in some wild grapevine accessions. Vitis 51: 161-165

Roggero, J.P., Coen, S. and Ragonnet, B. 1986: High performance liquid chromatography survey on changes in pigment content in ripening grapes of 'Syrah' an approach to anthocyanin metabolism. Am. J. Enol. Vitic. 37: 77-83

Rolle, L. and Guidoni, S. 2007: Color and anthocyanin

evaluation of red winegrapes by CIEL*, a*, b* parameters. J. Int. Sci. Vigne Vin 41: 193-201

Springob, K., Nakajima, J., Yamazaki, M. and Saito, K. 2003: Recent advances in the biosynthesis and accumulation of anthocyanins. Nat. Prod. Rep. 20: 288-303

Wu Dai, Z., Ollat, N., Gomès, E., Decroocq, S., Tandonnet, J.P., Bordenave, L., Pieri, P., Hilbert, G., Kappel, C., Van Leeuwen, C., Vivin, P. and Delrot, S. 2011: Ecophysiological, genetic, and molecular causes of variation in grape berry weight and composition: a review. Am. J. Enol. Vitic. 62: 413-425

Yamane, T., Jeong, T.S., Goto-Yamamoto, N., Koshita, Y. and Kobayashi, S. 2006: Effects of temperature on anthocyanin biosynthesis in grape berry skins. Am. J. Enol. Vitic. 57: 154-59

Yokotsuka, K., Nagao, A., Nakazawa, K. and Sato, M. 1999: Changes in anthocyanins in berry skins of 'Merlot' and 'Cabernet Sauvignon' grapes grown in two soils modified with limestone or oyster shell versus a native soil over two years. Am. J. Enol. Vitic. 50: 1-12

Zdunić, G., Maletić, E., Vokurka, A., Karoglan-Kontić, J., Pezo, I. and Pejić, I. 2007: Phenotypical, sanitary and ampelometric variability within the population of cv. 'Plavac mali' (*Vitis vinifera* L.). Agric. Conspec. Sci. 72: 117-128

Zdunić, G., Šimon, S., Malenica, N., Preiner, D., Maletić, E. and Pejić, I. 2012: Morphometric and molecular analysis of a pink berried mutant within the population of grape cultivar 'Plavac mali'. Vitis 51: 7-13

Will, F. and Dietrich, H. (2013): Processing and chemical composition of rhubarb (*Rheum rhabarbarum*) juice. Food Sci. Technol. 50: 673-678

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