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Influence of Foliar Fertilization with P and K on Chemical Constituents of Grape cv. 'Cardinal'

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ABSTRACT: The foliar fertilization has been used as an important agrotechnical measure to avoid deficiencies and to improve quality. During the two consecutive years, a study has been performed on *Vitis vinifera* L. (cv. 'Cardinal') to examine whether a grape berry quality has been affected by the foliar application of PK fertilizer. A liquid mineral fertilizer containing 15% P_2O_5, 20% K_2O with 0.1% B, 0.1% Mn and 0.01% Mo (% w/w) has been sprayed three times at rate of 8 L ha^{-1} every 14−15 days starting at about 15 days before veraison. The sugars, organic acids and flavonoids (anthocyanins, flavonols and flavan-3-ols) have been analyzed by the high performance liquid chromatography in the grape berries. The foliar fertilization of grapevine can accelerate the accumulation of sugars and anthocyanins, whereas climatic factors and yearly fluctuations influence the content of sugars, organic acids, and phenolic compounds in general. The effect of fertilizer spraying on flavonols and flavan-3-ols has not been found.

KEYWORDS: anthocyanins, flavan-3-ols, flavonols, foliar fertilizer, organic acids, sugars, table grape, *Vitis vinifera*

INTRODUCTION

The quality of table grapes depends on chemical constituents, i.e., the content of sugars, organic acids, and phenolic compounds. Phenolics include the nonflavonoids (hydroxybenzoic, hydroxycinnamic acids, and stilbenes) and flavonoids (anthocyanins, flavan-3-ols, and flavonols). Anthocyanins are responsible for the color of red grapes, flavonols are accumulated in the skin throughout berry development and act as "sunscreen" protecting the berry from harmful ultraviolet radiation, and flavan-3-ols are responsible for the astringent taste sensation of grapes. In recent years, phenolics have been of special interest, due to their antioxidant properties and potentially beneficial effects for human health.[1−3]

In order to improve grape quality, it is essential to know the factors that regulate biosynthesis and/or further turnover and catabolism of mentioned grape components during ripening. The availability of key macronutrients during the plant growth has a significant potential to affect their accumulation. Potassium stimulates photosynthetic activity and favors the translocation of sugars to the fruit. This indirectly benefits the synthesis of phenolic components during ripening, which is closely related to the presence of carbohydrates in the grape.[4,5] Phosphorus affects the synthesis of sugars and alcohol esters through the ATP activity.[6]

Fertilization of soil is one important agrotechnical measure with a great effect on vineyard yield and grape quality. Amiri and Fallahi[7] concluded that potassium alone or in combination with N or Mg increased the content of soluble solids in table grape cv. 'Bidaneh Qermez' and that the potassium application increased the yield. Delgado et al.[8] found that soil fertilization by potassium did not affect the soluble solids content, but because of strong N × K interaction, optimal nutritional N:K ratios might enhance the phenolic features of grape berries. Nowadays, the foliar fertilization of grapevine has been used to avoid deficiencies and to improve quality.[9]

In general, very little research has been undertaken on the effects of fertilization on phenolic composition of grape berries. Besides, there is little published information on identification and quantification of phenolics in table grape varieties, compared with wine grape varieties, which have been widely investigated.

The aim of this work was to evaluate both the influence of foliar application of PK fertilizer on chemical constituents of grape cv. 'Cardinal', as one of the leading table grapes, and their changes throughout ripening. PK foliar fertilizer was applied, considering the role of K and P on synthesis of carbohydrates, and indirectly on phenolic compounds. The effect of highly calcareous and skeletal soil was considered also where the concentration of P and K in most of the grapevine organs could be depressed.[10] Principal component analysis (PCA) and discriminant analysis (DA) as multivariate statistical analyses helpful in identification of the biochemical compounds responsible for the separation sample groups[11,12] were used to assess the effects of foliar spraying and growing season on grape quality (sugars, organic acids, and phenolics).

MATERIALS AND METHODS

Experimental Site, Design, and Treatment. The experimental trial was carried out in commercial vineyard (13 Jul - Plantaže a.d.) located about 10 km southeast of the town of Podgorica (latitude: 42° 27′ North, longitude: 19° 28′ East, elevation: 10−50 masl), Montenegro in the 2008 and 2009 growing seasons. *Vitis vinifera* L. cv. 'Cardinal' on SO4 rootstock (Selection Oppenheim Nr. 4) was planted in 1997 in 2.6 m row and 1.2 m vine spacing. The soil type is euthric brown on a fluvioglacial deposit consisting of carbonates.[13] In the fine soil fraction (<2 mm) of layer 0 − 30 cm, sand content is 45%, silt 27%, and clay 28%. This highly calcareous soil (22% $CaCO_3$) with a relatively high pH

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Table 1. Content of Nutrients in Leaf Blade of Grapevine (Mean ± Standard Error) before Fertilizer Was Applied[a]

nutrient	date of sampling	
	9 June 2008	9 June 2009
N (%)	2.99 ± 0.04a	2.66 ± 0.03b
S (%)	0.52 ± 0.05a	0.34 ± 0.02b
K (%)	1.20 ± 0.03a	0.77 ± 0.01b
P (%)	0.23 ± 0.00a	0.20 ± 0.00b
Mg (%)	0.24 ± 0.01a	0.26 ± 0.01b
Ca (%)	3.19 ± 0.11a	3.13 ± 0.08a
Fe (mg/kg)	94.46 ± 1.30a	72.36 ± 1.91b
Mn (mg/kg)	67.50 ± 1.16a	84.70 ± 3.25b
Zn (mg/kg)	11.76 ± 0.23a	15.46 ± 0.87b
Cu (mg/kg)	8.08 ± 0.37a	8.19 ± 0.29a
B (mg/kg)	49.29 ± 0.75a	49.13 ± 2.10a

[a] In each line, different letters indicate significant differences between means ($p < 0.05$).

(7.9) has an optimal level of organic matter (1.8% C) and plant-available nutrients in the upper layer (0−30 cm). The optimal level of nutrients in vineyard soil was being maintained through routine fertilizer applications. The grapevines were well-supplied with water. Water was being applied through the drip irrigation system at rate of 24 mm on every fifth day (regulated according to technical and weather conditions), meaning 4.8 mm of water per day for each vine.

The trial involved vines nonsprayed (marked as the Control) and vines sprayed with Hascon M 10 AD (marked as the Hascon) in four replications. Trial design was 4 replications with 5 vines within the same row.

Hascon M 10 AD (Green Has Italia S.p.A., Italy) is a liquid mineral fertilizer containing 15% P_2O_5, 20% K_2O with 0.1% B, 0.1% Mn and 0.01% Mo (% w/w). By manually spraying 2 L of 0.6% (v/v) solution (in level 8 L ha^{-1}) of this fertilizer were applied on five grapevines three times every 14−15 days starting on 10th June, i.e., about 15 days before veraison. The application of foliar fertilizer was completed on ninth July. The fertilizer was being sprayed early in the morning.

In order to check the nutritional status of the grapevines before the use of PK foliar fertilizer as well as possible changes after that, the leaf blade sampling was carried out. The results (Table 1) indicated an optimal level of grapevine nutrients[14] and also showed significant differences between growing seasons for all nutrients, with the exception of Ca, Cu, and B. Further, no effect of PK foliar fertilizer on the content of P and K of grapevine leaves was detected (the data not presented). A similar result for K in grapevine leaf was found by Knoll et al.[15] The grapevines have shown a rapid foliar absorption of nutrients and that the increase of nutrient concentrations due to fertilization is often not detected via foliar analysis because of the rapid movement to other organs.[16] Besides, Delgado et al.[8] found that the lower potassium supplementation of the soil (60 g K_2O per vine) did not significantly increase the nutrient levels in the leaf blades compared with the Control.

Sampling of Grape. The average sample of grape berries (about 2.5 kg) was taken from both sides of the Control vines which were not treated by foliar fertilizer (marked as C) as well as from vines sprayed with foliar fertilizer (marked as H), three times during the last month of ripening (in 2008 − date 1 (C1−08 and H1−08), 16th July; date 2 (C2−08 and H2−08), 30th July; date 3 (C3−08 and H3−08), 13th August; in 2009 − date 1 (C1−09 and H1−09), 17th July; date 2 (C2−09 and H2−09), 31st July; date 3 (C3−09 and H3−09), 14th August). The grape clusters chosen for the average sample had the total soluble solids (determined in 2−3 berries from cluster by hand refractometer) representative for five vines. During the period of investigation for both years, the total soluble solids increased from about 11−12 to 17−18 °Brix.

Berries were separated from clusters, frozen immediately and stored at −20 °C until the analyses of sugars, organic acids and flavonoids. Every sample was analyzed in four replicates.

Analysis of Individual Sugars and Organic Acids. The samples for HPLC analyses of sugars and organic acids were prepared according to the procedure described by Mikulič-Petkovšek et al.[17] Ten grams of homogenized grape berries were extracted with 50 mL of twice distilled water for 30 min at room temperatures. After extraction the homogenates were centrifuged for 7 min at 10 000 rpm at 5 °C. The supernatants were filtered through cellulose mixed esters filters 0.45 μm (Macherey Nagel, Düren, Germany), transferred to vials and stored at −20 °C until analyzed by HPLC.

The HPLC analysis of sugars was performed using a Thermo Finnigan Spectra HPLC System (Thermo Scientific, Waltham, MA, USA) with a refractive index (RI) detector. Separation of sugars was carried out using a Rezex RCM-monosaccharide column (300 × 7.8 mm; Phenomenex, Torrance, CA, USA) with the column temperature maintained at 65 °C and flow rate of 0.6 mL min^{-1}. The samples were eluted according to the isocratic method described by Mikulič-Petkovšek et al.[17] For the mobile phase, twice distilled water was used, and a refractive index detector for identification.

Organic acids were analyzed with HPLC, using Rezex ROA-organic acid column (300 × 7.8 mm; Phenomenex, Torrance, CA, USA) and a UV detector set at 210 nm, according to the method described by Mikulič-Petkovšek et al.[17] with a flow rate of 0.6 mL min^{-1} maintaining the column temperature at 65 °C. For the mobile phase, 4 mM sulfuric acid was used. The concentrations of carbohydrates and organic acids were calculated with the help of corresponding external standards.

Extraction and Determination of Individual Phenolic Compounds. The samples for HPLC analyses of phenolics were prepared according to Topalović and Mikulič-Petkovšek.[18] The sample of 1 g of skin was extracted with 10 mL of methanol containing 1% 2,6-di-*tert*-butyl-4-methylphenol (BHT) and 3% formic acid for 1 h in a cooled ultrasonic bath. After extraction, the samples were centrifuged for 7 min at 10 000 rpm at 0 °C. The supernatants were filtered through polyamide 0.45 μm (Macherey Nagel, Düren, Germany), transferred to vials and stored at −20 °C until analyzed by HPLC.

The HPLC analysis of flavonoids was performed using a Thermo Finnigan Surveyor HPLC system (San Jose, CA, USA) and a diode array detector (DAD). The flavan-3-ols were analyzed at 280 nm, flavonols at 350 nm and anthocyanins at 530 nm. The column used was a Gemini C18 (150 × 4.6 mm 3 μm; Phenomenex, Torrance, USA) protected with a Phenomenex security guard column operated at 25 °C. The elution solvents were 1% formic acid in twice distilled water (A) and 100% acetonitrile (B) and a flow rate was 1 mL min^{-1}. The samples were eluted in the linear gradient:[18] 0−5 min, 3−9% B; 5−15 min, 9−16% B, 15−45 min, 16−50% B; 45−50 min, 50%

isocratic; and finally, washing and reconditioning of the column. The injection volume was 20 μL of extract.

The identification of compounds was achieved by comparing retention times and their UV−vis spectra from 200 to 600 nm, as well as by the addition of an external standard. Mass Spectrometry analyses were performed using a LCQ Deca XP MAX (Thermo Scientific, Waltham, MA, USA) with an electrospray interface (ESI) operating in negative ion mode. The analyses were carried out using the full-scan data dependent MS^2 scanning from m/z 115 to 2000. The quantification was achieved according to the concentrations of a corresponding external standard.

The concentrations of phenolic compounds were calculated from the peak areas of the sample and the corresponding standards. For compounds lacking standards, quantification was carried out using similar compounds as standards. Procyanidin B2 was used for procyanidin dimers at retention times 11.8, 12.7, and 18.5 min (later marked as 1, 2, and 3) and procyanidin trimer; myricetin for myricetin hexosides at retention time 20.3 and 21.5 min (later as 1 and 2); quercetin 3-galactoside for quercetin glucuronide; kaempferol for kaempferol hexoside; delphinidin for delphinidin 3-glucoside; and peonidin for peonidin 3-glucoside and petunidin 3-glucoside.

Chemicals. The following standards were used for the quantification of sugars and organic acids: sucrose, glucose, and fructose; and tartaric, malic acids from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The following standards were used for the quantification of phenolic compounds: rutin (quercetin-3-*O*-rutinoside), myricetin, kaempferol, delphinidin, cyanidin-3-*O*-glucoside, peonidin and malvidin-3-*O*-glucoside from Sigma-Aldrich (Steinheim, Germany), (−)-epicatechin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, procyanidin B2 from Fluka Chemie (Buchs, Switzerland), and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for extraction of phenolics was acquired from Sigma-Aldrich (Steinheim, Germany). The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka (Buchs, Switzerland). Water for mobile phase was twice distilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA).

Statistical Analysis. The statistical elaboration of the data was performed using the SPSS 10.0 Program. The significant differences between the means were determined with the one-way ANOVA and Duncan's test at $p < 0.05$. Principal component analysis (PCA) with varimax rotation was used to examine the intrinsic variation in the data set. Discriminant analyses were performed on sums of sugars, organic acids, anthocyanins, flavonols, and flavan-3-ols in order to estimate differences in grape quality between certain dates and trials during two consecutive years.

RESULTS AND DISCUSSION

Climate. Water availability, light, and temperature have major roles in grape ripening dynamics. From the beginning of April to the end of August (in Podgorica) the amount of rainfall was 325.3 mm in 2008 and 330.5 mm in 2009, the number of the sunlight hours was 1406.7 and 1393.7 h, whereas the average temperatures were 23.5 and 23.8 °C, respectively. The mean daily temperatures during the trial period have been shown in Figure 1.

Changes of Content of Sugars and Organic Acids. During the last month of ripening, fructose and glucose content in grape berries increased, whereas that of sucrose decreased (Tables 2 and 3) at similar rate for both investigated years. The ratio between fructose and glucose ranged 1.04−1.16 in 2008 and 1.05−1.21 in 2009. In general, the influence of foliar spraying on the content of individual sugars was noticed in the middle of last month of ripening in 2008 and 2009, when the sum of sugars was significantly higher in berries of the Hascon compared with those of the Control.

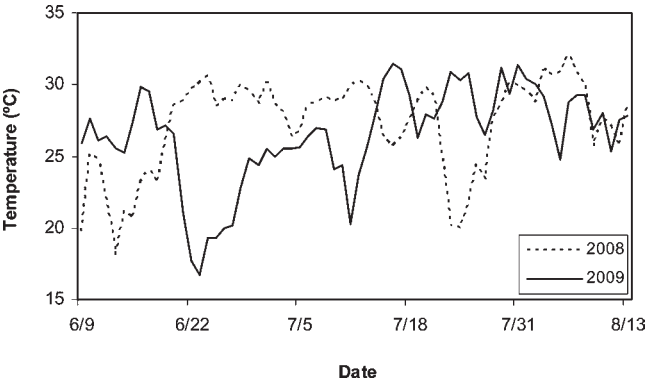

Figure 1. Mean daily temperature during the trial lasting.

Organic acids have important effects on the quality of table grapes, although their contents are low in comparison to sugars. The decrease of tartaric acid is mainly caused by changing from acid to salt forms and by a dilution effect as the berry weight increases, while the loss of malic acid occurs mainly through respiration.[19] Organic acids are sensitive to climate change.[11,20−23] The mentioned changes in tartaric and malic acid contents were detected in berries of 'Cardinal' during the last month of ripening (Tables 2 and 3). The ratio between tartaric and malic acid changed greatly in 2008 and was in the range of 7.4 to 22.1 in 2008, and of 2.1 to 2.6 in 2009. The very high value in 2008 was a consequence of a very low malic acid content, which could be explained by enhanced loss of malic acid in warm temperatures.[21] As seen in Figure 1, from 21st June to 13th July the mean temperature was 29.1 °C in 2008, and 23.1 °C in 2009.

Due to a lower tartaric acid content in berries of the Hascon trial compared with the Control, there was the difference in the sum of organic acids at the second sampling date in both years. It is probably caused by an excessive migration of K^+ cations to the berries, which produces potassium bitartrate from tartaric acid.[24] Delgado et al.[8] showed that potassium supplies had caused decreases in the total acidity of the 'Tempranillo' berries.

Consequently, the sugar/acid ratio increased during investigation. It ranged from 19.30 to 53.86 in 2008, whereas due to a higher content of organic acids it was 13.43−22.64 in 2009. Topalović and Mikulič-Petkovšek[18] found the sugar/acid ratio in the range of 26.09 to 62.56 during July 2007 for 'Cardinal' on Paulsen rootstock, when the ratio between °Brix and titratable acids ranged between 19.4 and 91.9.

PCA after varimax rotation (Table 4) has shown that two PCs (selected on eigenvalues 1 criterion) account for 93.6% of the variation in sugars and organic acids. PC1 (59.3% of the total variance) correlates with tartaric and malic acid as well as sucrose, but PC2 (34.3%) with monosaccharide fructose and glucose. The biplot of average scores on PCs (Figure 2) has been clearly divided by investigated years through PC1, on the basis of content of organic acids and sucrose which were higher in berries from 2009 than those from 2008. It is in agreement with investigations which have shown that acidity fell at higher rates at high temperature than at low temperature[22,23] and the grapes had lower concentration of tartaric and malic acid during warmer growing season.[11]

Table 2. Content (Mean ± Standard Error) of the Analyzed Compounds in Grape in mg/kg FW for Phenolics and g/kg FW for Sugars and Organic Acids in 2008[a]

	sampling date					
	C1−08	C2−08	C3−08	H1−08	H2−08	H3−08
sucrose	5.09 ± 0.17b	4.22 ± 0.30a	4.90 ± 0.13b	5.83 ± 0.14c	4.72 ± 0.11b	4.62 ± 0.05ab
glucose	44.45 ± 2.12a	45.58 ± 1.70a	76.20 ± 1.58c	44.88 ± 1.70a	62.63 ± 2.49b	76.92 ± 1.24c
fructose	46.22 ± 2.23a	52.82 ± 2.11b	85.99 ± 1.70d	48.15 ± 1.84ab	69.88 ± 3.01c	86.40 ± 1.42d
sum of sugars	**95.76 ± 4.34a**	**102.62 ± 3.83a**	**167.09 ± 3.26c**	**98.87 ± 3.58a**	**137.23 ± 5.51b**	**167.94 ± 2.61c**
tartaric acid	4.48 ± 0.21b	4.09 ± 0.15b	3.43 ± 0.21a	4.28 ± 0.16b	3.32 ± 0.30a	3.09 ± 0.17a
malic acid	0.53 ± 0.03c	0.27 ± 0.02b	0.17 ± 0.01a	0.58 ± 0.02c	0.22 ± 0.02b	0.14 ± 0.01a
sum of organic acids	**5.01 ± 0.23c**	**4.36 ± 0.15b**	**3.60 ± 0.22a**	**4.86 ± 0.17bc**	**3.54 ± 0.32a**	**3.23 ± 0.18a**
sugars/acids	**19.30 ± 1.12a**	**23.95 ± 1.00a**	**48.57 ± 4.00bc**	**21.09 ± 1.13a**	**43.20 ± 4.64b**	**53.86 ± 4.45c**
delphinidin 3-glucoside	14.72 ± 1.54a	50.17 ± 3.98b	55.37 ± 5.96bc	19.15 ± 2.19a	62.73 ± 3.43c	46.34 ± 3.61b
cyanidin 3-glucoside	21.62 ± 2.05a	75.14 ± 7.05b	75.08 ± 10.73b	28.78 ± 3.38a	100.63 ± 7.49c	67.00 ± 6.50b
petunidin 3-glucoside	29.99 ± 1.81a	79.89 ± 6.80b	82.60 ± 9.30b	34.10 ± 2.92a	104.62 ± 6.32c	77.89 ± 6.44b
peonidin 3-glucoside	182.12 ± 11.43a	684.66 ± 60.15b	802.38 ± 99.52b	250.09 ± 27.10a	872.33 ± 60.31b	822.92 ± 72.23b
malvidin 3-glucoside	125.23 ± 7.16a	394.93 ± 30.81b	457.77 ± 33.41b	144.59 ± 14.12a	449.13 ± 30.14b	463.31 ± 34.84b
sum of anthocyanins	**373.68 ± 20.98a**	**1284.79 ± 94.14b**	**1473.19 ± 153.00bc**	**476.71 ± 46.72a**	**1589.44 ± 97.53c**	**1477.46 ± 110.79bc**
myricetin hexoside 1	2.35 ± 0.14a	6.90 ± 0.54b	8.74 ± 1.14b	3.30 ± 0.29a	7.80 ± 0.54b	8.82 ± 0.90b
myricetin hexoside 2	3.29 ± 0.34a	8.11 ± 1.01b	14.32 ± 1.84c	2.77 ± 0.37a	9.06 ± 0.69b	12.50 ± 1.55c
rutin	9.90 ± 1.19a	17.59 ± 2.16b	12.42 ± 1.55a	8.83 ± 0.99a	11.60 ± 0.90a	12.78 ± 1.86a
quercetin 3-galactoside	4.92 ± 0.69a	18.15 ± 2.56b	34.95 ± 5.60c	3.85 ± 0.71a	23.65 ± 1.75b	34.32 ± 4.54c
quercetin 3-glucoside	162.50 ± 21.37a	617.62 ± 74.71b	1029.79 ± 147.61c	140.04 ± 22.59a	722.65 ± 47.87b	1033.69 ± 131.22c
quercetin glucuronide	518.30 ± 45.44a	841.07 ± 85.28b	677.42 ± 63.61ab	518.01 ± 70.31a	689.32 ± 51.11ab	626.36 ± 63.42a
kaempferol hexoside	93.81 ± 16.06a	323.91 ± 47.21b	577.09 ± 116.58c	74.29 ± 14.92a	392.11 ± 39.35bc	583.60 ± 87.33c
sum of flavonols	**795.06 ± 75.85a**	**1834.62 ± 215.51b**	**2354.73 ± 322.85b**	**707.20 ± 92.05a**	**1856.19 ± 130.26b**	**2312.06 ± 262.05b**
procyanidin dimer 1	110.90 ± 10.49b	76.27 ± 15.68a	89.70 ± 12.79ab	113.22 ± 10.24b	85.85 ± 6.10ab	68.86 ± 7.23a
procyanidin dimer 2	49.18 ± 2.95a	62.29 ± 3.84c	58.13 ± 4.66abc	50.56 ± 3.85ab	61.36 ± 2.95bc	55.07 ± 2.81abc
(+)-catechin	60.47 ± 7.90 cd	69.82 ± 9.13d	28.57 ± 4.06ab	55.56 ± 5.71 cd	44.36 ± 4.14bc	25.54 ± 1.78a
procyanidin trimer	38.38 ± 4.14a	154.72 ± 21.64b	162.62 ± 28.00b	55.21 ± 7.48a	221.15 ± 21.89c	147.66 ± 17.19b
(−)-epicatechin	351.91 ± 31.80a	510.84 ± 50.95b	438.55 ± 59.92ab	410.07 ± 45.34ab	526.59 ± 44.27b	411.96 ± 42.50ab
procyanidin dimer 3	12.73 ± 4.51	17.83 ± 4.52	7.90 ± 0.60	7.18 ± 2.82	18.04 ± 6.56	13.50 ± 2.90
sum of flavan-3-ols	**623.56 ± 57.20a**	**891.78 ± 83.77bc**	**785.47 ± 101.51abc**	**651.24 ± 55.63a**	**980.81 ± 72.93c**	**722.58 ± 66.42ab**

[a] Different letters in row indicate significantly different values at $p < 0.05$. C1−08, C2−08, C3−08, H1−08, H2−08, H3−08 are different sampling date for Control and Hascon trial, respectively (explained in Materials and Methods).

At second sampling date, PC1 and especially PC2 showed the difference between the berries of vines sprayed by foliar fertilizer and those nonsprayed.

Changes of Content of Flavonoids. Accumulation of phenolic compounds during grape ripening is subject to a considerable fluctuation, is closely related to physiological and biochemical changes that take place, and is the result of an equilibrium between biosynthesis and further turnover and catabolism.[25]

In grape skin, peonidin 3-glucoside had the highest concentration, followed by malvidin 3-glucoside, whereas delphinidin 3-glucoside was a minor analyzed anthocyanin (Tables 2 and 3) which is in agreement with the study by Carreno et al.[26] The contents of individual anthocyanins were similar to some values in red table grape varieties 'Red Globe', 'Flame Seedless', 'Crimson Seedless', except for petunidin 3-glucoside.[27] Namely, in comparison to the study of Cantos et al.[27] the content of petunidin 3-glucoside was more than 2-fold higher only in grape skin of 'Flame Seedless' compared with 'Cardinal', while other cultivars had considerably lower concentration of petunidin 3-glucoside (even 9−10 times in the grape skin of 'Crimson Seedless').

Comparison of individual anthocyanin contents (except of petunidin 3-glucoside in 2009) for the Control and the Hascon showed no significant difference at the beginning and the end of last month of ripening. However, by the second date, delphinidin 3-glucoside, cyanidin 3-glucoside, and petunidin 3-glucoside concentrations were significantly higher in grape skin of the Hascon trial than of the Control in 2008, as were concentrations of delphinidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside in 2009.

The sum of anthocyanins increased from the first to second sampling date, and thereafter was constant for the Control and the Hascon in 2008 and for the Hascon in 2009, but for the Control in 2009 it continually increased (Tables 2 and 3). In general, the Hascon trial had a significantly higher sum of anthocyanins in the middle of investigated period compared with the Control for both of years, indicating a positive effect of PK foliar fertilizer use on the accumulation of anthocyanins similar to that for sugars. He et al.[28] reported that there were two controversial opinions about the relationship between anthocyanins and sugars: (1) sugars in the skin played a role as regulators in the synthesis of anthocyanins; and (2) sugars were important

Table 3. Content (Mean ± Standard Error) of the Analyzed Compounds in Grape in mg/kg FW for Phenolics and g/kg FW for Sugars and Organic Acids in 2009[a]

	sampling date					
	C1−09	C2−09	C3−09	H1−09	H2−09	H3−09
sucrose	13.60 ± 0.23d	10.99 ± 0.22c	8.28 ± 0.12a	13.30 ± 0.29d	9.77 ± 0.20b	7.76 ± 0.13a
glucose	60.10 ± 3.74a	69.91 ± 2.32b	76.36 ± 2.92bc	62.40 ± 2.62a	77.42 ± 1.86bc	80.65 ± 1.75c
fructose	63.27 ± 3.73a	80.19 ± 2.71b	89.70 ± 3.40c	67.59 ± 2.68a	88.23 ± 2.20c	97.94 ± 1.92d
sum of sugars	**141.99 ± 5.97a**	**161.09 ± 5.13b**	**179.37 ± 4.14c**	**143.29 ± 5.49a**	**175.42 ± 4.22c**	**184.66 ± 3.56c**
tartaric acid	7.89 ± 0.26c	6.61 ± 0.33b	5.35 ± 0.24a	7.11 ± 0.32b	5.34 ± 0.16a	5.87 ± 0.16a
malic acid	3.03 ± 0.22ab	2.99 ± 0.25ab	2.44 ± 0.19a	3.20 ± 0.22b	2.58 ± 0.15a	2.55 ± 0.15a
sum of organic acids	**10.92 ± 0.44d**	**9.60 ± 0.54bc**	**7.93 ± 0.41a**	**10.32 ± 0.52 cd**	**7.92 ± 0.29a**	**8.41 ± 0.30ab**
sugars/acids	**13.43 ± 0.57a**	**17.93 ± 1.44b**	**22.64 ± 1.25c**	**14.49 ± 0.94a**	**22.55 ± 0.94c**	**22.43 ± 0.79c**
delphinidin 3-glucoside	36.17 ± 4.13a	43.38 ± 3.60ab	53.21 ± 3.65bc	38.10 ± 1.96a	56.62 ± 4.94c	52.99 ± 2.27bc
cyanidin 3-glucoside	64.91 ± 9.58a	68.63 ± 7.87ab	89.07 ± 8.11ab	86.60 ± 10.05ab	92.00 ± 5.57b	88.04 ± 6.08ab
petunidin 3-glucoside	80.78 ± 7.85a	77.26 ± 6.05a	80.56 ± 6.14a	123.59 ± 11.34b	100.08 ± 6.59a	90.69 ± 5.03a
peonidin 3-glucoside	471.23 ± 41.51a	758.51 ± 64.44b	1065.41 ± 94.14c	610.07 ± 59.80ab	1073.33 ± 74.35c	1166.94 ± 64.28c
malvidin 3-glucoside	158.05 ± 12.85a	386.21 ± 19.46b	460.77 ± 26.68c	184.54 ± 13.63a	508.82 ± 36.82c	520.64 ± 26.46c
sum of anthocyanins	**811.15 ± 63.25a**	**1333.99 ± 89.50b**	**1749.02 ± 121.08c**	**1029.50 ± 79.47a**	**1830.85 ± 120.45c**	**1919.30 ± 86.38c**
myricetin hexoside 1	4.11 ± 0.35a	7.05 ± 0.69b	10.39 ± 0.98 cd	5.98 ± 0.64ab	9.89 ± 0.72c	12.20 ± 0.71d
myricetin hexoside 2	7.17 ± 0.57a	14.33 ± 1.03b	19.85 ± 1.61c	7.46 ± 0.66a	17.37 ± 1.48bc	18.12 ± 1.17c
rutin	20.41 ± 1.52c	17.25 ± 1.92abc	14.69 ± 1.69ab	18.33 ± 1.16bc	13.23 ± 0.92a	13.17 ± 0.98a
quercetin 3-galactoside	9.83 ± 0.89a	14.92 ± 2.04ab	25.40 ± 3.48c	9.43 ± 0.77a	20.24 ± 3.19bc	19.97 ± 2.21bc
quercetin 3-glucoside	245.83 ± 26.03a	479.57 ± 63.67b	894.55 ± 115.29c	256.35 ± 25.73a	707.88 ± 103.08c	696.11 ± 75.26bc
quercetin glucuronide	860.96 ± 53.85c	747.14 ± 66.80abc	671.87 ± 46.42ab	814.62 ± 48.09bc	638.43 ± 40.03a	596.29 ± 38.76a
kaempferol hexoside	158.69 ± 25.78a	321.18 ± 59.68ab	618.01 ± 99.08c	186.83 ± 32.09ab	556.39 ± 138.39c	405.48 ± 54.35bc
sum of flavonols	**1306.99 ± 101.03a**	**1601.45 ± 185.49a**	**2254.77 ± 251.34b**	**1299.01 ± 90.28a**	**1838.75 ± 254.29ab**	**1761.34 ± 149.68ab**
procyanidin dimer 1	237.46 ± 17.80d	131.65 ± 21.97a	180.90 ± 10.60bc	199.10 ± 10.49 cd	155.91 ± 10.57ab	139.46 ± 4.98ab
procyanidin dimer 2	55.34 ± 1.90a	54.21 ± 2.75a	81.93 ± 5.13b	86.46 ± 6.29b	67.09 ± 6.02a	88.84 ± 2.60b
(+)-catechin	188.66 ± 15.02c	106.63 ± 10.79ab	148.98 ± 21.91bc	296.37 ± 25.17d	81.14 ± 4.85a	162.51 ± 12.97c
procyanidin trimer	39.02 ± 6.31a	79.89 ± 15.66bc	114.92 ± 18.01c	98.70 ± 13.45c	156.41 ± 11.44d	52.12 ± 2.87ab
(−)-epicatechin	1045.66 ± 61.54b	787.01 ± 78.68a	687.49 ± 51.29a	599.30 ± 113.08a	699.09 ± 41.20a	706.92 ± 47.43a
procyanidin dimer 3	5.50 ± 1.03a	13.01 ± 1.67bc	11.48 ± 1.52bc	8.27 ± 1.10ab	7.76 ± 2.30ab	14.80 ± 2.15c
sum of flavan-3-ols	**1489.54 ± 70.15b**	**1172.41 ± 120.72a**	**1225.69 ± 65.56a**	**1307.16 ± 107.79ab**	**1161.35 ± 60.75a**	**1152.25 ± 56.94a**

[a] Different letters in row indicate significantly different values at $p < 0.05$. C1−09, C2−09, C3−09, H1−09, H2−09, H3−09 are different sampling date for Control and Hascon trial, respectively (explained in Materials and Methods).

Table 4. Principal Component Analysis after Varimax Rotation for the Sugars and Organic Acids in 'Cardinal' Grape: Eigenvalues, Cummulative of the Total Variance, Factor Loading of the 2 Factors, and Communality Estimates of the 5 Parameters

	PC1	PC2	commun
eigenvalue	2.66	2.02	
cumul (%)	59.28	93.60	
tartaric acid	0.957	−0.014	0.916
malic acid	0.930	0.232	0.918
sucrose	0.922	0.124	0.865
fructose	0.096	0.991	0.991
glucose	0.127	0.986	0.989

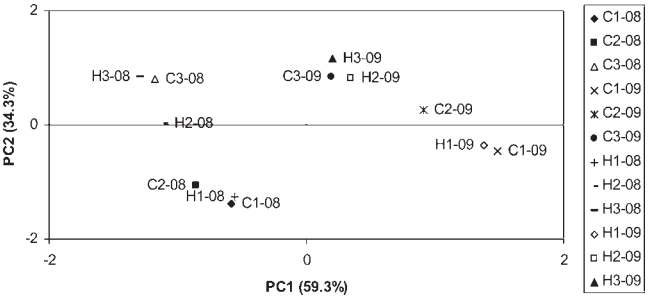

Figure 2. Rotated PC1 × PC2 scores scatter plot of sugars and organic acids in 'Cardinal' grape skin for 2008 and 2009.

only as substrates for anthocyanin formation. Champagnol[29] concluded that factors that affected the sugar content affected the berry color as well.

Among flavonols, quercetin 3-glucoside had the highest concentration at the end of ripening, followed by quercetin glucuronide which was the major flavonol at the beginning of sampling. The minor analyzed flavonol was myricetin hexoside 1 (Tables 2 and 3). In the previously mentioned red table varieties studied by Cantos et al.[27] the content of quercetin 3-glucuronide ranged from 5.0 to 34.2 mg/kg of fresh weight of grape berry (skin + pulp), and of sum of quercetin 3-glucoside and quercetin

Table 5. Principal Component Analysis after Varimax Rotation for the Phenolic Compounds in 'Cardinal' Grape Skin: Eigenvalues, Cummulative of the Total Variance, Factor Loading of the 3 Factors, and Communality Estimates of the 18 Parameters

	PC1	PC2	PC3	commun
eigenvalue	6.93	3.28	3.07	
cumul (%)	47.10	63.24	73.73	
peonidin 3-glucoside	0.930	0.243	0.024	0.925
myricetin hexoside 1	0.912	0.204	0.090	0.881
delphinidin 3-glucoside	0.900	0.113	0.190	0.859
cyanidin 3-glucoside	0.833	0.332	0.122	0.819
malvidin 3-glucoside	0.817	−0.103	0.231	0.731
petunidin 3-glucoside	0.755	0.446	0.067	0.773
myricetin hexoside 2	0.704	0.138	0.435	0.704
quercetin 3-glucoside	0.680	−0.199	0.653	0.929
procyanidin trimer	0.670	−0.140	0.147	0.490
procyanidin dimer 3	0.158	0.043	0.095	0.036
procyanidin dimer 1	−0.060	0.847	0.168	0.749
(+)-catechin	0.028	0.838	−0.055	0.706
(−)-epicatechin	0.304	0.719	0.135	0.627
procyanidin dimer 2	0.525	0.585	0.103	0.629
quercetin glucuronide	0.056	0.411	0.815	0.837
rutin	−0.010	0.503	0.777	0.856
kaempferol hexoside	0.565	−0.138	0.709	0.842
quercetin 3-galactoside	0.600	−0.214	0.688	0.879

3-rutinoside between 7.8 and 37.3 mg/kg, whereas kaempferol 3-galactoside and kaempferol 3-glucoside were found in traces. Pena-Neira et al.[30] determined a high concentration of quercetin 3-glucoside (675.34 mg/1000 g) in the skin of 'Syrah' berries at the harvest time. Thus, the grape skin of 'Cardinal' was very abundant with these compounds.

There was no difference in content of individual flavonols at the certain sampling dates between the Control and the Hascon, except in the content of myricetin hexoside 1, quercetin 3-glucoside, and kaempferol hexoside, which was higher at the second date in 2009 for the Hascon than for the Control. No difference in the sum of flavonols was detected between them. The trend of sum of flavonols was the same as for anthocyanins just for 2008, whereas in 2009 for the Control the sum of flavonols increased from second to third sampling date and for the Hascon it was not statistically different during the last month of ripening.

The content of (−)-epicatechin was the highest among the analyzed flavan-3-ols, and that of procyanidin dimer 3 was the lowest (Tables 2 and 3).

In general, in 2008 the sum of analyzed flavan-3-ols achieved the highest value at the second date, but in 2009 the highest value was detected at the first sampling date. From the aspect of sum of flavan-3-ols, there was no difference between the Control and the Hascon trial through sampling dates in the both of years. However, the differences are noticeable for (+)-catechin and procyanidin trimer at second sampling date in 2008; for procyanidin dimer 2, (+)-catechin and (−)-epicatechin at the beginning, for procyanidin dimer 1 at the third date as well as for procyanidin trimer during the whole last month of ripening in 2009.

PCA after varimax rotation (Table 5) showed that three PCs account for 73.7% of the variation in the flavonoid data. PC1 (47.1% of the total variance) has correlated with peonidin

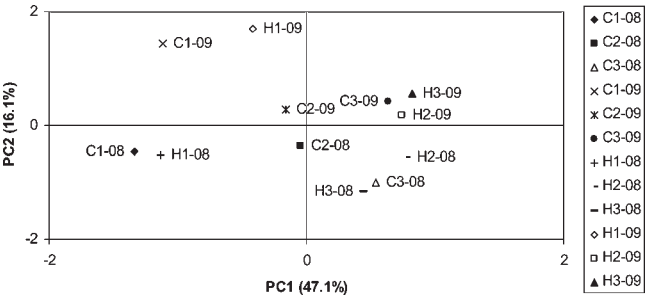

Figure 3. Rotated PC1 × PC2 scores scatter plot of all analyzed phenolic compounds in 'Cardinal' grape skin for 2008 and 2009.

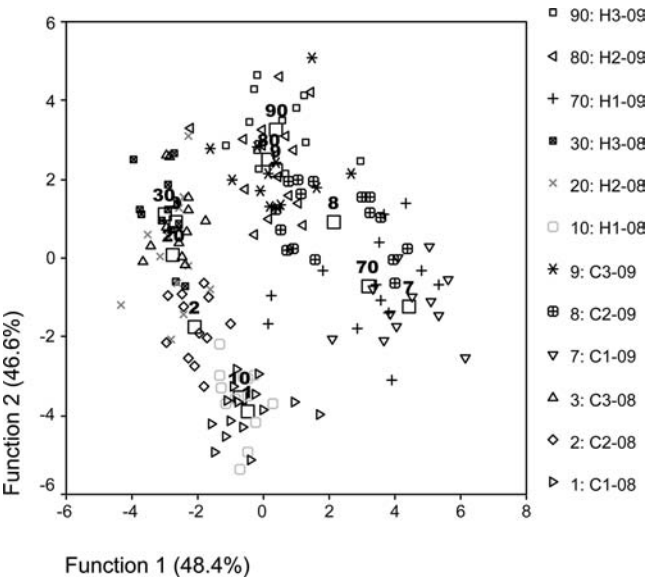

Figure 4. Application of stepwise discriminant analysis to sum of sugars, organic acids, anthocyanins, and flavan-3-ols (excluding of flavonols) at different times of grape ripening during 2008 and 2009.

3-glucoside, myricetin hexoside 1, delphinidin 3-glucoside, cyanidin 3-glucoside, malvidin 3-glucoside, petunidin 3-glucoside, myricetin hexoside 2, quercetin 3-glucoside and procyanidin trimer, but has had the high loadings of procyanidin dimer 2, kaempferol hexoside and quercetin 3-galactoside. PC2 (16.1%) has been described mostly by flavan-3-ols procyanidin dimer 1, (+)-catechin, (−)-epicatechin, procyanidin dimer 2 and has had the relatively high loadings of petunidin 3-glucoside, quercetin glucuronide and rutin. PC3 (10.5%) has correlated with the flavonols quercetin glucuronide, rutin, kaempferol hexoside and quercetin 3-galactoside and has had a high loading of quercetin 3-glucoside and a relatively high loading of myricetin hexoside 2.

In PCA scores plot (Figure 3) between skin flavonoids during two consecutive years, there has been a very good differentiation of berries of the growing season achieved by PC2. The upper section of the scores plot represents metabolites, mainly flavan-3-ols that were higher in 2009 than in 2008. Besides, the high content of flavan-3-ols at the first sampling date in 2009 (Table 3), differentiates grapes of C1−09 and H1−09 from the others in 2009 and from all in 2008 (Table 2).

However, the effect of foliar spraying can be observed through PC1. PC1 scores represent noticeable differences between the Control and the Hascon trial, at the second sampling date for

Table 6. Pairwise Group Comparisons (F and p statistics)

		sampling date										
		C1−08	C2−08	C3−08	C1−09	C2−09	C3−09	H1−08	H2−08	H3−08	H1−09	H2−09	H3−09
C1−08	F												
	p												
C2−08	F	15.493											
	p	0.000											
C3−08	F	46.981	19.636										
	p	0.000	0.000										
C1−09	F	52.759	65.522	81.595									
	p	0.000	0.000	0.000									
C2−09	F	57.045	43.186	41.053	18.472								
	p	0.000	0.000	0.000	0.000								
C3−09	F	62.859	32.843	18.932	42.488	8.251							
	p	0.000	0.000	0.000	0.000	0.000							
H1−08	F	0.324	10.821	35.826	48.101	46.955	50.728						
	p	0.861	0.000	0.000	0.000	0.000	0.000						
H2−08	F	37.447	6.834	7.288	79.606	44.241	22.617	27.833					
	p	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
H3−08	F	52.314	19.966	0.496	89.102	46.364	20.618	40.105	5.788				
	p	0.000	0.000	0.739	0.000	0.000	0.000	0.000	0.000				
H1−09	F	41.075	44.964	58.724	3.540	7.041	25.152	36.048	55.890	64.999			
	p	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000			
H2−09	F	73.602	36.475	20.081	52.217	11.898	0.234	58.918	24.250	21.500	32.144		
	p	0.000	0.000	0.000	0.000	0.000	0.919	0.000	0.000	0.000	0.000		
H3−09	F	88.429	47.771	25.548	57.175	14.781	1.792	72.093	33.162	27.143	37.576	1.185	
	p	0.000	0.000	0.000	0.000	0.000	0.134	0.000	0.000	0.000	0.000	0.320	

both of years, but a relatively similar result at the beginning for 2008 and at the end of investigation for 2008 and 2009. However, there is a relatively greater difference between H1−09 and C1−09, due to the significant differences in content of petunidin 3-glucoside, procyanidin dimer 2, and procyanidin trimer as previously mentioned for them, which have had the high loadings in PC1. It is unclear whether the use of foliar fertilizer had a more efficient impact on these phenolic compounds in 2009 than in 2008, considering the smaller difference between H1−08 and C1−08.

From the aspect of growing season, although the concentration of individual anthocyanins varied considerably at the beginning and was lower in 2008 than in 2009, by the end of last month of ripening it achieved a value which was not significantly different for delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside (the results of ANOVA not shown). The content of peonidin 3-glucoside was significantly lower at each sampling date in 2008 when compared with the 2009 results. Macheix et al.[31] stated that from chemotaxonomic point of view, the content of malvidin monoglucoside as well as ratio between malvidin 3-glucoside and peonidin 3-glucoside could be selected for cultivar characterization. Results from this study showed that among the ratios of analyzed anthocyanins, the ratio malvidin 3-glucoside: petunidin 3-glucoside had very similar values (5.5−5.9) at the third date regardless of the effect of growing season and foliar fertilization.

General Estimating Differences in Grape Quality. The higher values of the sum of sugars, anthocyanins and flavan-3-ols in 2009 than in 2008, and of flavonols at the beginning of last month of ripening, can be caused by more suitable climatic conditions. Huglin and Schneider[32] stated that at temperatures above 25 °C, net photosynthesis decreases even at constant sun exposure. He et al.[28] outlined that low temperatures, such as 25 °C, favored the anthocyanin biosynthesis. The daily temperature was constantly above 26.4 °C during 23 days after 21st June in 2008 (already mentioned in the case of organic acids). Taking into account that the sum of flavonols was not significantly different at the second and the third date for two consecutive years, the influence of some other factors overlapped the initial difference. It can be attributed to sunlight exposure.[12] The detailed examination of the effect of sunlight exposure on the contents of quercetin, myricetin, and kaempferol glycosides revealed that berries of cv. 'Merlot' from sun exposed clusters might contain as much as ten times the content found in samples obtained from shaded clusters.[33]

Discriminant function analysis is used for estimating overall differences in grape quality from the aspect of sum of sugars, organic acids, anthocyanins, flavonols, and flavan-3-ols between certain dates and trials during two consecutive years. Among the initial five variables, the sum of flavonols was excluded, because it had the least contribution to the discrimination between groups. The results have been shown on Figure 4. The first two canonical variables (or canonical discriminant function) account for 95.0% of the total dispersion; for the first canonical variable 48.4% and for the second 46.6%.

The F and p statistics describing which quality of grape berries is most alike or different has been given in Table 6. The grape quality of the control vines and the ones treated by foliar fertilizer is not different at the first and the third sampling date, but there is

a difference at the second date, i.e., about 21−22 days after foliar fertilizing with P and K. Initially, the value of significance for the pair C1−09 and H1−09 has been 0.009 indicating that the quality of grape berries does differ. However, considering the number of tested pairs (66 in total), in general no difference has been identified between the grape quality of nonsprayed and of sprayed grapevine at the first sampling date in 2009.

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