https://doi.org/10.17221/52/2025-HORTSCI

Effect of harvest time on total phenolic and flavonoid contents and antioxidant capacities of two grape varieties from the Trebinje vineyard area

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Citation: Delić M., Blesić M., Behmen F., Anđušić A., Topčić F., Murtić S. (2025): Effect of harvest time on total phenolic and flavonoid contents and antioxidant capacities of two grape varieties from the Trebinje vineyard area. Hort. Sci. (Prague), 52: 304–312.

Abstract: The aim of this study was to analyse the effects of harvest timing on the total phenolic and flavonoid contents, in addition to the total antioxidant capacity in the berries of 'Žilavka' and 'Vranac' varieties grown in the Trebinje vineyard area. Grape samples for both varieties were collected at three distinct points during their ripening. The chosen harvest dates were set 10 days apart, and the optimal date for grape harvesting was determined by assessing the sugar content and titratable acidity present in the grapes. Total phenolics, total flavonoids and total antioxidant activity of grape samples were determined by the Folin-Ciocalteu method, aluminium chloride method, and ferric reducing antioxidant power assay, respectively. The total phenolic and flavonoid contents, along with the total antioxidant capacity in the 'Vranac' grape, increased from the first to the second harvest date, but a decline was noted at the third harvest. 'Žilavka' grape exhibited an increase in total phenolic and flavonoid contents, as well as total antioxidant capacity from the first to the second harvest date, with no decrease recorded by the third harvest. As expected, the red grape variety 'Vranac' contained higher levels of phenolics and flavonoids than the light-skinned grape variety 'Žilavka'.

Keywords: berries; grape harvesting; phenolic compounds; ripening

Fruit ripeness is a key factor that affects the quality of grapes, thereby influencing the sensory properties of the resulting wine. Therefore, to ensure the highest quality of grapes for producing wine, it is vital to harvest them at the appropriate ripeness stage (Luo et al. 2024).

The optimal timing for grape harvest varies greatly depending on the grape variety, soil properties, climatic conditions, and the desired wine style (Zhou et al. 2019). It is widely recognised that grapes harvested early exhibit higher acidity and lower sugar content, resulting in wines that are usually crisp, fresh, and light-bodied. Conversely, grapes that remain on the vine for an extended period will accumulate more sugar, producing wines that are typically fuller-bodied with intense flavours and possess

https://doi.org/10.17221/52/2025-HORTSCI

a higher alcoholic volume. However, a prolonged delay in harvesting can result in overripe fruit, leading to wines that are excessively sweet or lacking in structure (Jahnke et al. 2023).

The phenolic and aromatic composition of grapes is also significantly influenced by harvest time, which, along with winemaking techniques, is crucial for producing high-quality wine (Allamy et al. 2023). Previous studies have shown that the quality of wine, particularly red wine, is significantly influenced by a crucial group of polyphenols known as flavonoids (Gutiérrez-Escobar et al. 2021; El Rayess et al. 2024). This group comprises various compounds, including flavones, flavonols, flavanones, flavanols, flavans, and anthocyanins, which are pivotal in determining the wine's aroma and colour. For instance, flavanols can lead to increased bitterness and astringency in wine, while anthocyanins are responsible for the red, purple, or blue colouration in grapes (Fernandes et al. 2017). Additionally, flavonoids are associated with numerous health benefits, including those found in grapes and wines, such as their antioxidant and cardioprotective effects (Nemzer et al. 2022). Consequently, viticulturists implement various agricultural techniques, such as adjusting harvesting times, to enhance the phenolic content and composition in grapes.

In light of the scarcity of information concerning the phenolic composition and antioxidant properties of grape varieties from the Trebinje winegrowing area, this study is designed to assess the effects of harvesting time on the total phenolic and total flavonoid content and total antioxidant activity in the grapes of the 'Žilavka' and 'Vranac' varieties, both of which are prominently cultivated in this region. The interval between the chosen harvest dates was set at 10 days, and the optimal harvest date for each grape variety was determined through a combination of sensory evaluation, visual inspections, and the measurement of total soluble solids and titratable acidity in the grapes.

MATERIAL AND METHODS

Plant material. 'Vranac' is an autochthonous grapevine variety of Montenegro and stands out as one of the key grape varieties used for producing red wines, not only in Montenegro but also in Herzegovina. 'Vranac' grape clusters are cylindrical-conical in shape and medium to large in size (Figure 1).

Figure 1. 'Vranac' and 'Žilavka' grape cluster

The weight of the grape cluster ranges from 150 g to 300 g and features a medium loose structure. The berries are medium to large in size, round or slightly oval in shape and dark blue in colour. The grape juice is colourless and has a neutral aroma. 'Vranac' grapes ripen in the third epoch (late ripening), but in the climatic conditions of Herzegovina and Montenegro, they ripen relatively early, in mid-September. The wines produced from these grapes are harmonious, offering a pleasant taste, velvety sweetness, and an intense dark red colour (Maraš et al. 2012; Šuković et al. 2020).

'Žilavka' is an autochthonous grapevine variety native to Herzegovina, where it is most prevalent. The grape clusters are medium to large in size and conical in shape. The weight of the grapes typically ranges from 150 to 200 g. The berries are medium-sized, either round or slightly oval, with a yellow-green and moderately thick skin (Figure 1). 'Žilavka' is classified as a late grape variety, indicating that its grapes reach maturity during the third epoch. The wines produced from 'Žilavka' grapes exhibit a striking green-yellow colour, a well-rounded and harmonious taste, along with aromatic notes of green apple and peach (Prusina, Herjavec 2008).

Experimental site. This study was conducted during 2024 at a commercial vineyard planted with 'Žilavka' and 'Vranac' vines. The vineyard was located in the Popovo polje valley near Trebinje in the Herzegovina region (42.96°N, 17.79°E). The Popovo polje valley is a longitudinal karst basin situated between Hutovo Blato and Trebinje, covering an area of 45.9 km^2 at an elevation of 250 meters above sea level (Juvanec 2016). It is surrounded by higher hills and lies not far from the Adriatic Sea (Figure 2).

The climate of this region is transitional between Mediterranean and continental (i.e. sub-Mediter-

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Figure 2. Study area: Popovo polje valley

ranean) with cool, rainy winters and hot summers. According to the Köppen and Geiger classification system, it is categorised as Csa, indicating a hot-summer Mediterranean climate (Kottek et al. 2006). The average annual temperature in the studied area in 2024 was 16.3 °C, and the average precipitation was 1 438.2 mm (Table 1). The Popovo polje valley receives the majority of its rainfall in winter, with minimal rain during the summer season (RHMS 2024).

The grapevines of both 'Žilavka' and 'Vranac' were planted in 2010, grafted onto 'Kober 5BB' rootstock (*Vitis berlandieri* × *Vitis riparia*), and trained to a Single Guyot training system. The vine had a between-row and within-row spacing of 2 m × 0.80 m. Winter pruning, for both varieties, was carried out, leaving 10 buds per vine (one long cane with 8 buds, which is horizontally tied to the wire and one spur with two buds on the other side of the vine). Throughout this trial, all vineyard management practices, including fertilisation, irrigation, weed control, pest management, and disease control, were implemented uniformly.

Experimental design. A trial site of three rows was selected for both varieties. Each row was divided into three blocks. One block contained 25 vines. At the same harvest time, one block per row was harvested for both varieties, resulting in three replicates. Grape samples were collected at three chosen time points during the ripening process (Table 2), comprising 10 clusters from 5 randomly selected vines of the 'Žilavka' and 'Vranac' varieties.

The interval between the chosen harvest dates was set at 10 days, and the optimal harvest date for each grape variety was identified by measuring the total soluble solids and titratable acidity in the grapes. In Herzegovina, the 'Vranac' and 'Žilavka' grapes are harvested when the total soluble solids (TSS) in the grape juice range between 22 and 23 °Brix, with titratable acidity (TA) values ranging from 0.5 to 0.6 g (tartaric acid)/100 mL. In this study, the first harvest date was established when the soluble sugar content in the berry juice was just below 22 °Brix, while the second and third harvest dates occurred approximately 10 days apart. The second harvest date for

Table 1. Meteorological monthly data of the study area

Year	Months												
	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	
Average air temperature (°C)													Annual average
2024	7.6	10.5	11.4	15.0	17.9	24.0	27.0	27.2	19.6	16.9	10.4	7.8	16.3
1961–2024	5.6	6.6	9.3	12.9	17.4	21.6	24.3	24.2	19.7	15.3	11.0	7.1	14.6
Total rainfall (mm)													Total annual rainfall
2024	101.5	164.3	156.8	55.6	91.7	100.0	68.2	44.8	306.9	79.3	115.3	153.8	1 438.2
1961–2024	186.5	163.7	149.6	132.6	92.1	77.3	49.7	77.5	136.4	175.2	234.7	225.5	1 700.8
Relative humidity (%)													Annual average
2024	67	69	71	72	70	62	53	50	68	74	66	72	66
1961–2024	70	67	68	67	67	63	53	55	63	70	72	72	66

https://doi.org/10.17221/52/2025-HORTSCI

Table 2. Harvest dates during the ripening process of 'Žilavka' and 'Vranac' grapes in 2024

Grape variety	Harvest		
	First	Second	Third
'Žilavka'	Aug 28	Sept 5	Sept 15
'Vranac'	Aug 25	Sept 3	Sept 12

both varieties can be regarded as optimal because, at that time, the sugar content in the grape juice and the TA were within the recommended ranges. The variations of the key chemical harvest maturity indices (TSS and TA) during ripening are shown for each variety separately (Tables 3 and 4).

As presented in Table 2, the 'Žilavka' grape harvest began on August 28, 2024, representing the early harvest. This was followed by a second harvest on September 5, 2024, which is considered the optimal harvest, and the final harvest took place on September 15, 2024, classified as the late harvest. The 'Vranac' grape harvesting started earlier, on August 25, with a second harvest occurring on September 3, and a third harvest on September 12, 2024.

Total soluble solids (TSS) and titratable acidity (TA) estimation. TSS and TA were analysed to assess the technological maturity of the grapes. TSS, expressed in °Brix, were determined using a digital refractometer (PAL-1, ATAGO, Tokyo, Japan) (ISO 2003). TA, expressed in g (tartaric acid)/100 mL of grape juice, was assessed via titration, utilising a 0.1 mol/L NaOH solution as the titrant and phenolphthalein as the indicator (AOAC 2000).

Extraction of phenolics from grape berries. Phenolic compounds were extracted from grape berries using a 50% aqueous ethanol solution. The extraction process began with fresh grape berries being dried in an oven (Memmert UM200, Oven lab, Germany) at 50 °C until they achieved a constant mass, followed by grinding and sieving through a 2-mm sieve. The dried grape sample, weighing 1 g, was added to a 100 mL Erlenmeyer flask and subsequently mixed with 40 mL of a 50% aqueous ethanol solution. Following this, the flask was heated in a water bath maintained at 35–37 °C for 1 hour. Afterwards, it was cooled to room temperature. The mixture was then filtered through filter paper into a 50 mL flask and adjusted to the mark with a 50% aqueous ethanol solution. The extract obtained from grape berries was used to assess the total phenolic (TP) content, total flavonoid (TF) content and total antioxidant capacity (TAC).

Total phenolic (TP) content estimation. The TP content in the grape extract was assessed using the Folin-Ciocalteu assay (Ough, Amerine 1988) with slight modifications. Briefly, 0.25 mL of the extract was added to a 25 mL flask containing 15 mL of distilled water and 1.25 mL of Folin-Ciocalteu reagent, which was diluted with distilled water at a 1 : 2 ratio. Following a 5-minute wait, 3.75 mL of a saturated sodium carbonate solution (21 g Na_2CO_3/100 mL of H_2O) was added. The flask was filled to the mark with a 50% aqueous ethanol solution and heated in a water bath set at 50 °C for 30 minutes. Following the heating process, the flask was cooled to room temperature, and the absorbance was recorded at 765 nm using a UV spectrophotometer (Ultrospec 2100 pro, Amersham, USA). The TP content was assessed based on a standard curve of gallic acid ranging from 20 to 500 mg/L, and the results were presented as mg of gallic acid equivalent per 100 g of fresh grape mass (mg GAE/100 g FM).

Total flavonoid (TF) content estimation. The TF content in the grape extract was assessed using the aluminium chloride colourimetric assay (Zhishen

Table 3. Chemical harvest maturity indices for 'Vranac' grapes

Parameters	5-day interval following veraison (Aug 13, 2024)						
	1	2	3	4	5	6	7
Total soluble solids (°Brix)	16.79	18.12	20.75	22.33	22.47	23.11	23.25
Titratable acidity (g/100 mL)	0.99	0.83	0.74	0.56	0.55	0.51	0.49

Table 4. Chemical harvest maturity indices for 'Žilavka' grapes

Parameters	5-day interval following veraison (Aug 15, 2024)						
	1	2	3	4	5	6	7
Total soluble solids (°Brix)	16.23	17.41	20.33	22.14	22.29	22.73	23.26
Titratable acidity (g/100 mL)	1.05	0.86	0.79	0.59	0.59	0.55	0.53

https://doi.org/10.17221/52/2025-HORTSCI

et al. 1999). Briefly, 1 mL of the extract was added to a 10 mL flask containing 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$. Thereafter, 0.3 mL of 10%$AlCl_3$ was added. Following a 6-minute incubation at room temperature, 2 mL of 1 mol/L NaOH was added to the flask. The flask was subsequently filled to the mark with distilled water. After 15 min, the absorbance was recorded at 510 nm using a UV spectrophotometer (Ultrospec 2100 pro, Amersham, USA). The TF content was assessed based on a standard curve of catechin ranging from 20 to 100 mg/L, and the results were presented as mg of catechin equivalent per 100 g of fresh grape mass (mg C/100 g FM).

Total antioxidant capacity (TAC) estimation. The TAC of the grape extract was assessed using the ferric reducing antioxidant power (FRAP) assay (Benzie, Strain 1996). The FRAP assay is based on the ability of antioxidants present in grape extract to reduce Fe^{3+} to Fe^{2+}. Briefly, 80 µL of the extract was added to a 10 mL flask containing 240 µL of distilled water. Thereafter, 2 080 µL of FRAP reagent (reagent was obtained by mixing 0.3 mol/L acetate buffer (pH = 3.6), 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mmol/L $FeCl_3 \times 6H_2O$ in a ratio of 10 : 1 : 1) was added to the flask and then heated in a water bath set at 37 °C for 5 minutes. Following the heating process, the flask was cooled to room temperature, and the absorbance was recorded at 595 nm using a UV spectrophotometer (Ultrospec 2100 pro, Amersham, USA). The TAC was assessed based on a standard curve of $FeSO_4 \times 7H_2O$ ranging from 0 to 2 mmol/L, and the results were presented as mmol Fe^{2+} per 100 g of fresh grape mass (mmol Fe^{2+}/100 g FM).

Statistical analysis. All the chemical measurements were conducted in triplicate, and the results were expressed as the mean ± standard deviation. The experimental data were subjected to analysis of variance (ANOVA) using the Microsoft Excel 2013 statistical software. Pearson's correlation coefficient analysis was carried out to examine the relationships between antioxidant substances (total phenolics and total flavonoids) and total antioxidant activity (FRAP).

RESULTS AND DISCUSSION

Table 5 illustrates the TP and TF contents, as well as the TAC of 'Žilavka' grapes based on the timing of their harvest.

The results showed that the timing of grape harvest significantly affects the TP contents of 'Žilavka' grapes. The TP content was found to be between 28.42 mg/100 g of fresh grapes at the first (initial) harvest and 49.06 mg/100 g at the third (late) harvest. An increase in TP contents in grape berries with ripening was also reported by Kurt-Celebi et al. (2020). The flavonoid content in 'Žilavka' grapes also exhibited an increasing trend during the ripening process; however, the differences in TF levels among the examined variants were not statistically significant.

Several studies have highlighted variations in phenolic content in grape berries during ripening, with a particular focus on the differences present in various parts of the berries (Awad et al. 2017; Roufas et al. 2023; Kamah et al. 2025). According to Garcia-Cabezon et al. (2020), the phenolic levels in grape skins generally increase with maturity, while those in the seeds decrease. In another study, Mbele et al. (2004) noted that the phenolic levels in grape skins declined from 33 to 44 days after flowering, but they remained largely unchanged for the rest of the berry maturation period. It is evident from these results that the variations in phenolic content and composition in grape berries and their parts (skin, pulp, and seeds) throughout the ripening process are not uniform and are greatly influenced by both the grape variety and the harvest time.

Similar to the TP contents, the TAC in 'Žilavka' grapes demonstrated an upward trend during the ripening process. 'Žilavka' grapes picked dur-

Table 5. Effect of harvest time on total phenolic and flavonoid contents and antioxidant capacities in 'Žilavka' grapes

Harvest time	Total phenolic content (mg/100 g)	Total flavonoid content (mg/100 g)	Total antioxidant capacity – FRAP (mmol Fe^{2+}/100 g)
First (Aug 28)	28.42 ± 5.49[b]	11.52 ± 3.52	0.42 ± 0.10[b]
Second (Sept 5)	39.51 ± 6.19[a]	15.74 ± 4.11	0.57 ± 0.09[a]
Third (Sept 15)	49.06 ± 17.03[a]	16.96 ± 5.45	0.68 ± 0.21[a]
$LSD_{0.05}$	10.66	–	0.14

FRAP – the ferric reducing antioxidant power; LSD – the least significant difference

[a,b]averages denoted by the same letter indicate no significant difference ($P \leq 0.05$)

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ing the initial harvest had considerably lower TAC values than those collected later, suggesting that an increase in TAC values is associated with a rise in TP content. These results are in agreement with the findings of Derradji-Benmeziane et al. (2014), who reported a strong and positive relationship between the levels of TP compounds and antioxidant power across five grape varieties.

Findings from this study indicated a distinct trend in the accumulation of phenolic compounds in 'Vranac' grapes throughout the ripening process, which differs from the patterns found in 'Žilavka' grapes (Table 6).

The highest contents of TP and TF in 'Vranac' grapes were found at the second harvest, which is regarded as the optimal harvest time from the technological grape maturity point of view. 'Vranac' grapes gathered during both the first and third harvest dates showed significantly lower levels of TP and TF. These results indicate that phenolic compounds tend to increase during the development of the berries until they reach their peak content, after which their levels begin to decline. The findings are in agreement with those reported by Delgado et al. (2004), who identified three distinct stages in the accumulation of phenolic compounds in grape berries throughout the ripening process: an initial slow increase, a sharp rise during the fifth and sixth weeks after veraison, and a subsequent decline leading up to harvest. According to Rouxinol et al. (2023), the concentrations of specific phenolic compounds, especially anthocyanins and tannins, in grape berries change during their ripening phase, which has a considerable impact on the TP and TF content in the grape berries. In particular, anthocyanins appear at veraison and steadily increase as the grapes ripen, achieving their peak concentration close to technological maturity, then declining during the over-ripening phase. Tannins in the grape skins show a similar pattern, although they are already present in considerable quantities at the time of veraison. The decline in TP and TF content in 'Vranac' grapes harvested 10 days after the recommended harvest date could be connected to these observations; however, this is only a hypothesis, as the anthocyanin and tannin content was not assessed.

The decrease in TP and TF levels in 'Vranac' grapes during ripening could potentially be explained by the activity of polyphenol oxidase (PPO). This enzyme facilitates the oxidation of polyphenolic compounds into quinones, which in turn decreases the level of phenolic compounds (Taranto et al. 2017). This observation aligns with the findings of Zhang (2023), which also demonstrate a negative correlation between PPO activity and the levels of phenolics and flavonoids present in plants. On the other hand, various studies have indicated that there is no clear relationship between PPO activity and the TP content in grapes (Valero et al. 1989; Wang et al. 2013). Additional studies will need to be conducted to explore this topic.

The findings of this study also indicated that the TP and TF content were notably higher in 'Vranac' grapes compared to 'Žilavka' grapes. These results were predictable as it is a widely recognised fact that red grape varieties are richer in phenolic compounds than white varieties. The key factor behind this difference is the higher anthocyanin content in black/red grape varieties, which are flavonoids that give the grapes their distinctive colour (Chen et al. 2024).

The study's findings also showed that the TAC of 'Vranac' grapes increased from the first to the second harvest, followed by a subsequent decline. This pattern suggests that fluctuations in TAC values in 'Vranac' grapes correspond to variations in TP content.

This study identified a significant correlation between the antioxidant capacity of grape extracts and their TP and TF levels in both the 'Vranac' and 'Žilavka' varieties (Figure 3), highlighting the impor-

Table 6. Effect of harvest time on total phenolic and flavonoid contents and antioxidant capacities in 'Vranac' grapes

Harvest time	Total phenolic content (mg/100 g)	Total flavonoid content (mg/100 g)	Total antioxidant capacity – FRAP (mmol Fe^{2+}/100 g)
First (Aug 25)	102.45 ± 22.63^{b}	51.01 ± 14.23^{b}	1.15 ± 0.38^{b}
Second (Sept 3)	135.52 ± 9.57^{a}	61.12 ± 4.81^{a}	1.62 ± 0.21^{a}
Third (Sept 12)	102.16 ± 7.94^{b}	47.12 ± 3.09^{b}	1.42 ± 0.35^{ab}
$LSD_{0.05}$	15.44	8.93	0.33

FRAP – the ferric reducing antioxidant power; LSD – the least significant difference

^{a,b}averages denoted by the same letter indicate no significant difference ($P \leq 0.05$)

https://doi.org/10.17221/52/2025-HORTSCI

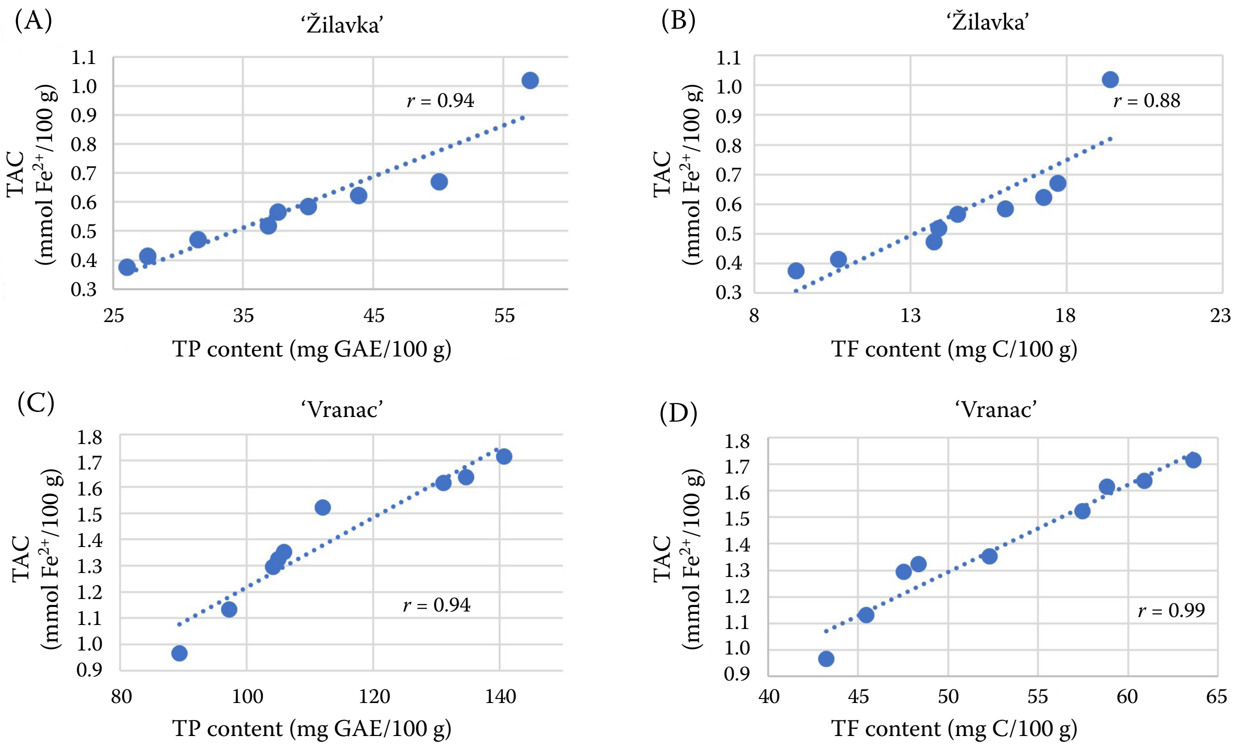

Figure 3. Correlation between total phenolic (TP)/total flavonoid (TF) content and total antioxidant capacity (TAC) in 'Žilavka' and 'Vranac' grape varieties

GAE – gallic acid equivalent; C – catechin equivalent; *r* – simple correlation coefficient

tant role of phenolic compounds in the antioxidant properties of these grape varieties. A strong and positive correlation between the content of phenolic compounds and the antioxidant capacity in grapes has been consistently confirmed by numerous scientific studies (Cosme et al. 2018; Neira-Ospina et al. 2024).

CONCLUSION

The 'Vranac' grape variety demonstrated an increase in TP and TF content, along with TAC, from the first to the second harvest date; however, a reduction was recorded at the third harvest. On the other hand, the 'Žilavka' variety showed a rise in TP and TF content, as well as TAC from the first to the second harvest, with no decrease noted by the third harvest. As expected, the levels of phenolics and flavonoids in the red grape variety 'Vranac' were significantly higher than those found in the light-skinned grape variety 'Žilavka'. This study found a strong and positive correlation between TAC and the levels of TP and TF present in 'Vranac' and 'Žilavka' grapes, highlighting the important role that phenolic compounds have in the antioxidant properties of these grape varieties.

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Received: April 9, 2025
Accepted: May 16, 2025
Published online: October 23, 2025