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Impact of Fruit Zone Leaf Removal on Anthocyanin Stability in Wine During Bottle Ageing

Running head: Stability of Anthocyanins During Wine Aging

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SUMMARY

Research background. Anthocyanins, the most abundant pigments in red wines, play an important role in the visual aspect of wine sensory properties. However, due to their unstable nature, their ability to polymerize with tannins is important for colour stability. Their content varies with grapevine variety, growing conditions, viticultural and winemaking practices. Leaf removal, a common viticultural practice, enhances anthocyanin accumulation in red grapevines, and partial fruit zone leaf removal at different phenological stages can significantly influence the anthocyanin content of grapes and wine. This two-year study examined how two different timings of fruit zone leaf removal at different

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phenological stages on the initial anthocyanins content in wine and their stability during aging in Merlot, Syrah and Cabernet Sauvignon wines grown in a Mediterranean climate.

Experimental approach. Partial leaf removal was performed during flowering (LRF) and during vérasion (LRV) and compared with an untreated control. Wines obtained from all treatments and varieties were bottled two months after the end of fermentation, and then stored and matured in cellar conditions for one year. To determine the influence of different times of leaf removal on the concentration of anthocyanins and their stability in the wine, wines were analyzed immediately after bottling, followed by 6 and 12 months of storage. For the determination of all phenolic compounds, high-performance liquid chromatography (HPLC) was used.

Results and conclusions. Leaf removal treatment increased the concentration of anthocyanins in all three cultivars. The obtained results showed that malvidin-3-*O*-glucoside (Mal-3-Glc) was the most abundant individual anthocyanin, while the most unstable anthocyanin was petunidin-3*O*-coumaroyl glucoside (Pet-3-Coum-Glc). Initial concentration of total anthocyanins in all wines were significantly affected by different conditions in two years of study, but with a significant impact of the defoliation treatments. Anthocyanin concentration decreased during the aging of the wine, and the degradation of anthocyanins ranged from 36 to 90%. The stability of anthocyanins in wine was most influenced by aging time, while year and treatment had no influence. The concentration of total phenolic acids increased during wine aging while concentration of total flavonol glycosides (TFG) decreased in all wines except Merlot from 2016.

Novelty and scientific contribution. The results of this study contribute to a better understanding of the stability of increased levels of anthocyanins obtained by grapevine leaf removal practice in the vineyard, in wines during aging.

Keywords: red wine; anthocyanin stability; phenolic compounds; wine ageing; Mediterranean climate

INTRODUCTION

Anthocyanins are water-soluble pigments, present in the vacuoles of the skin cells responsible for the red colour of the grape skin and are responsible for the intense colour of red wines (*1*). Besides being colour pigments, anthocyanins have other roles, such as protecting plants from excessive sun and UV radiation, collecting free radicals, increasing antioxidant capacity and protecting against numerous pathogenic organisms (*2*).

Anthocyanin biosynthesis is one of the most important biochemical processes during the growth and development of red grapevine cultivars. The accumulation of anthocyanins in the berry skin is influenced by agroecological factors, the most important of which are grapevine variety, climate, soil conditions, canopy management irrigation, and yield (*3*).

The accumulation of anthocyanins in grapes begins at vérasion and is characterized by a rapid increase in concentration in the first stage, followed by slower accumulation or even a drop in concentration by the end of the ripening period (*4,5*).

Leaf removal in the cluster zone, as a common viticultural practice, has a significant role in the synthesis of polyphenols in grapes. Due to excessive insolation and UV radiation, the plant synthesizes anthocyanins as a defence mechanism (*2*). Light positively affects the accumulation of anthocyanins in the berry (*6,7*). Excessive lighting can, indirectly by heating the berries, lead to their reduction (*8,9*) because temperatures above 30 °C led to inhibition of anthocyanin synthesis (*10*). This phenomenon is significantly dependent on the variety, so in certain varieties, partial defoliation positively affects the synthesis of polyphenols (*11,12*) without the negative influence of elevated temperature (*8,13*).

Regarding the time of leaf removal, the impact on specific grape qualitative (sugar level, titratable acidity, phenolic compounds, etc.) and quantitative (yield)parameters, and therefore on the wine, is different. The implementation of early leaf removal, before or during flowering, had the effect of increasing the concentration of total anthocyanins as was shown on different grapevine varieties such as Tempranillo (*12*), Carignan (*14*) Barbera and Lambrusco (*15*). According to Di Profio *et al.* (*16*) partial leaf removal by removing basal leaves on Merlot, Cabernet Sauvignon and Cabernet franc increases the concentration of total anthocyanins and colour intensity on all three cultivars. By performing leaf removal after vérasion, Palliotti *et al.* (*17*) determined that the anthocyanin content was not significantly different from that of the control vines without leaf removal. Late leaf removal, during vérasion, reduces anthocyanin content and increases the negative impact of sunburn, while leaf removal performed before flowering increases sugar and anthocyanin content (*11*).

The main drawback of anthocyanins is their extremely low stability, which is easily influenced by external factors, such as light and temperature (*18*). Thus, it is extremely important for the red wine colour stability that anthocyanins are found in more stable (glycoside) forms. Anthocyanins are initially found in grapes in monomeric forms. As they are highly reactive in nature, their forms change in various reactions and interactions during winemaking and wine aging (*1*). The stability of anthocyanins can be achieved in several ways, by copigmentation or polymerization with flavan-3-ols and

procyanidins, creating new pigments and polymeric anthocyanins that significantly affect the stability of wine colour (*19-21*). The stability of anthocyanins can be achieved by sugar acylation (*2*) because the rest of the sugar can be acylated with aromatic or aliphatic acids at the C-6 position. Although the initial concentration of anthocyanins in young wines are high immediately after fermentation, due to their instability, the concentration of these acylated anthocyanins drops just after fermentation, and they disappear after a few months (*22*). The concentration of anthocyanins in young wines after fermentation can vary from 100 mg/L to 1500 mg/L, depending on the cultivar (*22*).

The aim of this study was to investigate the influence of different timing of fruit zone leaf removal on the content and stability of anthocyanins in wines of Merlot, Syrah and Cabernet Sauvignon during aging in bottles.

MATERIALS AND METHODS

Vineyard site, plant material and weather conditions

The research was conducted in 2015 and 2016 on cultivars Merlot, Syrah, and Cabernet Sauvignon. Vineyard is located 20 km north of Zadar (Baštica, Suhovare) in Dalmatia region, subregion Dalmatian hinterland (latitude 44°06´N; longitude 15°13´E) and is a part of the University of Zadar. All three grapevine cultivars were grafted on Kober 5BB (*Vitis berlandieri* Planch. x *Vitis riparia* Michx.) rootstock planted in 2007 on anthropogenic soil called rigosol with a sandy-clay texture. Vines were planted with a spacing of 90 cm within the row and 280 cm between rows (plant density of 4100 vines/ha). All three grapevine cultivars are trained to vertical shoot-positioned, with single-cane-pruned Guyot, leaving about 12 to 14 buds per vine. Basal wire was set to 100 cm above the ground, with two sets of catch wires positioned 50 and 90 cm above the cordon. The maximum canopy height was 200 cm. The experimental field provided no irrigation system, and the space between the rows was grassed. Same vineyard management practices were applied to all treatments.

The beginning of the main phenophases was determined visually. Full flowering was estimated when 50 % flower caps fell off, which is stage 23 according to the modified Eichorn and Lorenz (E-L) system (*23*), while vérasion was estimated when berries begin to brighten in colour, which is stage 35 according to the same scale.

The harvest date was determined by measuring the total soluble solids (Brix), total acids (g/L), and pH. Harvesting began when the total soluble solids were above 19 Brix. Grapes were harvested manually at different times depending on the grapevine variety and measured parameters. Each treatment was harvested separately.

Weather conditions, including average temperature and precipitation for both seasons from April to September, were measured by the Croatian Meteorological and Hydrological Service (Weather station Benkovac), 25 km away from the experimental vineyard, and the data are shown in Table S1. The weather conditions were reflected at the beginning of the flowering and vérasion, and also during the leaf removal treatments. Harvest time differed by only a few days in both years. The Merlot harvest in 2016 was three days earlier (September 22) than in 2015 (September 25), probably due to the previously mentioned dry period during July, which affected the slightly earlier harvest. Syrah was harvested in 2015 on the same day as well as Merlot, but in 2016, 8 days later than Merlot on 30 September. Cabernet Sauvignon was harvested on 9 October in 2015, and on 13 October in 2016.

Experimental design

The experiment was a completely randomized block design with three treatments in three replications for each cultivar. Each replication consisted of 15 continuous plants, so there were 135 plants per cultivar and 405 plants in total. The cultivars were in the same vineyard, but one was next to the other, so the experiment was set up in the same way for different grapevine varieties. All treatments were repeated in the same position in the vineyard for two years.

The three treatments were: (1) leaf removal during flowering (LRF) (full flowering; 50 % open flowers); (2) leaf removal at vérasion (LRV) (beginning of vérasion; 30 % of the berries are coloured); and (3) control (C) - without leaf removal.

In both leaf removal treatments, the basal leaves were removed up to the height of the last cluster on the shoot (4 to 6 leaves).

Vinification

Manually harvested grapes were separately destemmed and crushed for each variety and treatment and put in an open plastic container (100 L) for maceration and fermentation. All vinifications were sulphited with 5 g K-metabisulfite/100 L, and after a few hours, *Saccharomyces cerevisiae* yeast (ICV, Lallemand, Montreal, Canada) was inoculated at a dose of 25 g/100 L. The pomace was manually mixed twice daily, and the temperature ranged between 25 °C and 28 °C. After seven days of maceration and fermentation, the wine was racked, and the fermentation continued in glass containers. At the end of fermentation, the wine was additionally sulphited with 5 g K-metabisulfite/100 L and racked again, bottled into 0.75 L bottles two months after the end of fermentation.

Musts samples were collected immediately after primary processing, for total soluble solids, titratable acidity and pH analysis. Total soluble solids in musts were measured by handheld refractometer (RHB 32 ATC; China) (expressed in Brix) and pH was determined with a pH meter (Lab 860; Schott Instruments; Mainz, Germany). Titratable acidity (g/L) was estimated using colouration pattern volumetric method according to the O.I.V. (*24*).

The wine was stored and matured in the cellar conditions for one year after bottling. Samples for analysis were taken randomly in triplicate after bottling, representing 0 months and after 6 and 12 months.

Analysis of phenolics content using HPLC-DAD

The concentration of anthocyanins and other phenolic compounds (phenolic acids, procyanidins, flavan-3-ols, and flavonol glycosides) was determined in all wine samples using High-Performance Liquid Chromatography (HPLC). The wine samples were filtered into glass vials through 0.45 µm syringe filters filter (Macherey-Nagel GmbH & Co. KG, Duren, Germany) and analysed by HPLC Agilent Infinity 1260 system equipped with Agilent 1260 photodiode array detector (PDA; Agilent, Santa Clara, CA, USA) with an automatic injector and Chemstation software (ver. C.01.03) for data processing and instrument control. Phenolic compounds were separated using Luna 100-5C18 column, 5 µm (250 x 4.6 mm; Phenomenex, Aschaffenburg, Germany). The injection volume was 5 µL, and solvent composition as gradient conditions were as previously described by Zorić *et al*. (*25*).

All anthocyanins were identified at 520 nm by comparing their retention times and absorption spectra with those of authentic standards. Quantifications were made by the external standard calculation, using calibration curves. Standards of delphinidin-3-glucoside (Del-3-Glc), cyanidin 3-glucoside (Cy-3-Glc), petunidin-3-glucoside (Pet-3-Glc), peonidin-3-glucoside (Peo-3-Glc) and malvidin-3-glucoside (Mal-3-Glc), were prepared as stock solutions in acidified methanol (1 % of formic acid in methanol, v/v) at the concentration of 100 mg/L and by diluting the stock solutions so as to obtain five concentrations ranging from 20 to 100 mg/L.

Phenolic acids, procyanidins, flavan-3-ols and flavonol glycosides were identified by comparing retention times and spectral data with those of authentic standards prepared in methanol, namely: chlorogenic acid, caffeic acid, *p*-coumaric acid, gallic acid, procyanidins B1 and B6, epigallocatechin gallate, catechin, quercetin-3-glucoside and kaempferol-3-rutinoside.

All results were expressed as mg/L in form of the mean value ± standard deviation.

Statistical analysis

Statistica v. 14.0 software (*26*) was applied for the statistical analysis. Descriptive statistics was employed to assess the basic information about the experimental data set, and the data are presented as mean values±SE. The normality and homoscedasticity of the data were analysed using the Shapiro–Wilk test and Levene's test, respectively, and were accordingly analysed by ANOVA coupled with the posthoc Tukey's HSD test with multiple comparisons of mean ranks. A statistically significant difference at the level of $p \leq 0.05$ was assigned for all tests.

RESULTS AND DISCUSSION

Basic chemical parameters of musts

The basic chemical parameters measured in the musts of three grape varieties (total soluble solids, titratable acidity, and pH) were influenced mostly by the experimental year, while there is no significant difference among varieties and leaf removal treatments (Table 1).

Two experimental years differed in the average temperature and precipitation during the vegetation period, with 2015 being warmer by 0.7 °C and having about 125 mm less precipitation. Furthermore, during the ripening period in August 2015 was, on average, 1.3°C warmer than in 2016. Higher temperatures impact increased cellular respiration, leading to malic acid breakdown (*27*) and decreased acidity. This was observed in 2015 samples, which had lower acidity and, consequently, higher pH than 2016 samples. Similar observations that experimental year was a significant factor influencing basic chemical parameters, compared to leaf removal treatments, were made by Mosetti *et al.* (*28*) on Sauvignon blanc, and Anic *et al.* (*29*) on Merlot, although in some cases, a mild impact of leaf removal treatments on basic chemical parameters was obtained (*30,31*). The timing of leaf removal also did not influence the basic chemical parameters of musts. There is no difference between the treatments in titratable acidity and pH, which is in line with other research (*32,33*).

Defoliation treatments, regardless of the time of implementation, did not affect the increase in total soluble solids. These observations are similar to other research (*29,32,33*).

Effects of leaf removal on anthocyanin content in wines

Leaf removal treatments positively influenced anthocyanin accumulation in all three grapevine varieties, which was expected and is in line with other research on different varieties (*29,34,35*). Similar results were obtained by other authors. For example, in the research on the Italian cultivar

Nebbiolo, the concentration of individual anthocyanins and polyphenols varied depending on the year and climatic conditions. Still, the total concentration was consistently higher in defoliated treatments compared to the control (*35*). The leaf removal effect on individual anthocyanins composition in Merlot, Syrah and Cabernet Sauvignon wine is shown in Table 2. In all three grapevine varieties, malvidin-3-*O*-glucoside (Mal-3-Glc) was the most abundant anthocyanin, with concentration varying depending on the year and leaf removal treatment, which is consistent with the studies of Shi *et al*. (*36*). The second most abundant anthocyanin for all three varieties was malvidin-3-*O*-acetyl--glucoside (Mal 3-Ac-Glc).

The influence of defoliation time on the concentration of total and individual anthocyanins differed depending on the variety and the year (Table S2, Table S3 and Table S4). Similar observations were obtained on Merlot, Pinot noir, and Gamay, where the experimental year had an important role in the success of the leaf removal treatments (*32-37*). In both years, there is significant influence of defoliation treatment on the concentration of Pet-3-Glc, Malv-3-Glc, Peo-3-Coum-Glc, Mal-3-Ac-Glc and Mal-3-Coum-Glc in Merlot, while for remaining individual anthocyanins the influence of defoliation is no significant. In contrast to Merlot, in Syrah, defoliation treatments consistently increased anthocyanin content in both years, with LRV having the most significant impact. Unlike Merlot, the effect of defoliation did not vary significantly between years. In 2016, Pet-3-Coum-Glc was undetectable across all treatments. In Cabernet Sauvignon, the impact of leaf removal depended on the experimental year. Only LRF in 2016 has significant influence on the individual anthocyanin concentration in Cabernet Sauvignon wines, while control wines had the highest anthocyanin content in 2015 (Table 2).

Regarding the time of leaf removal, different results are reported. According to some studies, a higher concentration of anthocyanins was found by applying early leaf removal in flowering compared to leaf removal at vérasion (*11,38,*), which is similar to our results in Merlot wines from 2015 and Cabernet Sauvignon wines from 2016 (Table 3 and Table 4). In contrast, in Merlot 2016 and in Syrah in both years, a significant influence on the increase in the concentration of total anthocyanins was recorded with LRV (Table 3 and Table 5). The highest concentration of total anthocyanins in Cabernet Sauvignon 2015 was recorded in the control sample (Table 4).

The positive influence of early defoliation on anthocyanin concentration due to increased UV radiation was also recorded on the Merlot in the research of Anić *et al.* (*29*). Due to increasingly warmer years and the influence of high temperatures on anthocyanin reduction, late leaf removal at vérasion loses its advantages compared to early leaf removal at flowering. Comparing the impact of

both defoliation treatments, Lemut *et al*. (*39*) measured a higher concentration of total anthocyanins in Pinot Noir wine by performing early leaf removal, contrary to our results obtained in Syrah from both years.

Anthocyanin content in wines during aging

The wine aging period had a significant effect on the reduction of the concentration of individual anthocyanins in all three researched varieties in both years (Table 6).

Anthocyanin concentration decreased during wine aging (Fig. 1), which agrees with previous studies (*40,41*). Although free anthocyanins are responsible for the red colour of young red wines, their concentration significantly decreases during wine aging to as little as 0–50 mg/L, thus causing a loss of colour in red wines (*22*).

The decrease in the concentration of anthocyanins in wine is partly influenced by external factors (temperature, light, precipitation). Still, a part of anthocyanins decreases due to their instability and strong reactivity with other compounds. This primarily refers to reactions of anthocyanins with other anthocyanins and their co-pigmentation and to polymerization reactions with flavan-3-ols and procyanidins, whereby new pigments of proanthocyanins and polymeric anthocyanins are formed that can stabilize wine colour (*19-21*).

According to the available literature data, earlier studies confirm a steady decrease in the total anthocyanin content during bottle aging for up to 42 months (*42-45*). Anthocyanin reduction during aging in all three wines was high, ranging from 36 to 90 %, depending on the year and treatment. In 2016, the degradation of anthocyanins ranged from 36 to 70 %, while in 2015, the degradation ranged from 65 to as high as 90 %, depending on the variety (Fig. 1). The highest reduction of anthocyanins was recorded in 2015 Merlot wine after 12 months of storage, in the treatment of leaf removal during flowering, and increased to a high 90 %.

Anthocyanin levels in Merlot declined during aging, with Pet-3-Glc, Pet-3-Coum-Glc, and peonidin-3-O-coumaroyl-glucoside (Peo-3-Coum-Glc) being the most unstable. Peo-3-Glc and Pet-3-Coum-Glc were no longer detectable in any treatment after 12 months. However, wines from the 2016 LRV treatment retained the highest total anthocyanin concentration after aging, even though some of the individual compounds were not detected, that is, were degraded in wines after 12 months of storage or were found in very low concentrations (Table S2).

In Syrah, in both years, the LRV had the most significant positive influence on the anthocyanin content (Table 5). Pet-3-Coum-Glc was undetectable across all treatments in 2016. Anthocyanin

stability in wines varied depending on the year and treatment. The treatment with the most stable anthocyanins in wine after 12 months of storage seems to be 2015 control. In 2016, the control and LRV had the same effect on the stability of anthocyanins in the wine (Fig. 1 and Table S3).

The effect of aging on the concentration of anthocyanin in Cabernet Sauvignon wines is presented in Table 6. In 2015, all varieties had a significant loss of anthocyanins during aging, while in 2016 the stability of anthocyanins in the stored wines was similar in control and LRF treatment. As in Syrah, Pet-3-Coum-Glc was undetectable in 2016, and Pet-3-Glc was the most unstable anthocyanin in wine, disappearing from all wines after 12 months (Fig. 1 and Table S4).

Only in Merlot from 2016 and Cabernet from 2015 did the implementation of leaf removal have a positive effect on the stability of anthocyanins. The degradation of anthocyanins in LRF and LRV was lower compared to the control. A lower percentage of degradation was recorded in the LRV treatment.

Although there are significant differences between the anthocyanins content in wines after 12 months of aging (Table 6), they cannot be related to the influence of the leaf removal treatments regardless of their effect on the increase of anthocyanin concentration in young wines. This can be explained by the fact that the stability of anthocyanins in wine is influenced by a number of factors, such as wine storage conditions, cultivars, but also by the different reactions that anthocyanins undergo during wine aging (*40*).

Furthermore, it seems that anthocyanin degradation was lower in the colder 2016 compared to the warmer 2015. This could be explained by the differences in the basic chemical parameters, that is in the pH differences, since the stability and colour shade of red wines are greatly influenced by pH and the amount of free sulphur dioxide (*20*). The red colour of the wine mostly comes fro m anthocyanins, which are in the flavylium state, and their concentration depends on the pH value and free sulphur dioxide. At low pH, the concentration of the flavylium state increases, the hydrolysis of anthocyanins slows down and the colour is more intense, while the colour intensity and the concentration of anthocyanins in the flavylium state decrease significantly with an increase in pH value (*20*). The grapevine variety significantly influenced the total anthocyanin content in wine. Syrah wine had the highest anthocyanin content compared to Merlot and Cabernet Sauvignon wines (Table 7).

Similar results were obtained on Merlot, Syrah, Cabernet Sauvignon and Marselan in the research by Shi *et al.* (*36*). Differences in the content of anthocyanins are cultivar characteristics. Still, the accumulation of anthocyanins in grapes is influenced by other factors, such as agroecological conditions, climate, soil conditions, canopy management and irrigation, agrotechnical practices and

yield (*2,46*). A significantly higher concentration of total anthocyanins was recorded in the drier and warmer 2015 compared to 2016 (Table 7), which is contrary to previous results (*7,29*), which confirm that increased solar radiation and temperature in the fruit zone reduce anthocyanin accumulation in the berry skin.

Effects of leaf removal on other phenolic compounds in wines

Considering that anthocyanins react with other phenolic compounds in polymerization reactions during wine aging, other groups of phenolic compounds were analyzed in all wines. In both researched years, both leaf removal treatments increased the total phenolic acids (TPA), total procyanidins (TPro), total flavan-3-ols (TFL-3-ols) and total flavonol glycosides (TFG) concentration in Merlot wines compared to the control (Table 3). At the same time, such an effect was not observed in Cabernet Sauvignon and Syrah wines (Table 4 and Table 5).

This result could be a consequence of cultivar characteristics and canopy porosity, which was earlier suggested by Tardaguila *et al.* (*14*). Differences were observed in the influence of the time of the leaf removal treatment, so earlier leaf removal during flowering affected the increase of TFG in Merlot and Syrah in both years which is consistent with other studies (*29,32*). Applying defoliation increases sun exposure and UV radiation in the grape zone. Flavonols protect plants from excessive UV radiation, and their accumulation is strongly influenced by environmental conditions (*6,47*). Together with anthocyanins in co-pigmentation processes, flavonols form more complex compounds that affect color stability and wine quality (*48).* Leaf removal at vérasion increase in TFL-3-ol also in Merlot and Syrah wines in 2015 and 2016, which is contrary to the results reported by Osrečak *et al*. (*33*). The end of the synthesis of flavan-3-ols in berry skin is around the vérasion, so it is considered that the application of late defoliation cannot be reflected in their concentration.

Differences in the results also exist between the two years of study, which can be connected to different meteorological and microclimatic conditions in the two vegetation seasons. Other authors also confirmed this, stating that the vintage effect plays an important role in the successful implementation of the treatment (*37*).

Other phenolic compounds during wine aging

The concentration of TPA increased during wine aging in all treatments in Syrah wines in both years (Table 5), and also in Merlot and Cabernet wines in 2015 (Table 3 and Table 4), which is in accordance with previous results (*40,49*). The highest concentration of phenolic acids is in Syrah wine

from 2016, and the lowest in Cabernet Sauvignon wines in 2015. TPro concentration in all wines decreased during aging, except for the Cabernet control wine from 2016, and their degradation is from 5 to 42 % depending on the variety, year and treatment (Table S5).

Concentration of total TFL-3-ols and FG in some varieties increased, while in others, they decreased during aging. The highest concentration of TFL-3-ols was recorded in Syrah wine from 2015 in LRV treatment, while the highest degradation of TFL-3-ols was 33 % in Syrah 2016 from LRV and Merlot 2015 in LRF (Table S5). Concentration of TFG decreased in all wines except Merlot from 2016. The lowest degradation percentage was recorded in Cabernet Sauvignon wine from 2016 in the LRF treatment, and the highest was over 70 % in Syrah from 2015 in the LRV treatment. The percentage of TFL-3-ols degradation during aging is largely influenced by the grapevine variety (*46*).

The interaction between the leaf removal treatment and wine aging showed that the leaf removal treatment and period of aging significantly influenced the phenolic compounds in all three wines (Table 3, Table 4 and Table 5).

CONCLUSIONS

Applied leaf removal treatments increased the concentration of anthocyanins in all three cultivars in both years, while the influence of leaf removal on the concentration of phenolic acids, procyanidins, flavan-3-ols and flavonol glycosides depended on the cultivar and year. Leaf removal treatments had the most significant effect on the increase in the concentration of total anthocyanins in Syrah in both years, especially the treatment of leaf removal at veraison (LRV).

Leaf removal treatments remain important viticultural practices for red grape and wine production. Although leaf removal significantly affected the initial concentration of anthocyanin in wine, this treatment did not affect the stability of anthocyanins in wine during aging. Anthocyanin concentration decreases with aging, and their stability in wine was most affected by the aging period and grapevine variety. Although the highest concentration of anthocyanins was recorded in Syrah wine, this did not affect their stability during wine aging.

Future studies should focus on how to preserve higher concentrations of anthocyanins obtained by leaf removal treatments in red wines during aging.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

M. Pavlović was in charge in the conception of the work, experimental investigation, data analysis, writing and drafting of the article. Z. Zorić took part in formal analysis, data analysis and in the writing, editing and final approval of the manuscript. Š. Marcelić was involved in investigation and data analysis obtained results. M. Repajić contributed to the review of statistical methods. I. Šikuten took part in the review and editing. D. Preiner took part in writing, editing and final approval of the version to be published.

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Table 1. Soluble solids, titratable acidity and pH influenced by year, cultivar and leaf removal effect

Type of influence	Soluble solid/°Brix	Titratable acidity/(g/L)	pH
Year	p<0.001*	p<0.001*	p<0.001*
2015	(20.33±0.16)^{b}	(4.96±0.13)^{b}	(3.58±0.02)^{a}
2016	(21.41±0.25)^{a}	(5.78±0.08)^{a}	(3.38±0.02)^{b}
Cultivar	p<0.001*	p=0.288	p=0.476
Merlot	(20.37±0.18)^{b}	(5.20±0.25)^{a}	(3.47±0.03)^{a}
Syrah	(20.74±0.19)^{b}	(5.35±0.12)^{a}	(3.47±0.04)^{a}
Cabernet Sauvignon	(21.50±0.39)^{a}	(5.56±0.05)^{a}	(3.51±0.02)^{a}
Leaf removal effect	p=0.387	p=0.942	p=0.621
Control	(20.56±0.29)^{a}	(5.41±0.18)^{a}	(3.46±0.04)^{a}
LRF	(21.12±0.28)^{a}	(5.33±0.16)^{a}	(3.49±0.03)^{a}
LRV	(20.93±0.29)^{a}	(5.38±0.15)^{a}	(3.50±0.02)^{a}

LRF=leaf removal flowering, LRV=leaf removal veraison. *Statistically significant variable at p≤0.05. Results are expressed as mean±SE. Values with different letters within column are statistically different at p≤0.05

Table 2. Leaf removal effect shown as average values of three ageing periods on Merlot, Syrah and Cabernet Sauvignon wine anthocyanin composition

Wine		*γ*/(mg/L)						
		Pet-3-Glc	Peo-3-Glc	Malv-3-Glc	Pet-3-Coum-Glc	Peo-3-Coum-Glc	Mal-3-Ac-Glc	Mal-3-Coum-Glc
Merlot	Treatment							
2015	Control	(1.90±0.50)^{c}	(0.78±0.19)^{a}	(35.88±5.39)^{b}	(0.47±0.12)^{a}	(0.97±0.06)^{b}	(13.34±2.15)^{b}	(6.93±1.32)^{c}
	LRF	(2.90±0.91)^{a}	(0.82±0.24)^{a}	(45.09±11.87)^{a}	(0.74±0.26)^{a}	(1.50±0.12)^{a}	(16.86±4.93)^{a}	(9.23±2.96)^{a}
	LRV	(2.43±0.68)^{b}	(0.79±0.21)^{a}	(42.09±8.50)^{a,b}	(0.65±0.19)^{a}	(1.46±0.11)^{a,b}	(16.29±3.62)^{a,b}	(8.16±2.07)^{b}
	Signif.	***	n.s.	**	n.s.	**	**	***
2016	Control	(2.10±0.25)^{c}	(1.30±0.49)^{a}	(39.30±5.38)^{c}	n.d.	(0.62±0.17)^{a}	(12.13±2.45)^{c}	(6.96±1.27)^{c}
	LRF	(2.91±0.12)^{b}	(1.70±0.63)^{a}	(52.47±4.14)^{b}	(0.39±0.20)^{a}	(0.61±0.15)^{a}	(15.29±2.16)^{b}	(10.10±1.03)^{a,b}
	LRV	(4.18±0.19)^{a}	(1.93±0.64)^{a}	(63.79±4.27)^{a}	(0.51±0.26)^{a}	(0.83±0.21)^{a}	(21.75±2.46)^{a}	(11.43±0.90)^{a}
	Signif.	***	n.s.	***	n.s.	n.s.	***	**
Syrah	Treatment							
2015	Control	(3.59±0.62)^{b}	(3.04±0.38)^{b}	(56.59±7.31)^{c}	(1.56±0.25)^{b}	(2.75±0.51)^{b}	(24.48±3.78)^{b}	(13.16±1.96)^{b}
	LRF	(3.79±0.70)^{b}	(2.59±0.39)^{c}	(63.55±9.55)^{b}	(2.09±0.47)^{a}	(2.82±0.40)^{b}	(26.66±4.55)^{a,b}	(13.75±2.14)^{b}
	LRV	(4.33±0.83)^{a}	(3.61±0.49)^{a}	(73.35±10.54)^{a}	(1.75±0.19)^{b}	(3.23±0.68)^{a}	(30.97±5.40)^{a}	(17.66±2.93)^{a}
	Signif.	**	***	***	**	**	**	**
2016	Control	(2.53±0.27)^{a,b}	(1.58±0.15)^{a,b}	(49.88±4.85)^{a,b}	n.d.	(1.74±0.49)^{a,b}	(19.49±3.20)^{a,b}	(11.33±1.62)^{a,b}
	LRF	(2.39±0.36)^{b}	(1.09±0.16)^{b}	(45.88±6.52)^{b}	n.d.	(1.15±0.34)^{b}	(17.27±3.24)^{b}	(9.30±1.84)^{b}
	LRV	(3.21±0.42)^{a}	(1.71±0.20)^{a}	(54.43±5.50)^{a}	n.d.	(2.39±0.47)^{a}	(20.79±3.48)^{a}	(12.13±1.81)^{a}
	Signif.	**	**	**	-	**	**	**
Cabernet Sauvignon	Treatment							
2015	Control	(1.85±0.52)^{a}	(0.27±0.14)^{a}	(59.54±10.73)^{a}	(0.81±0.18)^{b}	(0.87±0.16)^{a}	(26.60±5.00)^{a}	(5.17±1.23)^{a}
	LRF	(1.81±0.50)^{a}	(0.39±0.59)^{a}	(54.38±9.72)^{a,b}	(1.16±0.05)^{a,b}	(0.97±0.13)^{a}	(23.74±4.11)^{a,b}	(3.78±0.87)^{b}
	LRV	(1.57±0.41)^{b}	(0.48±0.39)^{a}	(47.53±6.56)^{b}	(1.26±0.11)^{a}	(0.83±0.08)^{a}	(20.07±3.06)^{b}	(4.48±0.76)^{a,b}
	Signif.	**	n.s.	**	**	n.s.	**	**

2016	Control	(0.38±0.19)^{b}	(2.34±0.24)^{b}	(59.11±3.39)^{a,b}	n.d.	(0.69±0.18)^{a}	(27.37±1.81)^{a}	(5.87±0.38)^{a}
	LRF	(0.68±0.95)^{a}	(3.99±0.23)^{a}	(67.12±4.01)^{a}	n.d.	(1.08±0.30)^{a}	(27.69±1.90)^{a}	(5.33±0.35)^{a}
	LRV	(0.60±0.90)^{a}	(2.71±0.39)^{b}	(53.10±6.36)^{b}	n.d.	(0.65±0.17)^{a}	(21.52±2.99)^{b}	(5.37±0.85)^{a}
	Signif.	**	**	**	-	n.s.	**	n.s.

Data was analyzed using one-way ANOVA model; ns,**, *** indicate not significant, significance at $p<0.01$ and >0.0001, respectively. LRF=leaf removal during flowering, LRV leaf removal during veraison. Means with different letter are significantly different within treatment. Abbreviations: Petunidin-3-O-glucoside (Pet-3-Glc); Peonidin-3-O-glucoside (Peo-3-Glc); Malvidin-3-O-glucoside (Malv-3-Glc) Petunidin-3-O-(coumaroyl) glucoside (Pet-3-Coum-Glc); Peonidin-3-O-(coumaroyl) glucoside (Peo-3-Coum-Glc); Malvidin-3-O-(acetyl) glucoside (Mal-3-Ac-Glc); Malvidin-3-O-(coumaroyl) glucoside (Mal-3-Coum-Glc)

Table 3. Leaf removal effect shown as average values on Merlot wine phenolic composition

Year		*γ*/(mg/L)				
		TA	TPA	TPro	TFL-3-ols	TFG
	Treatment					
2015	Control	64.23^{c}	52.40^{c}	85.95^{b}	19.76^{c}	26.15^{c}
	LRF	77.13^{a}	61.29^{b}	80.98^{c}	22.31^{b}	36.78^{a}
	LRV	71.87^{b}	62.81^{a}	95.45^{a}	24.18^{a}	33.54^{b}
	Signif	***	***	***	***	***
	Period					
	0	121.94^{a}	58.75^{a}	102.87^{a}	25.06^{a}	45.08^{a}
	6	64.59^{b}	57.98^{a}	86.27^{b}	22.15^{b}	31.32^{b}
	12	20.29^{c}	59.05^{a}	71.37^{c}	18.46^{c}	17.96^{c}
	Signif	***	ns	***	***	***
	T × P	***	ns	***	***	***
	Treatment					
2016	Control	62.41^{c}	65.47^{c}	92.50^{c}	26.28^{b}	18.98^{c}
	LRF	83.48^{b}	92.01^{a}	93.57^{b}	27.96^{a}	30.99^{a}
	LRV	104.43^{a}	83.28^{b}	96.65^{a}	24.61^{c}	22.11^{b}
	Signif.	**	***	***	***	***
	Period					
	0	114.31^{a}	73.77^{c}	117.88^{a}	19.44^{c}	23.19^{c}
	6	83.36^{b}	75.26^{b}	94.82^{b}	27.61^{b}	23.91^{b}
	12	52.65^{c}	91.72^{a}	70.02^{c}	31.81^{a}	24.98^{a}
	Signif.	**	***	***	***	***
	T × P	**	***	***	***	***

Data was analyzed using two-way ANOVA model; ns,**, *** indicate not significant, significance at p<0.01 and> 0.0001, respectively. TxP=significance of the treatment x period of aging interaction. LRF=leaf removal during flowering, LRV=leaf removal during veraison. Means with different letter are significantly different within treatments and period of aging. Abbreviations: TA: Total anthocyanins; HPA:Total phenolic acids; TPro: Total procyanidins;TFL-3-ols:Total Flavan-3-ols; TFG:Total flavonol glycosides

Table 4. Leaf removal effect shown as average values on Cabernet Sauvignon wine phenolic composition

Year		*γ*/(mg/L)				
		TA	TPA	TPro	TFL-3-ols	TFG
	Treatment					
2015	Control	95.12^{a}	47.37^{b}	54.44^{a}	19.23^{b}	25.42^{a}
	LRF	86.22^{b}	43.73^{c}	44.71^{c}	21.10^{a}	22.84^{b}
	LRV	76.23^{c}	53.42^{a}	47.13^{b}	19.34^{b}	21.91^{c}
	Signif	**	***	**	***	***
	Period					
	0	138.55^{a}	43.77^{c}	54.09^{a}	19.63^{b}	32.71^{a}
	6	82.86^{b}	49.81^{b}	48.91^{b}	19.46^{b}	24.92^{b}
	12	36.16^{c}	50.93^{a}	43.28^{c}	20.57^{a}	12.55^{c}
	Signif	**	***	**	***	***
	T × P	**	***	**	***	***
	Treatment					
2016	Control	95.75^{b}	56.20^{b}	39.09^{c}	44.79^{b}	11.30^{c}
	LRF	105.90^{a}	74.18^{a}	61.76^{a}	45.67^{a}	25.96^{a}
	LRV	83.95^{c}	45.81^{c}	40.44^{b}	37.43^{c}	17.88^{b}
	Signif.	**	**	**	**	**
	Period					
	0	124.72^{a}	85.36^{a}	51.60^{a}	17.05^{c}	22.55^{a}
	6	91.17^{b}	33.14^{c}	48.10^{b}	52.09^{b}	15.73^{c}
	12	69.71^{c}	57.68^{b}	41.59^{c}	58.74^{a}	16.87^{b}
	Signif.	**	**	**	**	**
	T × P	**	**	**	**	**

Data was analyzed using two-way ANOVA model; ns,**, *** indicate not significant,significance at p<0.01 and> 0.0001, respectively. TxP=significance of the treatment x period of aging interaction. LRF=leaf removal during flowering, LRV=leaf removal during veraison. Means with different letter are significantly different within treatments and period of aging. Abbreviations: TA: Total anthocyanins; HPA:Total phenolic acids; TPro: Total procyanidins;TFL-3-ols:Total Flavan-3-ols; TFG:Total flavonol glycosides

Table 5. Leaf removal effect shown as average values on Syrah wine phenolic composition (mg/L)

Year		*y*/(mg/L)				
		TA	TPA	TPro	TFL-3-ols	TFG
2015	Treatment					
	Control	105.17^{c}	97.53^{a}	84.97^{a}	73,67^{a}	69.83^{b}
	LRF	115.25^{b}	93.35^{b}	79.41^{b}	70.73^{b}	82.92^{a}
	LRV	134.90^{a}	83.27^{c}	77.34^{c}	67.06^{c}	68.55^{c}
	Signif	**	**	**	***	**
	Period					
	0	179.20^{a}	84.56^{c}	91.61^{a}	61.07^{b}	105.24^{a}
	6	120.67^{b}	93.45^{b}	84.84^{b}	58.82^{c}	73.83^{b}
	12	55.45^{c}	96.14^{a}	65.26^{c}	91.55^{a}	42.23^{c}
	Signif	**	**	**	***	**
	T × P	**	**	**	***	**
2016	Treatment					
	Control	86.55^{c}	90.71^{c}	74.99^{a}	27.45^{c}	40.15^{c}
	LRF	77.07^{b}	117.40^{a}	56.81^{b}	29.05^{b}	61.16^{a}
	LRV	94.67^{a}	91.19^{b}	52.46^{c}	31.48^{a}	52.03^{b}
	Signif.	**	***	**	***	**
	Period					
	0	128.43^{a}	87.83^{c}	76.72^{a}	33.21^{a}	61.56^{a}
	6	80.25^{b}	93.5^{b}	59.49^{b}	29.79^{b}	51.77^{b}
	12	49.61^{c}	117.97^{a}	48.05^{c}	24.99^{c}	43.02^{c}
	Signif.	**	***	**	***	**
	T × P	**	***	**	***	**

Data was analyzed using two-way ANOVA model; ns,**, *** indicate not significant,significance at p<0.01 and> 0.0001, respectively. TxP=significance of the treatment x period of aging interaction. LRF=leaf removal during flowering, LRV=leaf removal during veraison. Means with different letter are significantly different within treatments and period of aging. Abbreviations: TA: Total anthocyanins; HPA:Total phenolic acids; TPro: Total procyanidins;TFL-3-ols:Total Flavan-3-ols; TFG:Total flavonol glycosides

Table 6. Effect of aging period, shown as average values of three leaf removal treatments on Merlot, Syrah and Cabernet Sauvignon wine anthocyanins composition (mg/L)

Wine		*γ*/(mg/L)						
		Pet-3-Glc	Peo-3-Glc	Malv-3-Glc	Pet-3-Coum-Glc	Peo-3-Coum-Glc	Mal-3-Ac-Glc	Mal-3-Coum-Glc
Merlot	Period							
2015	0	(4.67±0.46)^{a}	(1.42±0.07)^{a}	(71.23±5.85)^{a}	(1.28±0.14)^{a}	(1.66±0.12)^{a}	(28.40±2.44)^{a}	(15.81±1.45)^{a}
	6	(2.64±0.07)^{b}	(0.97±0.05)^{b}	(38.61±1.24)^{b}	(0.57±0.02)^{b}	(1.25±0.09)^{b}	(13.66±0.65)^{b}	(6.91±0.35)^{b}
	12	(0.00±0.00)^{c}	(0.00±0.00)^{c}	(13.22±0.68)^{c}	(0.00±0.00)^{c}	(1.01±0.06)^{b}	(4.44±0.29)^{c}	(1.62±0.11)^{c}
	Signif.	***	***	***	***	**	***	***
2016	0	(3.63±0.29)^{a}	(3.91±0.17)^{a}	(66.47±2.21)^{a}	(0.90±0.23)^{a}	(1.19±0.07)^{a}	(24.85±1.28)^{a}	(13.35±0.40)^{a}
	6	(3.18±0.31)^{a,b}	(1.03±0.12)^{b}	(53.64±4.75)^{a}	(0.00±0.00)^{b}	(0.87±0.06)^{b}	(15.59±1.84)^{b}	(9.05±0.87)^{b}
	12	(2.38±0.33)^{b}	(0.00±0.00)^{c}	(35.46±3.79)^{b}	(0.00±0.00)^{b}	(0.00±0.00)^{c}	(8.72±1.15)^{c}	(6.09±0.75)^{c}
	Signif.	**	***	**	**	***	***	***
Syrah	Period							
2015	0	(6.02±0.24)^{a}	(4.44±0.21)^{a}	(94.52±4.33)^{a}	(2.96±0.25)^{a}	(4.87±0.21)^{a}	(44.11±1.89)^{a}	(22.28±4.26)^{a}
	6	(4.51±0.09)^{b}	(3.26±0.15)^{b}	(67.41±1.90)^{b}	(1.48±0.06)^{b}	(2.72±0.07)^{b}	(25.35±0.65)^{b}	(15.94±1.72)^{b}
	12	(1.18±0.04)^{c}	(1.54±0.10)^{c}	(31.56±1.31)^{c}	(0.96±0.04)^{b}	(1.21±0.10)^{c}	(12.65±0.42)^{c}	(6.36±1.04)^{c}
	Signif.	***	***	***	**	***	***	***
2016	0	(3.98±0.21)^{a}	(2.04±0.11)^{a}	(69.57±1.12)^{a}	n.d.	(3.32±0.27)^{a}	(31.98±0.60)^{a}	(17.53±0.35)^{a}
	6	(2.54±0.12)^{b}	(1.42±0.12)^{b}	(49.48±2.43)^{b}	n.d.	(1.59±0.12)^{b}	(15.60±0.71)^{b}	(9.62±0.65)^{b}
	12	(1.61±0.09)^{c}	(0.92±0.08)^{c}	(31.14±1.15)^{c}	n.d.	(0.37±0.18)^{c}	(9.96±0.26)^{c}	(5.61±0.31)^{c}
	Signif.	***	***	***	-	***	***	***
Cabernet Sauvignon	Period							
2015	0	(3.27±0.13)^{a}	(0.95±0.06)^{a}	(85.51±4.35)^{a}	(1.43±0.06)^{a}	(1.33±0.05)^{a}	(38.11±2.08)^{a}	(7.95±0.47)^{a}
	6	(1.97±0.03)^{b}	(0.19±0.10)^{b}	(52.55±1.09)^{b}	(1.13±0.04)^{b}	(0.84±0.04)^{b}	(22.15±0.59)^{b}	(4.03±0.24)^{b}

	12	(0.00±0.00)^{c}	(0.00±0.00)^{b}	(23.39±0.14)^{c}	(0.67±0.14)^{c}	(0.50±0.03)^{c}	(10.15±0.19)^{c}	(1.45±0.09)^{c}
	Signif.	***	**	***	***	***	***	***
2016	0	(1.63±0.13)^{a}	(4.10±0.24)^{a}	(76.45±1.59)^{a}	n.d.	(1.46±0.15)^{a}	(33.54±0.34)^{a}	(7.53±0.30)^{a}
	6	(0.03±0.03)^{b}	(2.73±0.23)^{b}	(57.99±2.18)^{b}	n.d.	(0.96±0.06)^{b}	(24.88±1.19)^{b}	(4.57±0.10)^{b}
	12	(0.00±0.00)^{b}	(2.20±0.32)^{b}	(44.88±3.19)^{c}	n.d.	(0.00±0.00)^{c}	(18.16±1.57)^{c}	(4.46±0.38)^{b}
	Signif.	**	**	***	-	***	***	**

Data was analyzed using one-way ANOVA model; ns,**, *** indicate not significant, significance at p<0.01 and >0.0001, respectively. Means with different letter are significantly different within period of aging. Abbreviations: Petunidin-3-O-glucoside (Pet-3-Glc); Peonidin-3-O-glucoside (Peo-3-Glc); Malvidin-3-O-glucoside (Malv-3-Glc) Petunidin-3-O-(coumaroyl) glucoside (Pet-3-Coum-Glc); Peonidin-3-O-(coumaroyl) glucoside (Peo-3-Coum-Glc); Malvidin-3-O-(acetyl) glucoside (Mal-3-Ac-Glc); Malvidin-3-O-(coumaroyl) glucoside (Mal-3-Coum-Glc)

Table 7. Total anthocyanins content (mg/L) in wine after fermentation influenced by cultivar and year

Source of variation	Total anthocyanins/(mg/L)
Cultivar	p<0.001*
Merlot	(119.39±5.67)^{b}
Syrah	(153.81±7.36)^{a}
Cabernet Sauvignon	(131.63±3.88)^{a,b}
Year	p<0.001*
2015	(147.40±6.60)^{a}
2016	(122.49±2.09)^{b}

*Statistically significant variable at p≤0.05. Results are expressed as mean±standard error. Values with different letters within column are statistically different at p≤0.05

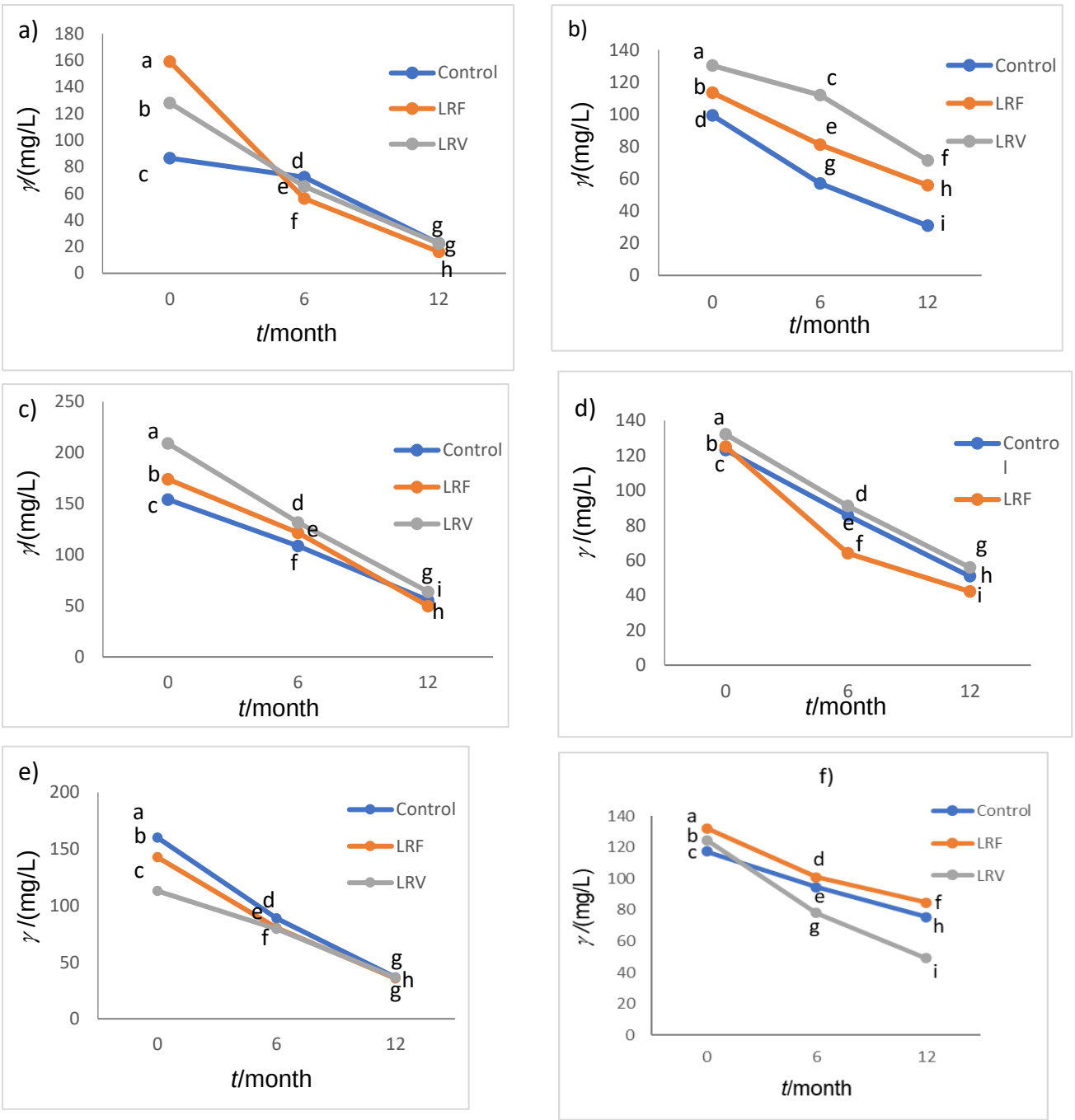

Fig. 1. Effect of aging and leaf removal treatment on anthocyanin content in: a) Merlot 2015, b) Merlot 2016, c) Syrah 2015, d) Syrah 2016, e) Cabernet Sauvignon 2015 and f) Cabernet Sauvignon 2016 vines

Table S1. Weather conditions during vegetation period (April - September) in 2015 and 2016 (Weather station Benkovac)

Month	Average temperature/°C		Precipitation/mm	
	2015	2016	2015	2016
April	12.0	13.8	30.0	53.4
May	18.0	16.1	89.3	93.2
June	22.3	20.8	16.5	141.3
July	26.5	25.2	34.3	1.2
August	24.8	23.4	76.2	57.5
September	19.6	19.6	75.1	99.7
Mean temperature	20.5	19.8		
Cumulative precipitation			321.4	446.2

Table S2. Concentration of anthocyanins in Merlot wine obtained from grapes affected by leaf removal flowering (LRF) and leaf removal vérasion (LRV) at different storage period (bottling, 6 and 12 months) for 2015 and 2016

			γ/(mg/L)							
Year	Treatment	*t*/month	Pet-3-Glc	Peo-3-Glc	Malv-3-Glc	Pet-3-Coum-Glc	Peo-3-Coum-Glc	Mal-3-Ac-Glc	Mal-3-Coum-Glc	Total
2015	Control	0	3.06±0.10	1.18±0.02	50.14±0.04	0.77±0.04	1.19±0.04	19.32±0.10	10.75±0.07	86.41±0.14
		6	2.87±0.04	1.15±0.03	42.75±0.06	0.62±0.03	0.92±0.09	15.73±0.06	8.17±0.02	72.21±0.33
		12	n.d.	n.d.	14.74±0.09	n.d.	0.78±0.04	4.98±0.07	1.88±0.04	22.38±0.15
	LRF	0	6.27±0.03	1.66±0.05	90.55±0.07	1.73±0.05	1.96±0.07	36.04±0.09	20.77±0.05	158.98±0.21
		6	2.39±0.05	0.78±0.05	34.19±0.15	0.51±0.06	1.35±0.07	11.26±0.10	5.74±0.07	56.22±0.33
		12	n.d.	n.d.	10.52±0.16	n.d.	1.19±0.03	3.29 ± 0.05	1.19±0.03	16.19±0.11
	LRV	0	4.67±0.21	1.41±0.21	73.01±0.21	1.34±0.21	1.82±0.21	29.84±0.21	15.90±0.21	127.99±0.21
		6	2.63±0.05	0.96±0.05	38.87±0.13	0.60±0.03	1.48±0.02	13.98±0.11	6.82±0.04	65.34±0.34
		12	n.d.	n.d.	14.40±0.04	n.d.	1.07±0.05	5.05±0.10	1.77±0.14	22.29±0.06
2016	Control	0	2.95±0.20	3.22±0.08	58.73±0.29	n.d.	1.20±0.06	21.46±0.07	11.77±0.03	99.66±0.69
		6	2.16±0.06	0.67±0.08	37.59±0.23	n.d.	0.67±0.06	10.03±0.17	5.96±0.11	57.08±0.67
		12	1.21±0.04	n.d.	21.58±0.06	n.d.	n.d.	4.89±0.14	3.14±0.11	30.82±0.28
	LRF	0	3.20±0.13	4.16±0.08	66.61±0.13	1.18±0.05	0.96±0.11	23.25±0.24	14.04±0.06	113.40±0.54
		6	3.07±0.09	0.95±0.07	52.86±0.09	n.d.	0.87±0.08	14.20±0.03	9.23±0.10	81.18±0.46
		12	2.45±0.07	n.d.	37.95±0.13	n.d.	n.d.	8.42±0.13	7.04±0.10	55.86±0.22
	LRV	0	4.76±0.07	4.33±0.05	74.07±0.10	1.53±0.08	1.41±0.06	29.86±0.21	14.25±0.04	130.21±0.42
		6	4.31±0.04	1.46±0.06	70.46±0.07	n.d.	1.09±0.06	22.54±0.06	11.96±0.13	111.82±0.30
		12	3.47±0.06	n.d.	46.85±0.06	n.d.	n.d.	12.86±0.06	8.08±0.09	71.26±0.10

Results are expressed as mean±S.D. (*N*=3); n.d.=not detected. Abbreviations: Petunidin-3-*O*-glucoside (Pet-3-Glc); Peonidin-3-*O*-glucoside (Peo-3-Glc); Malvidin-3-*O*-glucoside (Malv-3-Glc) Petunidin-3-*O*-(coumaroyl) glucoside (Pet-3-Coum-Glc); Peonidin-3-*O*-(coumaroyl) glucoside (Peo-3-Coum-Glc); Malvidin-3-*O*-(acetyl) glucoside (Mal-3-Ac-Glc); Malvidin-3-*O*-(coumaroyl) glucoside (Mal-3-Coum-Glc)

Table S3. Concentration of anthocyanins in Syrah wine obtained from grapes affected by leaf removal flowering (LRF) and leaf removal vérasion (LRV) at different storage period (bottling, 6 and 12 months) for 2015 and 2016

			γ/(mg/L)							
Year	Treatment	*t*/month	Pet-3-Glc[A]	Peo-3-Glc	Malv-3-Glc	Pet-3-Coum-Glc	Peo-3-Coum-Glc	Mal-3-Ac-Glc	Mal-3-Coum-Glc	Total
2015	Control	0	5.38±0.05	4.16±0.06	79.72 ± 0.41	2.52±0.03	4.56±0.03	38.23±0.10	19.70±0.02	154.27±0.47
		6	4.25±0.10	3.37±0.03	60.51 ± 0.17	1.32±0.02	2.67±0.06	23.01±0.05	13.66±0.03	108.79±0.20
		12	1.21±0.03	1.59±0.04	29.54 ± 0.65	0.84±0.04	1.01±0.06	12.20±0.02	6.12 ± 0.13	52.51 ± 0.55
	LRF	0	5.72±0.04	3.90±0.04	94.11 ± 0.35	3.94±0.09	4.35±0.05	42.94±0.08	19.20±0.07	174.16±0.33
		6	4.57±0.07	2.70±0.02	68.11 ± 0.16	1.41±0.02	2.52±0.08	25.57±0.08	16.77±0.08	121.65±0.08
		12	1.07±0.08	1.18±0.02	28.43 ± 0.44	0.91±0.02	1.59±0.06	11.48±0.04	5.30 ± 0.05	49.96 ± 0.43
	LRV	0	6.95±0.08	5.27±0.04	109.73±0.41	2.41±0.02	5.70±0.14	51.16±0.06	27.94±0.10	209.16±0.74
		6	4.76±0.07	3.69±0.04	73.61 ± 0.53	1.72±0.11	2.99±0.05	27.45±0.13	17.38±0.04	131.60±0.57
		12	1.26±0.10	1.86±0.04	36.71 ± 0.39	1.12±0.06	1.02±0.03	14.27±0.06	7.66 ± 0.05	63.90 ± 0.39
2016	Control	0	3.41±0.06	2.05±0.10	65.25 ± 0.33	n.d.	3.42±0.06	31.76±0.08	17.26±0.04	123.15±0.27
		6	2.63±0.07	1.64±0.06	52.40 ± 0.53	n.d	1.80±0.02	16.54±0.05	10.68±0.02	85.69 ± 0.67
		12	1.55±0.07	1.06±0.18	31.98 ± 0.55	n.d	n.d	10.16±0.08	6.06 ± 0.10	50.81 ± 0.77
	LRF	0	3.74±0.04	1.66±0.19	70.82 ± 0.20	n.d	2.35±0.09	30.04±0.06	16.48±0.03	125.09±0.45
		6	2.08±0.08	0.95±0.09	40.01 ± 0.40	n.d	1.10±0.08	12.80±0.11	7.04 ± 0.06	63.98 ± 0.43
		12	1.34±0.06	0.64±0.07	26.82 ± 0.21	n.d	n.d	8.97 ± 0.07	4.38 ± 0.04	42.15 ± 0.17
	LRV	0	4.79±0.02	2.41±0.04	72.65 ± 0.39	n.d	4.20±0.05	34.15±0.06	18.85±0.04	137.05±0.41
		6	2.91±0.04	1.66±0.06	56.02 ± 0.10	n.d	1.87±0.03	17.47±0.05	11.14±0.08	91.07 ± 0.16
		12	1.93±0.09	1.06±0.09	34.62 ± 0.54	n.d	1.07±0.06	10.75±0.05	6.40 ± 0.05	55.83 ± 0.58

Results are expressed as mean±S.D. (N=3); n.d.=not detected. Abbreviations: Petunidin-3-O-glucoside (Pet-3-Glc); Peonidin-3-O-glucoside (Peo-3-Glc); Malvidin-3-O-glucoside (Malv-3-Glc) Petunidin-3-O-(coumaroyl) glucoside (Pet-3-Coum-Glc); Peonidin-3-O-(coumaroyl) glucoside (Peo-3-Coum-Glc); Malvidin-3-O-(acetyl) glucoside (Mal-3-Ac-Glc); Malvidin-3-O-(coumaroyl) glucoside (Mal-3-Coum-Glc)

Table S4. Concentration of anthocyanins in Cabernet Sauvignon wine obtained from grapes affected by leaf removal flowering (LRF) and leaf removal vérasion (LRV) at different storage period (bottling, 6 and 12 months) for 2015 and 2016

			y/(mg/L)							
Year	Treatment	*t*/month	Pet-3-Glc	Peo-3-Glc	Malv-3-Glc	Pet-3-Coum-Glc	Peo-3-Coum-Glc	Mal-3-Ac-Glc	Mal-3-Coum-Glc	Total
2015	Control	0	3.57±0.04	0.82±0.04	97.90±0.26	1.29±0.05	1.46±0.05	45.12±0.24	9.84±0.07	160.00±0.23
		6	1.98±0.11	n.d	56.90±0.12	1.02±0.07	0.76±0.07	23.86±0.17	4.20±0.18	88.72 ± 0.17
		12	n.d.	n.d	23.76±0.04	0.12±0.02	0.38±0.03	10.82±0.26	1.47±0.11	36.55 ± 0.31
	LRF	0	3.46±0.16	1.16±0.13	89.80±0.47	1.32±0.03	1.40±0.02	38.47±0.33	7.06±0.09	142.67±0.84
		6	1.97±0.08	n.d	50.51±0.15	1.14±0.01	0.97±0.08	22.68±0.08	3.15±0.11	80.62 ± 0.29
		12	n.d	n.d	22.83±0.06	1.02±0.04	0.53±0.04	10.06±0.16	1.13±0.06	35.57 ± 0.23
	LRV	0	2.77±0.04	0.87±0.04	68.76±0.04	1.67±0.04	1.12±0.10	30.73±0.66	6.96±0.14	112.88±0.67
		6	1.96±0.08	0.58±0.04	50.24±0.07	1.23±0.08	0.79±0.02	19.92±0.32	4.75±0.06	79.47 ± 0.28
		12	n.d	n.d	23.58±0.09	0.89±0.03	0.59±0.04	9.57 ± 0.08	1.74±0.03	36.37 ± 0.10
2016	Control	0	1.14±0.08	3.25±0.09	70.93±0.13	n.d	1.18±0.08	33.45±0.51	7.33±0.34	117.28±0.25
		6	n.d	2.16±0.09	58.94±0.08	n.d	0.87±0.04	27.73±0.44	4.92±0.13	94.62 ± 0.43
		12	n.d	1.60±0.05	47.45±0.11	n.d	n.d	20.93±0.19	5.36±0.09	75.34 ± 0.31
	LRF	0	1.95±0.07	4.88±0.03	81.93±0.15	n.d	2.05±0.10	34.70±0.30	6.64±0.04	132.15±0.53
		6	0.10±0.17	3.62±0.03	65.03±0.12	n.d	1.20±0.03	26.71±0.36	4.28±0.15	100.94±0.60
		12	n.d	3.46±0.06	54.41±0.26	n.d	n.d	21.67±0.10	5.07±0.16	84.61 ± 0.24
	LRV	0	1.80±0.04	4.17±0.16	76.50±0.39	n.d	1.14±0.02	32.48±0.08	8.63±0.14	124.72±0.74
		6	n.d	2.41±0.04	50.02±0.17	n.d	0.82±0.03	20.19±0.18	4.52±0.05	77.96 ± 0.18
		12	n.d	1.54±0.06	32.79±0.20	n.d	n.d	11.89±0.07	2.96±0.10	49.18 ± 0.13

Results are expressed as mean±S.D. (*N*=3); n.d.=not detected. Abbreviations: Petunidin-3-*O*-glucoside (Pet-3-Glc); Peonidin-3-*O*-glucoside (Peo-3-Glc); Malvidin-3-*O*-glucoside (Malv-3-Glc) Petunidin-3-*O*-(coumaroyl) glucoside (Pet-3-Coum-Glc); Peonidin-3-*O*-(coumaroyl) glucoside (Peo-3-Coum-Glc); Malvidin-3-*O*-(acetyl) glucoside (Mal-3-Ac-Glc); Malvidin-3-*O*-(coumaroyl) glucoside (Mal-3-Coum-Glc)

Table S5. Leaf removal effect on total phenolic acids, total procyanidins, total flavan-3-ols and total flavonol glycosides concentration during different aging times (0, 6 and 12 months), shown as average values in Merlot, Syrah and Cabernet Sauvignon wines (2015/2016)

Year	Treatment	*t*/month	*γ*(total phenolic acids)/(mg/L)			*γ*(total procyanidins)/(mg/L)			*γ*(total flavan-3-ols)/(mg/L)			*γ*(total flavonol glycosides)/(mg/L)		
			Merlot	Syrah	Cabernet Sauvignon	Merlot	Syrah	Cabernet Sauvignon	Merlot	Syrah	Cabernet Sauvignon	Merlot	Syrah	Cabernet Sauvignon
2015	Control	0	52.48±0.22	83.36±0.05	43.17±0.21	93.06±0.38	92.55±0.14	59.97±0.26	22.04±0.05	56.78±0.24	21.14±0.11	37.29±0.35	91.15±0.55	35.81±0.32
		6	50.63±0.13	102.54±0.05	48.59±0.14	89.18±0.45	87.37±0.37	57.88±0.09	20.74±0.19	58.73±0.07	17.94±0.06	28.37±0.33	75.57±0.46	26.65±0.22
		12	51.36±0.10	106.70±0.11	50.34±0.10	67.30±0.31	74.98±0.44	45.49±0.19	17.31±0.22	105.49±0.47	18.61±0.51	16.28±0.41	42.76±0.10	13.80±0.21
	LRF	0	59.91±0.14	87.38±0.21	41.14±0.22	100.13±0.56	88.64±0.27	46.14±0.25	26.87±0.37	61.88±0.58	20.41±0.22	58.38±0.52	112.84±0.25	33.14±0.10
		6	60.41±0.56	95.74±0.18	44.74±0.78	77.29±0.59	83.09±0.49	43.77±0.35	22.02±0.04	59.27±0.57	20.33±0.22	32.70±0.20	83.89±0.41	23.01±0.13
		12	63.54±0.45	96.94±0.07	45.30±0.14	65.46±0.45	66.50±0.23	44.22±0.27	18.06±0.15	91.03±0.55	22.57±0.30	19.26±0.46	52.04±0.65	12.37±0.77
	LRV	0	63.27±0.43	82.95±0.11	47.00±0.39	112.67±0.26	93.64±0.03	56.16±0.35	28.84±0.08	64.56±0.44	17.35±0.14	49.39±0.17	111.74±0.66	29.17±0.43
		6	62.89±0.63	82.06±0.08	56.11±0.26	92.36±0.39	84.06±0.15	45.08±0.35	23.69±0.15	58.47±0.17	20.13±0.15	32.89±0.58	62.03±0.41	25.09±0.29
		12	62.26±0.03	84.80±0.37	57.13±0.42	81.34±0.57	54.32±0.17	40.14±0.20	20.00±0.13	78.14±0.81	20.54±0.20	18.34±0.33	31.89±0.09	11.48±0.43
2016	Control	0	60.50±0.22	79.89±0.09	80.98±0.69	110.71±0.59	98.17±0.06	34.71±0.29	17.22±0.15	30.48±0.08	14.67±0.23	15.34±0.25	41.44±0.39	13.28±0.17
		6	60.91±0.17	83.95±0.06	27.33±0.26	99.60±0.34	70.10±0.21	40.72±0.34	29.04±0.41	27.31±0.19	54.46±0.23	20.14±0.03	41.57±0.13	11.44±0.05
		12	74.99±0.34	108.29±0.34	60.28±0.27	67.21±0.34	56.69±0.49	41.83±0.11	32.58±0.24	24.57±0.18	65.24±0.11	21.47±0.07	37.45±0.50	9.19±0.24
	LRB	0	86.06±0.34	104.92±0.18	93.66±0.36	118.89±0.06	66.18±0.37	73.70±0.12	18.94±0.05	31.80±0.36	18.81±0.28	31.92±0.16	81.91±0.46	30.13±0.10
		6	84.09±0.54	110.49±0.12	38.94±0.18	92.80±0.16	58.76±0.86	60.65±0.38	28.97±0.02	29.96±0.48	55.36±0.65	30.24±0.12	60.68±0.11	20.36±0.27
		12	105.87±0.46	136.79±0.81	89.93±0.41	69.01±0.36	45.50±0.20	50.93±0.22	35.97±0.22	25.41±0.11	62.83±0.44	30.80±0.34	49.89±0.29	27.40±0.41
	LRV	0	74.75±0.39	78.67±0.64	81.45±0.09	124.04±0.14	65.81±0.18	46.38±0.81	22.15±0.17	37.35±0.36	17.69±0.31	22.31±0.77	61.33±0.17	24.25±0.32
		6	80.78±0.62	86.06±0.48	33.16±0.44	92.07±0.15	49.62±0.78	42.92±0.33	24.80±0.48	32.10±0.81	46.46±0.43	21.35±0.12	53.05±0.40	15.39±0.37
		12	94.30±0.79	108.85±0.39	22.83±0.24	73.83±0.51	41.95±0.22	32.02±0.38	26.87±0.64	24.99±0.29	48.14±0.22	22.67±0.64	41.72±0.09	14.02±0.18

Results are expressed as mean±S.D. (*N*=3)