# Anthocyanin and flavonol composition response to veraison leaf removal on Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese Vitis vinifera L. cultivars



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**Anthocyanin and flavonol composition response to veraison leaf removal on Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese *Vitis vinifera* L. cultivars.**

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Abstract

The elimination of a certain number of leaves around bunches before veraison is a common practice in vineyards to increase berries sunlight exposure, which, if acting in synergy with temperature increase, may affect grape anthocyanin and flavonol composition and give rise to contradictory results. The aim of this study was to analyze the effect over two years of leaf removal on anthocyanin and flavonol composition at harvest in four red *Vitis vinifera* L. varieties: Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese, characterized by different anthocyanin and flavonol profiles. The concentration of total anthocyanins in berries did not vary among control and defoliated vines in all varieties in both vintages, while total flavonols strongly increased after the treatment. Our results showed a genotype-dependent response to leaf removal that may induce a strong enhancement of the di-substituted branch of the flavonoid pathway, with consequences on anthocyanins and flavonols profile.

**Keywords:** grapevine;defoliation;anthocyanins;flavonols;sun exposure; temperature.

## 1. Introduction

Defoliation is a common crop management practice on grapevine in many viticultural regions. The elimination of a certain number of basal leaves conventionally applied in the fruiting zone from berry set to veraison, enhances air circulation, berries sunlight exposure and increases berry temperature, while reduces Botrytis bunch rot infection and increases fungicide spray penetration (English et al., 1989; Stapleton and Grant, 1992; Zoecklein et al., 1992). Especially the effects of veraison defoliation on grape composition have been shown to be strongly influenced by intensity of treatment, genotype and climatic conditions (Downey et al., 2006; Guidoni et al., 2007; Hunter et al., 1991, Matus et al., 2009). Leaf removal applied at veraison has a strong impact on bunch microclimate and a limited impact on the vine source–sink balance due to the lower photosynthetic activity of basal leaves compared to the intermediate and apical leaves at that stage

(Poni et al., 1994). In general after leaf removal, bunches are subjected to synergistic effects due to increase of light and temperature that, depending on the seasonal and climatic conditions, may affect grape composition. Several authors, mainly reporting the effects of shading on grape color, agreed that low light reduces anthocyanin and other flavonoid concentrations, while increasing light increases the flavonoid content of grapes (Crippen and Morrison, 1986 a, b; Dokoozlian and Kliewer, 1996; Hale and Buttrose, 1974; Hunter et al., 1991; Iland, 1988; Kliewer and Lider, 1968; Kliewer, 1970; Matus et al., 2009; Zoecklein et al., 1992). Further investigations into the effects of increasing light exposure on grape color gave rise to contradictory results. Some studies reported that high light levels resulted in decreased anthocyanin levels (Bergqvist et al., 2001; Pastore et al., 2013; Spayd et al., 2002), while in other cases no change was observed in total anthocyanin concentration (Downey et al., 2004; Haselgrove et al., 2000; Price et al., 1995; Spayd et al., 2002). When exposure to sunlight is associated with excessive berry temperature, as occurs in warm conditions, this may often lead to berry sunburn that has a negative impact on the color of some red berry grapevine varieties (Kliewer and Torres, 1972; Mori et al., 2005; Mori et al., 2007). It has been pointed out that the lower anthocyanin content in berries under high temperature reflects the combined impact of reduced biosynthesis and increased degradation in which the role of peroxidase enzymes in anthocyanin catabolism is probably involved (Movahed et al., 2016). The modification of bunch light exposure around veraison can also affect anthocyanin composition. As is well-known, grape anthocyanins are based on cyanidin, peonidin, delphinidin, petunidin and malvidin that are glycosylated at the third position of the B ring. The glucoside portion can be esterified with acetyl and coumaroyl compounds, giving origin to the different anthocyanins commonly found in *V. vinifera* varieties (Mazza, 1995). Several researches have shown shifts in anthocyanin composition after bunches microclimatic variation, with an increase in the di-substituted anthocyanin concentration (cyanidin and peonidin) in shaded bunches giving rise to an increased di-substituted to tri-substituted anthocyanins (delphinidin, petunidin and malvidin) ratio (Downey et al., 2004; Ristic et al., 2007; Spayd et al., 2002). Although other authors showed opposite results since bunch light exposure increased the proportion of di- respect to tri-substituted anthocyanins (Chorty et al., 2010; Guidoni et al., 2008; Tarara et al., 2008), there is agreement in the literature that greater bunch shading results in a shift toward acylated anthocyanins (Downey et al., 2004; Le Guan et al, 2016). These contradictory results also in terms of composition may be probably ascribe again to both light and temperature effects, which frequently coexist, playing a conflicting role especially in warm climatic conditions. Cabernet Sauvignon berries under high temperature showed an anthocyanin shift with a decreased proportion of di-substituted anthocyanins (Mori et al., 2005; Mori et al., 2007; Tarara et al., 2008), which are considered less stable than tri-substituted ones at high temperature. In Sangiovese these results are only slightly confirmed. In fact, berries ripened under high temperature showed similar profiles at harvest with respect to control berries, but the proportional depletion of malvidin 3-glucoside was the lowest compared to all the other glycosylate anthocyanin forms (Pastore et al., 2013).

Sunlight is known to enhance flavonol accumulation in berries (Downey et al., 2006) and recent papers focused on the effects of solar UV radiation, suggest a strong positive correlation between illumination and flavonol levels, reflecting their role as UV protectants (Carbonell-Bejerano et al., 2014, Price et al., 1995; Spayd et al., 2002). High accumulation of flavonols was also observed in different varieties subjected to leaf removal compared to controls (Lemut et al., 2013; Pereira et al., 2006 ) and this was also supported by an increase in flavonol synthase gene expression in the berries (Pastore et al., 2013).

Although in Sangiovese berries a shift in flavonol composition was registered after veraison defoliation due to higher accumulation of quercetin and kaempferol than myricetin compared to control berries (Pastore et al., 2013), studies on other cultivars have shown that the abundance of all flavonol compounds increases with the same intensity following defoliation (Spayd et al., 2002).

Considering that the profile of anthocyanins (Mattivi et al., 2006) and flavonols (Downey et al., 2003) in each variety are relatively stable over seasons and that distinctive varietal responses to light and temperature may be observed in flavonol and anthocyanin accumulation and composition in berry skin (Mattivi et al., 2006), the aim of this study was to analyze anthocyanin and flavonol composition of berries at harvest by describing the response of four red varieties, characterized by different anthocyanin and flavonol profiles, to veraison leaf removal over two years.

## 2. Material and methods

The trial was conducted in 2008 and 2009 on adult *Vitis vinifera L.* Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese vines grafted to SO4, in a vineyard with no irrigation system located in Bologna, Italy (44°30'N, 11°24'E), with north–south oriented rows. Vine spacing was 1.0 m x 3.0 m and the training system was a vertical shoot positioned spur pruned cordon (12 buds per vine), with cordon height at

1.0 m above the ground and canopy height of about 1.3-1.4 m. Pest management followed local practices in the Emilia Romagna Region. Each vine in the trial was uniformed for bud load and bunch number at flowering. Nine vines per treatment in three blocks were selected in a single uniform row and each vine was randomly assigned to the following treatments: a) control (C), no treatment; b) veraison defoliation (D), hand defoliation of six basal leaves at veraison. In the defoliation treatments, any laterals growing in the 6 basal node of the main shoot were also removed.

Defoliation treatments and harvest were performed according to the berry ripening trend in each cultivar and year as reported in Table 1.

Weather data (mean daily air temperature and rainfall) were recorded from April to September in both years, by a meteorological station located close to the experimental site.

### 2.1. Agronomic parameters at harvest

At harvest the number and weight of bunches per vine were measured. For each bunch we determined the surface areas infected by Botrytis and damaged by sunburn. During winter, the wood pruned from each vine was weighed.

### 2.2. Temperature monitoring

Berry skin temperature was monitored in 2008 and 2009 in four selected bunches on control and defoliated vines of each tested variety. For each treatment, temperature data were collected from stage 33 (beginning of bunch closure, berries touching, according to Lorenz et al., (1995) until harvest and this fluctuated for each cv: Cabernet Sauvignon and Nero d’Avola from JD 226 to 276 in 2008 and from JD 217 to 271 in 2009; Raboso Piave from JD 226 to 287 in 2008 and from JD 225 to 281 in 2009; Sangiovese from JD 211 to 265 in 2008 and from JD 210 to 261 in 2009. Eight T-type thermocouples (RS components, MI, Italy) were positioned in the sub-cuticular tissues of the berry skin. Four were positioned on two different bunches, two on the east side and two on the west side of the cordon. For each side, one thermocouple was inserted in a berry located in the external part of the bunch and the other in the internal part. Each probe was then connected to a CR10X data logger (Campbell Scientific Ltd., Leicestershire, UK) that registered temperature data every 15 minutes. In three days during August in 2008 and in 2009 for each bunch, the percentage of bunch exposure was visually estimated in three moments of the day: in the morning (9.00-9.30 a.m.), when the sun position is at its Zenith (1.30- 2.00 p.m.) and in late afternoon (5.30-6.00 p.m.).

### 2.3 Biochemical analysis

For each treatment, we collected 40 berries from each of the three vines in each block at harvest. The samples were divided into two parts. Twenty berries were weighed and immediately tested for ripening by crushing and filtering the must through a strainer for the evaluation of °Brix, titratable acidity and pH. The remaining 20 berries were used to extract anthocyanins and flavonols for HPLC analysis according to Mattivi et al. (2006).

### 2.4 Statistical analyses

Yield components and grape composition parameters were processed for each variety by analysis of variance using the mixed procedure available in SAS v9.0 (SAS Institute, Inc., Cary, NC, USA). Treatment comparisons were analyzed using the Tukey test with a cut-off at $P \leq 0.05$.

To compare anthocyanin and flavonol composition in different varieties, treatments and years multivariate analysis was applied on the data of each compound. An exploratory principal component analysis was performed separately on anthocyanins and flavonols to point out differences and any gradients.

## 3. Results

### 3.1. Climatic data and impact of defoliation on berry skin temperature

The weather during 2008 and 2009 was on the average of the area and total rainfall from April through September was very similar in the two seasons (320 mm and 317.4 mm respectively). Mean and maximum temperature (Figure 1) during the growing season in 2008 ((19.8 °C and 35.9 °C respectively) was lower than in 2009 (20.9 °C and 36.8° C respectively) and this reflected on total active heat summation calculated using base 10 °C days from April through September (1758 °C in 2008 and 2006 °C in 2009).

Sangiovese was the earliest variety for both veraison and harvest, while Raboso Piave was the latest. It should be noticed that the number of days between veraison and harvest was similar among varieties and ranged from 50 to 61.

We monitored the berry skin temperature from the application of leaf removal until harvest in the control and defoliated vines of each variety. The berries of all tested varieties in the control treatment were exposed to temperatures >30 °C for less time than in the defoliated samples with differences between the two treatments ranging from up to 70 hours to a minimum of 31 hours (for the same cv Sangiovese respectively in 2009 and 2008, Table 2). In both treatments, the number of hours with berry temperature above 30 °C was higher in

2009 than in 2008. The estimation of the percentage of bunch exposure after defoliation showed in both years an increase of around 20 % in the daily average (Table 2).

### 3.2 Vegetative and productive traits

There were only minor differences between the two years in vegetative and productive measurements at harvest following the leaf removal in all tested varieties. Starting from a uniform bunch number per vine, no differences were detected after defoliation in yield per vine or berry mass at harvest, for either variety or year. Vintage had an influence on berry mass in all varieties with higher values in 2009 than 2008 and only in Cabernet Sauvignon, an increase in yield per vine was registered in the second year (Table 3). In 2009 Raboso Piave and Sangiovese showed a significant increase in the percentage of sunburned bunches on defoliated compared with control vines, whereas only Nero d'Avola had significantly fewer bunches attacked by *Botrytis* on defoliated vines in 2009 (Table 3). It should be noted that the untreated Nero d'Avola was the most sensitive cultivar to *Botrytis*, showing the highest level of attack in 2009. Surprisingly, Sangiovese cv, despite a strong *Botrytis* incidence in 2009, did not respond to leaf removal with significant rot reduction (Table 3). Sugar concentration in must at harvest was not affected by veraison defoliation, but differed in the two vintages, while total acidity and pH in Cabernet Sauvignon, Nero d'Avola and Sangiovese were reduced and increased respectively by defoliation (Table 3).

### 3.3. Anthocyanins and flavonols

#### 3.3.1. Univariate analyses

The concentration of total anthocyanins in the berries (mg/g) did not vary among treatments at harvest in both vintages and in all varieties (Table 4). In Sangiovese, where the profile showed only traces of acetate and coumarate anthocyanins, the total concentration corresponded mainly to glycosylate anthocyanins. In Cabernet Sauvignon, Nero d'Avola and Raboso Piave the concentration of glycosylate, acetate and coumarate anthocyanins was not modified following leaf removal treatments compared to the control (Table 4).

The di-substituted to tri-substituted anthocyanins ratio significantly increased with defoliation in Nero d' Avola and Sangiovese cultivars. Raboso Piave showed a similar tendency but without significant differences between treatments, while Cabernet Sauvignon revealed an opposite trend in each year and a strong Year x Treatment interaction effect.

There were significant differences between vintages in Cabernet Sauvignon and Raboso Piave anthocyanin concentrations, with the highest level recorded in 2008. Moreover, Raboso Piave showed a clear Year x Treatment interaction for all measured compounds except acetate anthocyanins (Table 4).

The concentration of total flavonols at harvest increased significantly in defoliated berries of all varieties compared to controls in both years (Table 4).

Each variety showed a characteristic composition in control berries as quercetin is the main component in Sangiovese, myricetin is in Nero d'Avola, while Raboso Piave and Cabernet Sauvignon showed similar proportions of quercetin and myricetin. The total flavonols increase was quite similar in all varieties but each flavonol compound showed a different increment following leaf removal. The highest proportional increase concerned quercetin in Raboso Piave (Table 5).

### 3.3.2. Multivariate quantitative data

Comprehensive analysis of the total data set of anthocyanin (Figure 2) and flavonol (Figure 3) concentrations in mg per gram of berry skin of the varieties Cabernet Sauvignon, Nero D'Avola, Raboso Piave and Sangiovese in 2008 and 2009, was conducted, applying an exploratory principal component analysis separately on anthocyanins and flavonols to evaluate the distribution of single observations and rank the data.

As presented in Fig. 2, 90% of the variability due to anthocyanin concentration is accounted for the two discriminant functions. The first one accounts for 55% of the information and is mainly correlated with the concentration of cyanidin 3-glucoside and peonidin 3-glucoside on one side and malvidin 3-glucoside on the other. Sangiovese and Raboso Piave are close to each other and clearly separated from Nero d'Avola, which is near Cabernet Sauvignon, according to the first component (PC1), by bunching at positive and negative PC1 values, respectively (Figure 1). The second function (PC2) accounts for 35% of the variability and seems to be responsible for the differences between treatments and years.

Raboso Piave shows high variability and treatments are not clearly separated, while it is possible to identify a separation in Sangiovese between defoliated and control vines independently of the season. In Cabernet

Sauvignon the two years appear grouped and in Nero d'Avola the two treatments are distinguished mainly according to the second component (PC2).

The same approach was applied for flavonol concentration and the results are reported in Figure 3 where the two discriminant functions account for more than 99% of the variability. The PC1 accounts for 70.9% of the variability mainly linked to the variation in quercetin. For all varieties, it is possible to separate the control from defoliated vines according to the PC1.

The second function (PC2), which accounts for 28.8% of the variability, is dependent mainly on myricetin. According to this function, the observations allow genotype separation with Nero d'Avola and Cabernet Sauvignon mainly matched with positive values, while Sangiovese and Raboso Piave with the negative values of PC2 (Figure 3).

## 4. Discussion

### 4.1. Vegetative and productive traits

Leaves removal around bunches at veraison, implying modification in light and temperature exposure, is a powerful and widely-used strategy to improve berry bunch microclimate and to reduce rot susceptibility. The responses in berries anthocyanin and flavonol accumulation and composition following veraison defoliation could be very different and dependent on several factors including climatic conditions, leaf removal intensity, temperature increase and genotype (Bergvist et al., 2001; Spayd et al., 2002).

The four varieties included in this research, Sangiovese, Cabernet Sauvignon, Nero d'Avola and Raboso Piave, as expected did not modify vegetative and yield traits as a result of veraison leaf removal. In fact veraison defoliation, with the elimination of already senescent basal leaves, may have a limited effect on the vine source-sink balance and on berries sugar accumulation (Bledsoe et al., 1988; Pastore et al., 2013; Percival et al., 1994).

On the other hand, veraison defoliation usually had strong impact on bunches microclimatic conditions. In our study actually we estimated an average daily increase of 20% of bunch exposure in defoliated compared to control vines, in both years, while the berry temperature difference between the treatments within all cultivars and years, expressed as number of hours in which the berries overcome 30°C from veraison to harvest, never exceeded 70 hours. Moreover, during the two seasons the maximum air temperature was around 36.5 °C.

Although we did not measure the individual malic and tartaric acid fractions, we could argue that the decrease in total acidity registered following defoliation in three of the four varieties, Cabernet Sauvignon, Nero d'Avola and Sangiovese, independently of sugar concentration, is correlated to the thermal increase due to higher bunch exposure to light, since light is not known to influence malic and tartaric acid accumulation in grape tissues (Crippen and Morrison, 1986 a; Kliewer and Lider, 1968). On the contrary, temperature has been known for some time to have significant effects on berry acidity, accelerating the breakdown of malic acid (Lakso and Kliewer, 1975; Kliewer and Schultz, 1964). This hypothesis is supported by the significant differences registered in the total acidity between 2008 and 2009 in these varieties. As previously described, the temperature during the 2009 season was higher than in 2008 and consequently the acidity was lower in the second year. The fact that the acidity concentration in Raboso Piave did not decrease as a result of defoliation treatment, suggests a cultivar-dependent thermal response of acidity, as previously reported on different cultivars subjected to increased temperature regime (Bergqvist et al., 2001; Sadras et al., 2013).

The overall increase in berry mass registered in all four varieties in 2009 could be linked to the higher rainfall recorded in July of that year compared to the same period in 2008, which may have conditioned the berry cell division stage of growth and final berry mass.

### 4.2. Anthocyanins and Flavonols

The concentration of total anthocyanins in the berries did not vary among treatments at harvest in both vintages in all varieties, so it could be assumed that light conditions were appropriate for anthocyanin biosynthesis in control vines and no improvement arose from bunch light exposure at veraison. At the same time in the current study, the temperature increase following leaf removal recorded in both years did not induce a negative impact on the berry color. On the contrary, anthocyanins reduction in berries under temperature rise is reported in several articles (Downey et al., 2006; Kliewer and Torres, 1972; Mohvaed et al., 2016; Mori et al., 2005; Mori et al., 2007).

The mechanism that suppresses anthocyanin accumulation in berry skins under high-temperature ripening conditions is not completely clear, but recent evidence suggests that the low anthocyanin content in berries ripened at high temperature reflects the combined impact of reduced biosynthesis and increased degradation verified in Sangiovese (Movahed et al., 2016) and in other varieties (Yamane et al., 2006; Mori et al., 2007).

At least for Sangiovese cv which usually shows great sensitivity to thermic condition variation, we may ascribe the lack of response in terms of anthocyanins concentrations to several reasons: first in our study the temperature condition of control berries already reached a high level of heat accumulation, corresponding to more than 250 hours over 30°C degree during the ripening period, secondly the temperature differences between control and defoliated berries were quite low with a maximum of 70 hours over 30 °C. In fact in a previous research a strong anthocyanins reduction, in Sangiovese berries ripened at more than 140 hours over 30 °C in comparison to control, was found (Mohaved et al., 2016). The multivariate approach applied on the complete anthocyanin concentration data sets allowed the varieties to be differentiated independently of treatments and seasons. The association of Sangiovese and Raboso Piave and their separation from Cabernet Sauvignon and Nero d'Avola is mainly driven by their typical anthocyanin profile, featuring a higher concentration of peonidin 3-glucoside and cyanidin 3-glucoside and a lower concentration of malvidin 3-glucoside in comparison to the other two varieties. In Sangiovese, the effect of veraison defoliation on anthocyanin concentration was stable between the two vintages, causing a clear separation between control and defoliated vines due to the increase in the di-substituted to tri-substituted ratio. Instead, in Raboso Piave the effect of veraison defoliation on total anthocyanin concentration seems to be vintage dependent, with opposite behavior in each year, but with di/tri ratio followed a general tendency to increase under defoliation treatment.

Cabernet Sauvignon and Nero d'Avola share a similar anthocyanin profile characterized by a high concentration of the three forms of malvidin present in grapevine and low level of di/tri ratio and showed a general higher stability to treatments and seasons compared to Sangiovese and Raboso Piave. Despite this, the Nero d'Avola response to veraison defoliation showed an increasing trend of the di/tri ratio as verified in Sangiovese, while not steady effects were registered in Cabernet Sauvignon according to multivariate analyses. In fact, it showed a more stable behavior under the defoliation treatments but revealed slight variations according to season with lower anthocyanins concentration. This last aspect is likely due to the connection between sugar accumulation in berry flesh and anthocyanin concentration in the skin, previously pointed out in several papers regarding in vivo and in vitro experiments (Gollop et al., 2002; Pirie and Mullins, 1976; Roubelakis-Angelakis and Kliewer, 1986). In fact Cabernet Sauvignon showed a general delay in sugar accumulation in 2009 clearly linked with the higher yield level, which may be responsible for the lower anthocyanins level recorded in that year.

On the contrary in Raboso Piave, a late ripening variety, since yield level was similar in the two seasons, the lower anthocyanin concentration at harvest in 2009 may be attributed to a strong sensitivity to temperature as shown by the high level of sunburned berries registered in defoliated vines in the season with highest air temperature (Table 3). Although we did not sample sunburned berries, which often exhibit poor color development (Krasnow et al., 2010), we may argue that the same conditions of higher irradiance and temperature that induced the sunburn may be responsible for a decrease in anthocyanins as previously reported in several red berry varieties (Pastore et al., 2013; Spayd et al., 2002).

The increase of di/tri ratio after defoliation in Nero d'Avola, Sangiovese and partially in Raboso Piave cultivars seems to disagree with previous findings referring to both light and temperature increases effects (Mori et al., 2005, Tarara et al, 2008), or with other research reported that light exclusion induces an increase of the di/tri ratio compared to control bunches (Downey et al., 2004). It should be considered that in our experimental vineyard, bunches of control vines were naturally shaded and that conditions were not comparable to the one obtained through the light exclusion imposed in the cited research. Moreover, the increase of di-substituted anthocyanins we registered is not in agreement with their supposed lower stability at high temperature due to the chemical degradation hypothesis reported by several authors (Cohen et al, 2012; Mori et al., 2007). Anyway our biochemical results were supported by other researches in Sangiovese (Pastore et al., 2013) and in Nebbiolo (Guidoni et al., 2008). Moreover the hypothesis that climate variables, such as light or temperature, could repress or enhance the biosynthesis of di-substituted or tri-substituted anthocyanins is confirmed by molecular studies on Sangiovese and Kyoho grapes, in which specific responses were recorded on main genes at the split-up point of the biosynthesis of di- and tri-substituted anthocyanins (F3'H and F3'5'H) under light exposure or high temperature (Azuma et al., 2012; Movahed et al., 2016; Pastore et al., 2013). Since we did not separate the effect of temperature and light, it is not clear which of them could be responsible.

Despite the total flavonol concentration appeared very variable among the four cultivars in the study, it was very different between control and defoliated vines in all varieties in both vintages. The higher bunch exposure induced by leaf removal in comparison to control berries resulted in an increase of total flavonols in all varieties, and this effect was more evident in 2009 than in 2008. Sunlight is known to enhance flavonol accumulation in berries (Downey et al., 2006) and there is a strong positive correlation between illumination and flavonol levels, reflecting their role as UV protectants (Pastore et al., 2013; Price et al., 1995; Spayd et al., 2002). Moreover, coherently with our results, several papers reported that the level of flavonols in berries was almost negligible when they had not been exposed to light and that the subsequent exposure of those tissues to sunlight determined the rise of flavonol accumulation after the increase in the expression of the gene encoding flavonol synthase (Downey et al., 2004; Pastore et al., 2013). Previous research on Sangiovese showed that in similar light conditions, temperature increase caused strong flavonol concentration reduction, suggesting a negative effect of high temperature on flavonol synthase (Mohaved et al., 2016). In our research, the temperature rise was associated with an increase in light exposure and flavonol concentration, revealing that the influence of light is dominant on the synthesis of these compounds compared to the thermal effect, at least under the observed temperature range.

As previously described the total content and pattern of flavonols is highly variable across genotypes and our results confirm that red grape varieties like Sangiovese synthesize mainly di-substituted derivatives like quercetin (Flamini et al., 2013). In control vines, Cabernet Sauvignon and Raboso Piave have similar proportions of myricetin and quercetin, while Nero d'Avola exhibits a high concentration of myricetin. Kaempferol is present in no or low concentration in all the varieties included in this study.

The multivariate approach applied on the complete flavonol concentration data sets separated the control from defoliated vines due to the significant increase in the latter, mainly driven by the rise of quercetin which appears the compound more responsive to light, as previously reported by other authors on Tempranillo (Carbonell-Bejerano et al., 2014). In our experimental conditions, this response drives towards a reduction in the differences between the original flavonol profiles of the four varieties.

## 5. Conclusion

In our conditions, where control berries were naturally shaded and subjected to quite high level of temperature which overcome 30° C for several hours, the response of four varieties to veraison defoliation in terms of anthocyanins accumulation remain unclear. We could not exclude that the similar anthocyanin content between treatments in all varieties is caused by the higher berry temperature on defoliated vines, which may have reduced anthocyanin concentration counterbalancing the supposed enhancement due to light exposure increase.

The strong increase in flavonol concentration in all varieties under defoliation suggests that the influence of light is dominant on the synthesis of these compounds compared to the thermal effect and that they may represent a marker of berries sun exposure. Furthermore, the stimulation of the synthesis of quercetin, derived from the di-substituted branch of the flavonoids pathway, also triggers the production of cyanidin, suggesting that defoliation may induce, according to genotypes, a specific response at the split-up point of the biosynthesis of di- and tri-substituted flavonoids with consequences on the profile of both anthocyanins and flavonols. Based on the overall results obtained from univariate and multivariate analyses it appears that the relationship between anthocyanin and flavonols and veraison defoliation is very complex and depends on many factors including genotype and the synergistic or antagonistic effect of different levels and extent of both temperature and light intensity experienced by the berries.

## Figure Captions:

**Figure 1.** Seasonal trends (1 April–30 September) of diurnal air mean, maximum and minimum temperature recorded close to the trial site in (A) 2008 and (B) 2009. Vertical bars indicate daily rainfall. The Degree Days and total rainfall from 1 April to 30 September were, respectively, 1768 and 332 mm in 2008 and 2006 and 317 mm in 2009.

**Figure 2.** Principal component analysis of the total data set of anthocyanin concentrations (mg per gram of berry skin) of control (red) and defoliated (green) of Cabernet Sauvignon, Nero D'Avola, Raboso Piave and Sangiovese in 2008 (empty) and 2009 (full). The name of single anthocyanin compound responsible of cultivars, treatments and seasons scattering, are represented with arrows and asterisks. In particular each name correspond to: Malv-3-G, malvidin 3-glucoside; Malv3-G ac, malvidin-3-acetyl-glucoside; Malv 3-G coum, malvidin 3-coumaroyl glucoside; Del 3-G, delphinidin 3-Glucoside; Peo3-G, peonidin 3-glucoside; Peo3-G coum, peonidin 3-coumaroyl glucoside; Cyan 3-G, cyanidin 3-Glucoside.

**Figure 3**. Principal component analysis of the total data set of flavonols concentrations (mg per gram of berry skin) of control (red) and defoliated (green) of Cabernet Sauvignon, Nero D'Avola, Raboso Piave and Sangiovese in 2008 (empty) and 2009 (full). The name of single flavonol compound (myricetin, kaempherol and quercetin) responsible of cultivars, treatments and seasons scattering, are represented with arrows and asterisks.

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Table 1. Julian Day on which veraison defoliation treatment and harvest took place in 2008 and 2009 for Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese.

| | 2008 | | 2009 | |
|---|---|---|---|---|
| | Defoliation | Harvest | Defoliation | Harvest |
| Cabernet Sauvignon | 226 | 276 | 217 | 271 |
| Nero d'Avola | 226 | 276 | 217 | 271 |
| Raboso Piave | 226 | 287 | 225 | 281 |
| Sangiovese | 211 | 266 | 210 | 261 |

Table 2. Number of hours during which berry temperature was higher than 30 °C on control (C) and defoliated (D) vines during the experimental period. For each variety and year the period of measurements ranges from leaf removal to harvest and are as follows: Cabernet Sauvignon and Nero d'Avola from JD 226 to 276 in 2008 and from JD 217 to 271 in 2009; Raboso Piave from JD 226 to 287 in 2008 and from JD 225 to 281 in 2009; Sangiovese from JD 211 to 265 in 2008 and from JD 210 to 261 in 2009. Values represent means of eight replicates. Average of percentage of bunch exposure estimated in 2008 and 2009. For each variety and year the measurements were performed in three days during August at 9.00 am, 1.30 pm and 5.30 pm.

| Parameter | 2008 | | 2009 | | Significance | | |
|---|---|---|---|---|---|---|---|
| | C | D | C | D | Treat. | Year | Treat. x Year |
| Cabernet Sauvignon | | | | | | | |
| h>30 °C | 147 | 202 | 214 | 270 | ** | * | ns |
| Average bunch exposure (%) | 5.2 | 24.8 | 6.2 | 26.4 | ** | ns | ns |
| Nero d'Avola | | | | | | | |
| h>30 °C | 145 | 205 | 212 | 263 | ** | * | ns |
| Average bunch exposure (%) | 3.3 | 23.4 | 4.2 | 25.3 | ** | ns | ns |
| Raboso Piave | | | | | | | |
| h>30 °C | 147 | 202 | 164 | 206 | ** | * | ns |
| Average bunch exposure (%) | 2.1 | 23.8 | 3.2 | 24.6 | ** | ns | ns |
| Sangiovese | | | | | | | |
| h>30 °C | 269 | 300 | 256 | 324 | ** | * | ns |
| Average bunch exposure (%) | 5.4 | 26.4 | 6.7 | 26.3 | ** | ns | ns |

Table 3. Yield components and main grape composition parameters recorded at harvest in Cabernet Sauvignon vines subjected to defoliation at veraison (D) in comparison to control vines (C) in 2008 and 2009. Botrytis and sunburn were expressed as average percentage of surface area with symptoms for each bunch at harvest.

| Parameter | 2008 | | 2009 | | Average 2008-2009 | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | D | C | D | C | D | T | Y | T x Y |
| **Cabernet Sauvignon** | | | | | | | | | |
| Bunches /vine | 24 | 24 | 24 | 25 | 24 | 25 | ns | ns | ns |
| Yield /vine (kg) | 2.71 | 3.17 | 3.48 | 3.91 | 3.09 | 3.54 | ns | * | ns |
| Berry mass (g) | 1.27 | 1.39 | 1.69 | 1.62 | 1.48 | 1.50 | ns | *** | ** |
| Botrytis (% ) | 0.00 | 0.00 | 5.00 | 0.00 | 2.50 | 0.00 | ns | ns | ns |
| Sunburn (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | ns | ns | ns |
| Total Soluble Solids (°Brix) | 22.83 | 22.54 | 21.49 | 20.99 | 22.16 | 21.77 | ns | *** | ns |
| Titratable acidity (g/L) | 7.35 | 6.25 | 6.80 | 5.57 | 7.07 | 5.91 | *** | ** | ns |
| pH | 3.61 | 3.69 | 3.60 | 3.71 | 3.61 | 3.70 | * | ns | ns |
| **Nero d'Avola** | | | | | | | | | |
| Bunches /vine | 23 | 21 | 20 | 18 | 22 | 20 | ns | ns | ns |
| Yield /vine (kg) | 4.3 | 4.42 | 4.71 | 3.92 | 4.51 | 4.17 | ns | ns | ns |
| Berry mass (g) | 2.01 | 2.01 | 2.80 | 2.60 | 2.40 | 2.31 | ns | *** | ns |
| Botrytis (% ) | 0.00 | 0.40 | 19.00 | 3.00 | 9.50 | 1.70 | * | * | ** |
| Sunburn (%) | 0.00 | 0.00 | 0.00 | 2.00 | 0.00 | 1.00 | ns | ns | ns |
| Total Soluble Solids (° Brix) | 22.92 | 22.49 | 21.22 | 21.29 | 22.07 | 21.89 | ns | *** | ns |
| Titratable acidity (g/L) | 8.24 | 7.73 | 7.33 | 6.39 | 7.79 | 7.06 | * | *** | ns |
| pH | 3.33 | 3.36 | 3.38 | 3.48 | 3.36 | 3.42 | * | *** | ns |
| **Raboso Piave** | | | | | | | | | |
| Bunches /vine | 11 | 11 | 12 | 11 | 12 | 11 | ns | ns | ns |
| Yield /vine (kg) | 3.81 | 3.82 | 4.59 | 2.67 | 4.20 | 3.24 | ns | ns | ** |
| Berry mass (g) | 1.88 | 1.69 | 2.10 | 2.06 | 1.99 | 1.87 | * | *** | ns |
| Botrytis (% ) | 0.00 | 0.00 | 1.00 | 0.00 | 0.50 | 0.00 | ns | ns | ns |
| Sunburn (%) | 0.00 | 0.00 | 2.20 | 37.20 | 1.10 | 18.50 | ** | ** | ** |
| Total Soluble Solids (° Brix) | 22.19 | 21.50 | 22.50 | 22.26 | 22.34 | 21.88 | ns | * | ns |
| Titratable acidity (g/L) | 12.02 | 12.69 | 10.39 | 10.62 | 11.21 | 11.65 | ns | *** | ns |
| pH | 3.15 | 3.16 | 3.27 | 3.30 | 3.21 | 3.23 | ns | *** | ns |
| **Sangiovese** | | | | | | | | | |
| Bunches /vine | 17 | 16 | 16 | 16 | 16 | 16 | ns | ns | ns |
| Yield /vine (kg) | 6.33 | 5.55 | 7.08 | 5.88 | 6.71 | 5.72 | ns | ns | ns |
| Berry mass (g) | 2.37 | 2.34 | 2.65 | 2.50 | 2.51 | 2.42 | ns | * | ns |
| Botrytis (% ) | 4.90 | 2.20 | 14.50 | 11.7 | 9.70 | 6.95 | ns | ** | ns |
| Sunburn (%) | 0.30 | 6.00 | 1.20 | 13.10 | 0.75 | 9.55 | ** | ** | ns |
| Total Soluble Solids (° Brix) | 20.77 | 20.67 | 21.01 | 22.17 | 20.89 | 21.42 | ns | * | ns |
| Titratable acidity (g/L) | 7.62 | 6.65 | 6.94 | 6.20 | 7.28 | 6.42 | *** | ** | ns |
| pH | 3.38 | 3.45 | 3.43 | 3.52 | 3.41 | 3.49 | *** | ** | ns |

*, **, ***, ns indicate significance at P< 0.05. P< 0.01 and P < 0.001 or not significant, respectively.

**Tables 4**

Table. 4. Concentration of total anthocyanins, sum of glycosylate, acetate and coumarate anthocyanins (mg/g skin) and ratio between di-substituted and tri-substituted anthocyanins at harvest in Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese vines subjected to defoliation at veraison (D) in comparison to control vines (C) in 2008 and 2009.

| Parameter | 2008 | | 2009 | | Average 2008-2009 | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | D | C | D | C | D | Treat. | Year | Treat. X Year |
| **Cabernet Sauvignon** | | | | | | | | | |
| Total anthocyanins | 7.19 | 6.58 | 4.74 | 4.08 | 5.96 | 5.33 | ns | *** | ns |
| Sum of glycosylate | 4.71 | 4.14 | 2.99 | 2.54 | 3.84 | 3.34 | ns | *** | ns |
| Sum of acetate | 1.88 | 1.73 | 1.32 | 1.08 | 1.60 | 1.40 | ns | *** | ns |
| Sum of coumarate | 0.60 | 0.71 | 0.43 | 0.46 | 0.52 | 0.59 | ns | ** | ns |
| Di-Tri substituted | 0.098 | 0.113 | 0.312 | 0.120 | 0.205 | 0.117 | *** | *** | ** |
| **Nero d'Avola** | | | | | | | | | |
| Total anthocyanins | 8.30 | 8.14 | 8.53 | 8.88 | 8.42 | 8.51 | ns | ns | ns |
| Sum of glycosylate | 5.92 | 5.98 | 5.65 | 6.21 | 5.80 | 6.09 | ns | ns | ns |
| Sum of acetate | 1.12 | 1.06 | 1.13 | 1.05 | 1.12 | 1.06 | ns | ns | ns |
| Sum of coumarate | 1.26 | 1.10 | 1.75 | 1.62 | 1.50 | 1.36 | ns | *** | ns |
| Di-Tri substituted ratio | 0.076 | 0.101 | 0.059 | 0.099 | 0.068 | 0.101 | *** | * | ns |
| **Raboso Piave** | | | | | | | | | |
| Total anthocyanins | 13.39 | 10.83 | 5.45 | 8.65 | 9.42 | 9.68 | ns | *** | *** |
| Sum of glycosylate | 11.39 | 9.17 | 4.69 | 7.54 | 8.03 | 8.32 | ns | *** | *** |
| Sum of acetate | 1.21 | 1.09 | 0.34 | 0.49 | 0.78 | 0.77 | ns | ** | ns |
| Sum of coumarate | 0.79 | 0.57 | 0.42 | 0.62 | 0.61 | 0.59 | ns | ** | *** |
| Di-Tri substituted ratio | 1.031 | 1.212 | 1.387 | 1.482 | 1.209 | 1.347 | ns | ** | ns |
| **Sangiovese (1)** | | | | | | | | | |
| Total anthocyanins | 4.87 | 4.33 | 4.30 | 4.71 | 4.58 | 4.52 | ns | ns | ns |
| Di-Tri substituted ratio | 0.709 | 1.273 | 0.951 | 1.639 | 0.830 | 1.456 | *** | ** | ns |

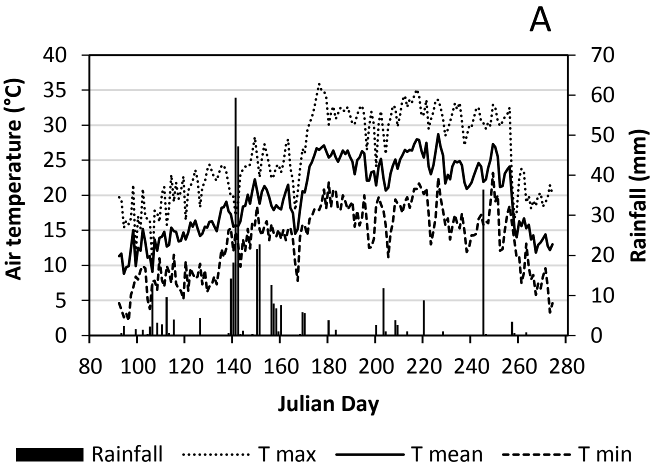
*, **, ***, ns indicate significance at P< 0.05. P< 0.01 and P < 0.001 or not significant, respectively. (1) Sangiovese has only traces of acetate anthocyanins, so the total anthocyanins are mostly glycosylate anthocyanins.

Table 5. Concentration of total and single flavonol compounds (mg/g skin) at harvest in Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese vines subjected to defoliation at veraison (D) and in control vines (C) in 2008 and 2009.

| Parameter | 2008 | | 2009 | | Average 2008-2009 | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | D | C | D | C | D | Treat. | Year | Treat. X Year |
| **Cabernet Sauvignon** | | | | | | | | | |
| Total flavonols | 0.16 | 0.35 | 0.24 | 0.62 | 0.20 | 0.48 | * | ** | ns |
| Myricetin | 0.08 | 0.16 | 0.13 | 0.26 | 0.10 | 0.21 | * | ** | ns |
| Quercetin | 0.08 | 0.17 | 0.10 | 0.30 | 0.09 | 0.23 | * | * | ns |
| Kaempferol | 0.00 | 0.02 | 0.01 | 0.06 | 0.01 | 0.04 | * | *** | ** |
| **Nero d'Avola** | | | | | | | | | |
| Total flavonols | 0.33 | 0.60 | 0.32 | 0.95 | 0.32 | 0.77 | * | *** | *** |
| Myricetin | 0.18 | 0.30 | 0.22 | 0.46 | 0.20 | 0.38 | * | *** | ** |
| Quercetin | 0.13 | 0.26 | 0.09 | 0.40 | 0.11 | 0.33 | * | * | *** |
| Kaempferol | 0.01 | 0.03 | 0.01 | 0.09 | 0.01 | 0.06 | * | *** | *** |
| **Raboso Piave** | | | | | | | | | |
| Total flavonols | 0.17 | 0.44 | 0.11 | 0.54 | 0.14 | 0.49 | ** | ns | ns |
| Myricetin | 0.10 | 0.10 | 0.04 | 0.12 | 0.07 | 0.11 | * | * | ** |
| Quercetin | 0.07 | 0.32 | 0.07 | 0.38 | 0.07 | 0.36 | ** | ns | ns |
| Kaempferol | 0.00 | 0.02 | 0.00 | 0.04 | 0.00 | 0.03 | * | * | ns |
| **Sangiovese** | | | | | | | | | |
| Total flavonols | 0.32 | 0.67 | 0.40 | 0.69 | 0.36 | 0.68 | *** | ns | ns |
| Myricetin | 0.06 | 0.07 | 0.06 | 0.08 | 0.06 | 0.07 | * | ns | ns |
| Quercetin | 0.25 | 0.57 | 0.32 | 0.55 | 0.28 | 0.56 | *** | ns | ns |
| Kaempferol | 0.01 | 0.03 | 0.02 | 0.06 | 0.02 | 0.05 | *** | ** | ns |

*, **, ***, ns indicate significance at P< 0.05. P< 0.01 and P < 0.001 or not significant, respectively.

**Figure 1**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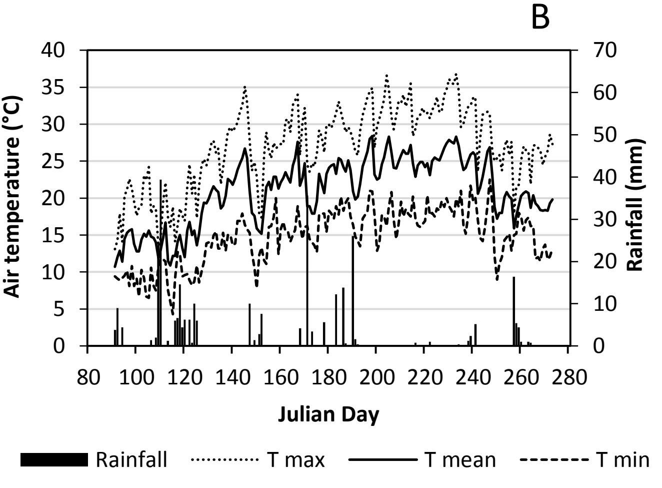
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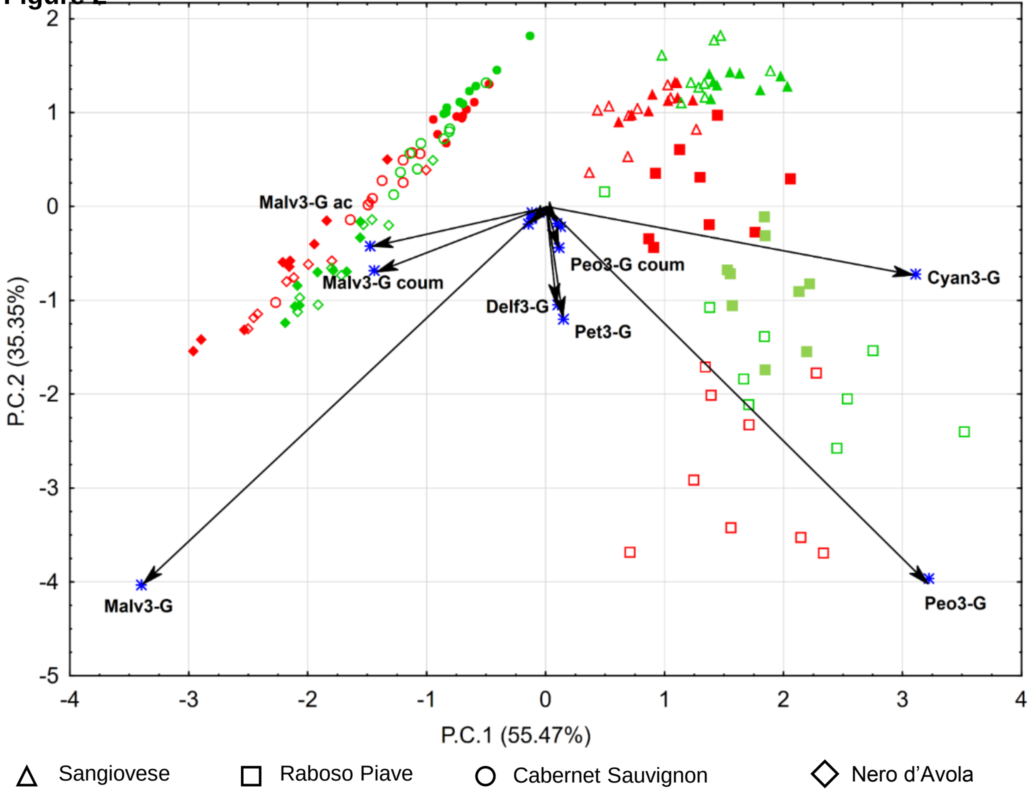
# Figure 2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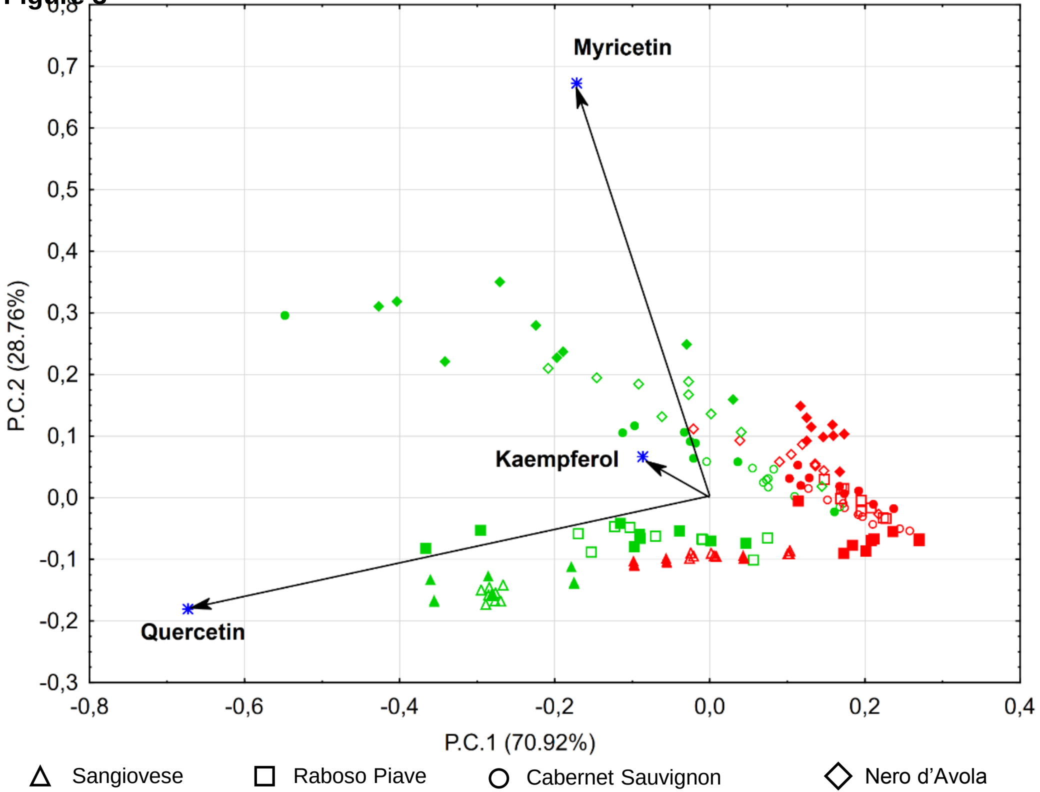
P.C.2 (35.35%)
P.C.1 (55.47%)
Malv3-G ac
Malv3-G coum
Peo3-G coum
Cyan3-G
Delf3-G
Pet3-G
Malv3-G
Peo3-G
Sangiovese
Raboso Piave
Cabernet Sauvignon
Nero d'Avola

Figure 3



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Kaempferol
Quercetin
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0,0
-0,1
-0,2
-0,3
P.C.2 (28.76%)
-0,8
-0,6
-0,4
-0,2
0,0
0,2
0,4
P.C.1 (70.92%)
Sangiovese
Raboso Piave
Cabernet Sauvignon
Nero d'Avola