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

3

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7 Abstract

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

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

19 1. Introduction

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

29 (Poni et al., 1994). In general after leaf removal, bunches are subjected to synergistic effects due to increase  
30 of light and temperature that, depending on the seasonal and climatic conditions, may affect grape  
31 composition. Several authors, mainly reporting the effects of shading on grape color, agreed that low light  
32 reduces anthocyanin and other flavonoid concentrations, while increasing light increases the flavonoid  
33 content of grapes (Crippen and Morrison, 1986 a, b; Dokoozlian and Kliewer, 1996; Hale and Buttrose,  
34 1974; Hunter et al., 1991; Iland, 1988; Kliewer and Lider, 1968; Kliewer, 1970; Matus et al., 2009;  
35 Zoecklein et al., 1992). Further investigations into the effects of increasing light exposure on grape color  
36 gave rise to contradictory results. Some studies reported that high light levels resulted in decreased  
37 anthocyanin levels (Bergqvist et al., 2001; Pastore et al., 2013; Spayd et al., 2002), while in other cases no  
38 change was observed in total anthocyanin concentration (Downey et al., 2004; Haselgrove et al., 2000; Price  
39 et al., 1995; Spayd et al., 2002). When exposure to sunlight is associated with excessive berry temperature,  
40 as occurs in warm conditions, this may often lead to berry sunburn that has a negative impact on the color of  
41 some red berry grapevine varieties (Kliewer and Torres, 1972; Mori et al., 2005; Mori et al., 2007). It has  
42 been pointed out that the lower anthocyanin content in berries under high temperature reflects the combined  
43 impact of reduced biosynthesis and increased degradation in which the role of peroxidase enzymes in  
44 anthocyanin catabolism is probably involved (Movahed et al., 2016). The modification of bunch light  
45 exposure around veraison can also affect anthocyanin composition. As is well-known, grape anthocyanins  
46 are based on cyanidin, peonidin, delphinidin, petunidin and malvidin that are glycosylated at the third  
47 position of the B ring. The glucoside portion can be esterified with acetyl and coumaroyl compounds, giving  
48 origin to the different anthocyanins commonly found in *V. vinifera* varieties (Mazza, 1995). Several  
49 researches have shown shifts in anthocyanin composition after bunches microclimatic variation, with an  
50 increase in the di-substituted anthocyanin concentration (cyanidin and peonidin) in shaded bunches giving  
51 rise to an increased di-substituted to tri-substituted anthocyanins (delphinidin, petunidin and malvidin) ratio  
52 (Downey et al., 2004; Ristic et al., 2007; Spayd et al., 2002). Although other authors showed opposite results  
53 since bunch light exposure increased the proportion of di- respect to tri-substituted anthocyanins (Chorty et  
54 al., 2010; Guidoni et al., 2008; Tarara et al., 2008), there is agreement in the literature that greater bunch  
55 shading results in a shift toward acylated anthocyanins (Downey et al., 2004; Le Guan et al, 2016). These  
56 contradictory results also in terms of composition may be probably ascribe again to both light and

57 temperature effects, which frequently coexist, playing a conflicting role especially in warm climatic  
58 conditions. Cabernet Sauvignon berries under high temperature showed an anthocyanin shift with a  
59 decreased proportion of di-substituted anthocyanins (Mori et al., 2005; Mori et al., 2007; Tarara et al., 2008),  
60 which are considered less stable than tri-substituted ones at high temperature. In Sangiovese these results are  
61 only slightly confirmed. In fact, berries ripened under high temperature showed similar profiles at harvest  
62 with respect to control berries, but the proportional depletion of malvidin 3-glucoside was the lowest  
63 compared to all the other glycosylate anthocyanin forms (Pastore et al., 2013).

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

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

74 Considering that the profile of anthocyanins (Mattivi et al., 2006) and flavonols (Downey et al., 2003) in  
75 each variety are relatively stable over seasons and that distinctive varietal responses to light and temperature  
76 may be observed in flavonol and anthocyanin accumulation and composition in berry skin (Mattivi et al.,  
77 2006), the aim of this study was to analyze anthocyanin and flavonol composition of berries at harvest by  
78 describing the response of four red varieties, characterized by different anthocyanin and flavonol profiles, to  
79 veraison leaf removal over two years.

## 80 **2. Material and methods**

81 The trial was conducted in 2008 and 2009 on adult *Vitis vinifera L.* Cabernet Sauvignon, Nero d'Avola,  
82 Raboso Piave and Sangiovese vines grafted to SO4, in a vineyard with no irrigation system located in  
83 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  
84 training system was a vertical shoot positioned spur pruned cordon (12 buds per vine), with cordon height at

85 1.0 m above the ground and canopy height of about 1.3-1.4 m. Pest management followed local practices in  
86 the Emilia Romagna Region. Each vine in the trial was uniformed for bud load and bunch number at  
87 flowering. Nine vines per treatment in three blocks were selected in a single uniform row and each vine was  
88 randomly assigned to the following treatments: a) control (C), no treatment; b) veraison defoliation (D), hand  
89 defoliation of six basal leaves at veraison. In the defoliation treatments, any laterals growing in the 6 basal  
90 node of the main shoot were also removed.

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

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

### 95 **2.1. Agronomic parameters at harvest**

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

### 99 **2.2. Temperature monitoring**

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

### 113 **2.3 Biochemical analysis**

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

### 119 **2.4 Statistical analyses**

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

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

## 126 **3. Results**

### 127 **3.1. Climatic data and impact of defoliation on berry skin temperature**

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

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

136 We monitored the berry skin temperature from the application of leaf removal until harvest in the control and  
137 defoliated vines of each variety. The berries of all tested varieties in the control treatment were exposed to  
138 temperatures  $>30$  °C for less time than in the defoliated samples with differences between the two treatments  
139 ranging from up to 70 hours to a minimum of 31 hours (for the same cv Sangiovese respectively in 2009 and  
140 2008, Table 2). In both treatments, the number of hours with berry temperature above 30 °C was higher in

141 2009 than in 2008. The estimation of the percentage of bunch exposure after defoliation showed in both  
142 years an increase of around 20 % in the daily average (Table 2).

143

### 144 **3.2 Vegetative and productive traits**

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

### 158 **3.3. Anthocyanins and flavonols**

#### 159 **3.3.1. Univariate analyses**

160 The concentration of total anthocyanins in the berries (mg/g) did not vary among treatments at harvest in  
161 both vintages and in all varieties (Table 4). In Sangiovese, where the profile showed only traces of acetate  
162 and coumarate anthocyanins, the total concentration corresponded mainly to glycosylate anthocyanins. In  
163 Cabernet Sauvignon, Nero d'Avola and Raboso Piave the concentration of glycosylate, acetate and  
164 coumarate anthocyanins was not modified following leaf removal treatments compared to the control (Table  
165 4).

166 The di-substituted to tri-substituted anthocyanins ratio significantly increased with defoliation in Nero d'  
167 Avola and Sangiovese cultivars. Raboso Piave showed a similar tendency but without significant differences

168 between treatments, while Cabernet Sauvignon revealed an opposite trend in each year and a strong Year x  
169 Treatment interaction effect.

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

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

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

### 180 **3.3.2. Multivariate quantitative data**

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

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

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

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

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

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

#### 205 **4. Discussion**

##### 206 **4.1. Vegetative and productive traits**

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

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

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

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

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

#### 239 **4.2. Anthocyanins and Flavonols**

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

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

251 At least for Sangiovese cv which usually shows great sensitivity to thermic condition variation, we may  
252 ascribe the lack of response in terms of anthocyanins concentrations to several reasons: first in our study the  
253 temperature condition of control berries already reached a high level of heat accumulation, corresponding to  
254 more than 250 hours over 30°C degree during the ripening period, secondly the temperature differences  
255 between control and defoliated berries were quite low with a maximum of 70 hours over 30 °C. In fact in a  
256 previous research a strong anthocyanins reduction, in Sangiovese berries ripened at more than 140 hours  
257 over 30 °C in comparison to control, was found (Mohaved et al., 2016). The multivariate approach applied  
258 on the complete anthocyanin concentration data sets allowed the varieties to be differentiated independently  
259 of treatments and seasons. The association of Sangiovese and Raboso Piave and their separation from  
260 Cabernet Sauvignon and Nero d'Avola is mainly driven by their typical anthocyanin profile, featuring a  
261 higher concentration of peonidin 3-glucoside and cyanidin 3-glucoside and a lower concentration of malvidin  
262 3-glucoside in comparison to the other two varieties. In Sangiovese, the effect of veraison defoliation on  
263 anthocyanin concentration was stable between the two vintages, causing a clear separation between control  
264 and defoliated vines due to the increase in the di-substituted to tri-substituted ratio. Instead, in Raboso Piave  
265 the effect of veraison defoliation on total anthocyanin concentration seems to be vintage dependent, with  
266 opposite behavior in each year, but with di/tri ratio followed a general tendency to increase under defoliation  
267 treatment.

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

278 delay in sugar accumulation in 2009 clearly linked with the higher yield level, which may be responsible for  
279 the lower anthocyanins level recorded in that year.

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

287 The increase of di/tri ratio after defoliation in Nero d'Avola, Sangiovese and partially in Raboso Piave  
288 cultivars seems to disagree with previous findings referring to both light and temperature increases effects  
289 (Mori et al., 2005, Tarara et al, 2008), or with other research reported that light exclusion induces an increase  
290 of the di/tri ratio compared to control bunches (Downey et al., 2004). It should be considered that in our  
291 experimental vineyard, bunches of control vines were naturally shaded and that conditions were not  
292 comparable to the one obtained through the light exclusion imposed in the cited research. Moreover, the  
293 increase of di-substituted anthocyanins we registered is not in agreement with their supposed lower stability  
294 at high temperature due to the chemical degradation hypothesis reported by several authors (Cohen et al,  
295 2012; Mori et al., 2007). Anyway our biochemical results were supported by other researches in Sangiovese  
296 (Pastore et al., 2013) and in Nebbiolo (Guidoni et al., 2008). Moreover the hypothesis that climate variables,  
297 such as light or temperature, could repress or enhance the biosynthesis of di-substituted or tri-substituted  
298 anthocyanins is confirmed by molecular studies on Sangiovese and Kyoho grapes, in which specific  
299 responses were recorded on main genes at the split-up point of the biosynthesis of di- and tri-substituted  
300 anthocyanins (F3'H and F3'5'H) under light exposure or high temperature (Azuma et al., 2012; Movahed et  
301 al., 2016; Pastore et al., 2013). Since we did not separate the effect of temperature and light, it is not clear  
302 which of them could be responsible.

303 Despite the total flavonol concentration appeared very variable among the four cultivars in the study, it was  
304 very different between control and defoliated vines in all varieties in both vintages. The higher bunch  
305 exposure induced by leaf removal in comparison to control berries resulted in an increase of total flavonols

306 in all varieties, and this effect was more evident in 2009 than in 2008. Sunlight is known to enhance flavonol  
307 accumulation in berries (Downey et al., 2006) and there is a strong positive correlation between illumination  
308 and flavonol levels, reflecting their role as UV protectants (Pastore et al., 2013; Price et al., 1995; Spayd et  
309 al., 2002). Moreover, coherently with our results, several papers reported that the level of flavonols in berries  
310 was almost negligible when they had not been exposed to light and that the subsequent exposure of those  
311 tissues to sunlight determined the rise of flavonol accumulation after the increase in the expression of the  
312 gene encoding flavonol synthase (Downey et al., 2004; Pastore et al., 2013). Previous research on  
313 Sangiovese showed that in similar light conditions, temperature increase caused strong flavonol  
314 concentration reduction, suggesting a negative effect of high temperature on flavonol synthase (Mohaved et  
315 al., 2016). In our research, the temperature rise was associated with an increase in light exposure and  
316 flavonol concentration, revealing that the influence of light is dominant on the synthesis of these compounds  
317 compared to the thermal effect, at least under the observed temperature range.

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

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

## 328 **5. Conclusion**

329 In our conditions, where control berries were naturally shaded and subjected to quite high level of  
330 temperature which overcome 30° C for several hours, the response of four varieties to veraison defoliation in  
331 terms of anthocyanins accumulation remain unclear. We could not exclude that the similar anthocyanin  
332 content between treatments in all varieties is caused by the higher berry temperature on defoliated vines,

333 which may have reduced anthocyanin concentration counterbalancing the supposed enhancement due to light  
334 exposure increase.

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

345

#### 346 **Figure Captions:**

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

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

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

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- 1 Table 1. Julian Day on which veraison defoliation treatment and harvest took place in 2008 and 2009 for
- 2 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

3

1 Table 2. Number of hours during which berry temperature was higher than 30 °C on control (C) and  
 2 defoliated (D) vines during the experimental period. For each variety and year the period of measurements  
 3 ranges from leaf removal to harvest and are as follows: Cabernet Sauvignon and Nero d'Avola from JD 226  
 4 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  
 5 to 281 in 2009; Sangiovese from JD 211 to 265 in 2008 and from JD 210 to 261 in 2009. Values represent  
 6 means of eight replicates. Average of percentage of bunch exposure estimated in 2008 and 2009. For each  
 7 variety and year the measurements were performed in three days during August at 9.00 am, 1.30 pm and 5.30  
 8 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

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

5

Parameter	2008		2009		Average 2008-2009		Significance		
	C	D	C	D	C	D	T	Y	T x Y
<b>Cabernet Sauvignon</b>									
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
Titrateable 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
<b>Nero d'Avola</b>									
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
Titrateable 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
<b>Raboso Piave</b>									
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
Titrateable 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
<b>Sangiovese</b>									
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
Titrateable 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

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

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1 Table. 4. Concentration of total anthocyanins, sum of glycosylate, acetate and coumarate anthocyanins (mg/g  
 2 skin) and ratio between di-substituted and tri-substituted anthocyanins at harvest in Cabernet Sauvignon,  
 3 Nero d'Avola, Raboso Piave and Sangiovese vines subjected to defoliation at veraison (D) in comparison to  
 4 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
<b>Cabernet Sauvignon</b>									
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	***	***	**
<b>Nero d'Avola</b>									
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
<b>Raboso Piave</b>									
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
<b>Sangiovese (1)</b>									
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

5 \*, \*\*, \*\*\*, ns indicate significance at  $P < 0.05$ .  $P < 0.01$  and  $P < 0.001$  or not significant, respectively. (1) Sangiovese has  
 6 only traces of acetate anthocyanins, so the total anthocyanins are mostly glycosylate anthocyanins.  
 7

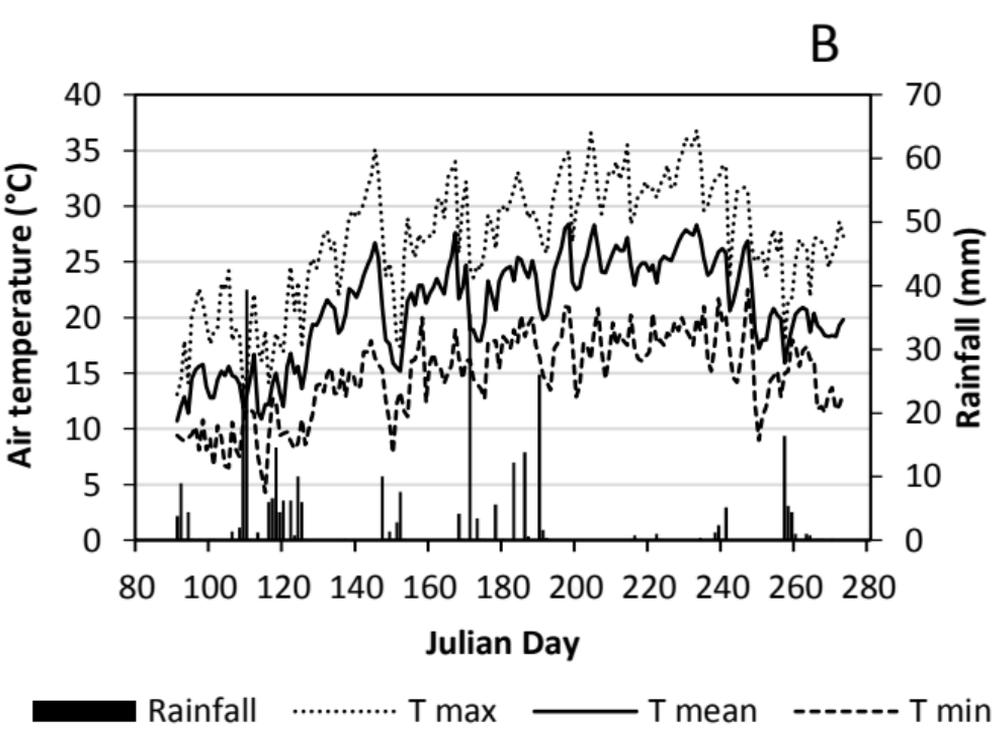
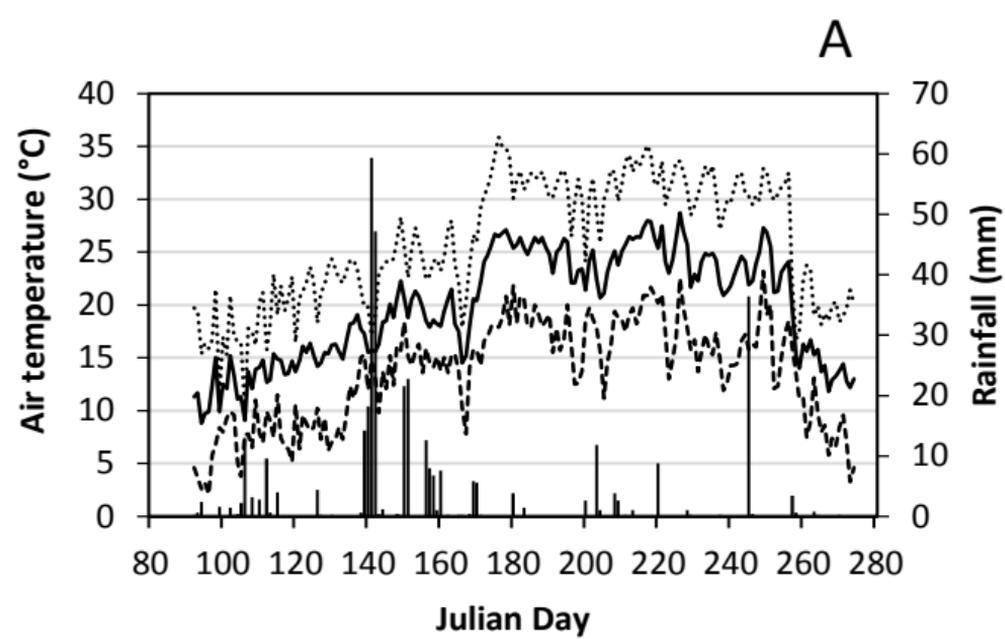
1 Table 5. Concentration of total and single flavonol compounds (mg/g skin) at harvest in Cabernet Sauvignon,  
 2 Nero d'Avola, Raboso Piave and Sangiovese vines subjected to defoliation at veraison (D) and in control  
 3 vines (C) in 2008 and 2009.

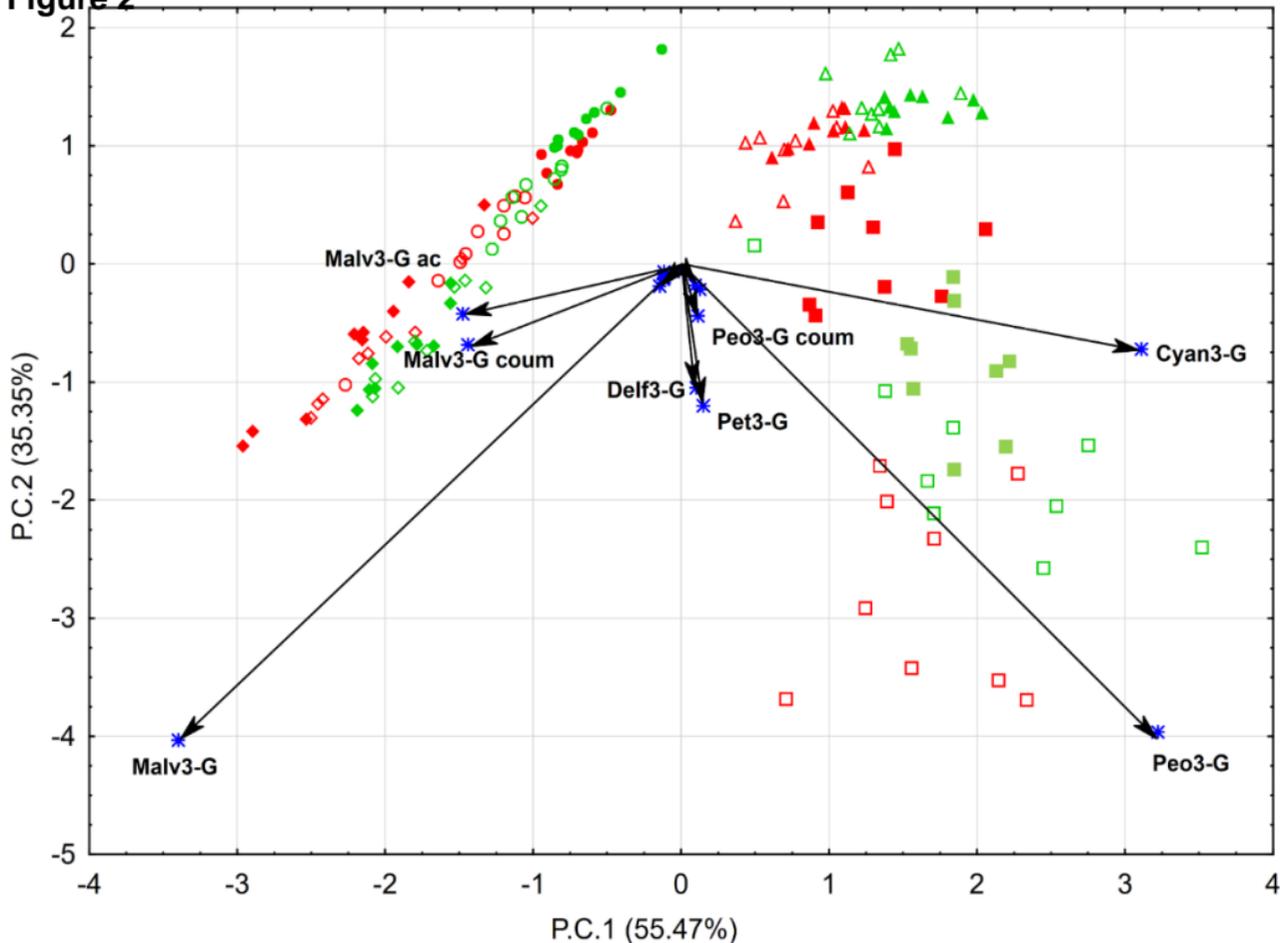
Parameter	2008		2009		Average 2008-2009		Significance		
	C	D	C	D	C	D	Treat.	Year	Treat. X Year
<b>Cabernet Sauvignon</b>									
Total flavonols	0.16	0.35	0.24	0.62	0.20	0.48	*	**	ns
Myricetin	0.08	0.16	0.13	0.26	0.10	0.21	*	**	ns
Quercetin	0.08	0.17	0.10	0.30	0.09	0.23	*	*	ns
Kaempferol	0.00	0.02	0.01	0.06	0.01	0.04	*	***	**
<b>Nero d'Avola</b>									
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	*	***	***
<b>Raboso Piave</b>									
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
<b>Sangiovese</b>									
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

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

5

**Figure 1**



**Figure 2**

△ Sangiovese

□ Raboso Piave

○ Cabernet Sauvignon

◇ Nero d'Avola

**Figure 3**

