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**CONSTITUTIVE POLYPHENOLS IN BLADES AND VEINS OF GRAPEVINE (*Vitis vinifera*, L.) HEALTHY LEAVES.**

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1 ABSTRACT

2 Despite the economic importance and the diffusion of grapevine cultivation worldwide, little is known  
3 about leaf chemical composition. We characterized the phenolic composition of Nebbiolo, Barbera,  
4 Pinot noir, Cabernet Sauvignon, Grenache and Shiraz (*Vitis vinifera* L.) healthy leaves (separating blades  
5 and veins) during the season. Quantitative and qualitative differences were found between leaf sectors  
6 and among genotypes. In healthy grapevine leaves, anthocyanins, dihydromyricetin-rhamnoside,  
7 hexosides of dihydroquercetin and dihydrokaempferol exclusively accumulated in veins. Astilbin was  
8 the only flavanone detected in blades and the prevalent flavanone in veins. Barbera distinguished for  
9 the lowest proanthocyanidin and the highest hydroxycinnamate content; Pinot noir for the absence of  
10 acylated-anthocyanins. Nebbiolo, Pinot noir and Cabernet Sauvignon displayed high concentration of  
11 epigallocatechin gallate. Nebbiolo leaves showed the highest concentrations of flavanones and the  
12 widest profile differentiation. Knowledge derived from the present work is a contribution to find out leaf  
13 polyphenol potential as a part of grapevine defense mechanisms and to dissect genotype-related  
14 susceptibility to pathogens; moreover, it represents a starting point for future deepening about grapevine  
15 and vineyard by-products as a source of bioactive phenolic compounds.

16 **KEYWORDS:** anthocyanins, flavonols, hydroxycinnamic acids, flavan-3-ols, flavanones, HPLC-  
17 DAD-UV-MS/MS.

## 18 INTRODUCTION

19 Grapevine (*Vitis vinifera* L.), one of the most widely cultivated plant species worldwide,  
20 comprises 5000 to 10000 varieties<sup>1</sup> and it plays important role in the economy of many countries due to  
21 wine, and fresh and dry grape production. Grapevine vegetative organs (shoots, stems and leaves) are  
22 used in traditional plant-based medicine as a source of bioactive compounds.<sup>2,3</sup> According to recent  
23 studies, grapevine leaves have beneficial effect on human health due to their anti-inflammatory,  
24 antibacterial, anticancerogenic, antiviral, antioxidant properties.<sup>4</sup> In the Middle East and Mediterranean  
25 regions, grapevine leaves are commonly used as food both in fresh and brined forms.<sup>5</sup>

26 Grapevines produce large amount of secondary metabolites, including chemically heterogeneous  
27 phenolic compounds. Due to this huge diversity, each group of phenolic compounds displays various  
28 roles in grapevine biology and ecology, conferring them a key role in grapevine adaptation to the  
29 environment. Polyphenols are part of the plant-defence mechanisms relying on molecular  
30 communication among plants and pathogens, involving signals for the establishment of infection, the  
31 activation of plant disease- resistance genes, the formation of elicitors, the activation of elicitor receptors  
32 and, finally gene regulation. In many of these steps phenylalanine ammonia lyase (PAL) and the  
33 chalcone synthase genes (CHs) are suppressed or over-expressed, resulting in the modulation of the  
34 accumulation of main classes of polyphenols.<sup>6</sup> Accumulation of polyphenols varies among plant organs,  
35 tissues and phenological stages. Many traits of the phenolic compound biosynthesis in grapevine berries  
36 are well detailed and it is well-known that they are under genetic control, even though external abiotic  
37 or biotic factors can influence polyphenolic concentrations and, sometimes, profiles. At the berry level  
38 the wide differences in the polyphenolic composition of *Vitis vinifera* varieties and clones have been  
39 investigated.<sup>7-9</sup> Polyphenol accumulation and profiles are influenced by seasonal climatic conditions,  
40 biotic and abiotic stressors, soil and cultural practices. Nevertheless, some traits are genetically  
41 determined, thus specific quantitative and qualitative chemical patterns characterize *Vitis vinifera*  
42 varieties. In berries, the ratio between tri-hydroxylated and di-hydroxylated anthocyanins and the ratio

43 between caftaric acid and coumaric acid are stable and they have long time been proposed as tools to  
44 classify *Vitis vinifera* varieties and clones.<sup>7-9</sup> Much less is known about vegetative organ polyphenolic  
45 composition, even though specific molecules or groups of molecules could be responsible of the inner  
46 and constitutive biochemical protection of the vine against various abiotic and biotic stressors.<sup>10</sup>  
47 Increasing knowledge about constitutive leaf polyphenols could be pivotal to explain the different level  
48 of susceptibility to pathogens displayed by *Vitis vinifera* genotypes. Different compositional traits and  
49 changes during the season in leaf compartments (blades and veins) can provide new insights about the  
50 interpretation of plant interaction with pathogens specifically accumulating in these two different leaf  
51 sectors. The present work investigates the polyphenolic concentration and profiles of grapevine leaves  
52 during the vegetative season to individuate characteristic chemical patterns in some *Vitis vinifera*  
53 varieties and to explore their constitutive accumulation as a part of grapevine defense potential  
54 mechanism. To provide new insights about concentrations, profiles and trends of main polyphenols in  
55 different leaf tissue, we analyzed blades and veins separately to spread light, in particular, on the vein  
56 constitutive polyphenols that could help to understand the different susceptibility of *Vitis vinifera*  
57 varieties to pathogens with vascular localization. To our knowledge, little is known about the  
58 polyphenolic characterization of *Vitis vinifera* leaf blades and veins analyzed separately and about their  
59 evolution during the vegetative season. Leaf polyphenols were analyzed spectrophotometrically and by  
60 targeted analytical approach using HPLC-DAD for quantitative or semi-quantitative purposes and  
61 HPLC-ESI-MS/MS for molecular identification.

## 62 MATERIALS AND METHODS

### 63 **Plant material**

64 The leaves of two major Italian varieties (Barbera - BR and Nebbiolo - NE) and of four  
65 international varieties (Pinot noir - PN, Cabernet Sauvignon - CS, Grenache - GR and Shiraz - SH) were  
66 sampled in the collection vineyard of DISAFA, University of Turin located at Grugliasco (45°03'N,  
67 7°35'E; in Piedmont, Italy), in 2015. Vine density was 4400 vines/ha (0.90 m x 2.50 m), vines were

68 planted in 2008, vertical shoot positioned and trained to the Guyot pruning system. The vineyard is  
69 located at 293 m above s.l., in a plain area. A detailed soil description is reported in Catoni et al.<sup>11</sup>  
70 Briefly, the A horizon pH was 7.9, organic C was 14.8 g kg<sup>-1</sup>, sand was 882 g kg<sup>-1</sup>, silt was 101 g kg<sup>-1</sup>  
71 and clay 17 g kg<sup>-1</sup>. The vineyard was organized in randomized blocks of maximum twelve vines each.  
72 Leaf samples were collected at five different time points: 1 = 22<sup>th</sup> of May (142 day of the year, DOY  
73 142), 2 = 2<sup>nd</sup> of July (DOY 183), 3 = 16<sup>th</sup> of July (DOY 197), 4 = 29<sup>th</sup> of July (DOY 210), 5 = 26<sup>th</sup> of  
74 August (DOY 238) in 2015. The general meteorological parameters of the vineyard are reported in  
75 Supplementary Table 1. Three adult healthy leaves between the fourth and the seventh node of main  
76 shoots per each block were collected from the west side of the row and immediately transported to the  
77 laboratory where leaves were rinsed, dried with a paper before blades and veins separation and  
78 extraction.

#### 79 **Dry matter content**

80 Leaf tissue dry matter was measured gravimetrically by drying inside an oven at 110 °C for 72  
81 hours.

#### 82 **Sample extraction**

83 Notwithstanding the well-known effects of water content on polyphenol final concentrations, we  
84 decided to work on fresh leaves, immersing blades and veins in an appropriate and specifically chosen  
85 extraction solvent (see below) soon after picking as freeze drying, including lyophilisation, can  
86 imperfectly preserve plant secondary metabolites, particularly polyphenols, as previously reviewed.<sup>12</sup>  
87 To ascertain the most adequate extraction solvent for leaf polyphenol analyses, we extracted three  
88 biological replicates of Nebbiolo blades and veins in seven different solvents: CH<sub>3</sub>OH 80%; CH<sub>3</sub>OH  
89 80%/HCl 0.1%; acetone 50%; acetone 50%/HCl 0.1%; phosphate-citrate buffer (pH 3.6); hydroalcoholic  
90 buffer (ethanol 12%, pH 3.2) and hydroalcoholic buffer (ethanol 40%, pH 3.9). This last gave the best  
91 results (see Results) thus two grams of leaf blades and two grams of leaf veins were extracted in 25 mL  
92 of this pH 3.9 hydroalcoholic buffer (40% ethanol, 2 g/L of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 5g/L of tartaric acid, 22 mL/L of

93 1 N NaOH). The samples were homogenized with an Ultraturrax dispersing machine (IKA, Staufen,  
94 Germany), centrifuged for 10 min at 4000 rpm. The supernatant was separated and kept in the dark. The  
95 pellet was re-suspended in 20 mL of the same buffer; the resuspension was macerated for 30 minutes at  
96 room temperature in the dark and then centrifuged again. The two extracts were combined and brought  
97 to a final volume of 50 mL. Extracts were stored at -20 °C until further analysis.

## 98 **Reagents and Standards**

99 Bovine serum albumin (BSA), sodium hydroxide, triethanolamine (TEA), and urea were  
100 purchased from Sigma-Aldrich S.r.l. (Milan, Italy). Folin-Ciocalteu reagent and tartaric acid were  
101 purchased from Merck (Darmstadt, Germany). Sodium sulfate and sodium metabisulfite were purchased  
102 from BDH Laboratory Supplies (Poole, England). Quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide,  
103 kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucuronide, myricetin 3-*O*-glucoside, isorhamnetin 3-*O*-  
104 glucoside, malvidin 3-*O*-glucoside, (+)- catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-  
105 epigallocatechin gallate, proanthocyanidin B<sub>1</sub> and proanthocyanidin B<sub>2</sub> were purchased from  
106 Extrasynthèse (Genay, France). Astilbin and *trans*-caftaric acid were purchased from Sigma-Aldrich  
107 S.r.l. (Milan, Italy); *trans*-fertaric acid and *trans*-coutaric acid were purchased from Phytolab  
108 (Vestenbergsgreuth, Germany).

## 109 **Spectrophotometric analyses**

110 Total polyphenols (TP) in grapevine leaves were measured with the Folin-Ciocalteu reagent.  
111 Absorbance was read at 760 nm in a UV/Vis spectrophotometer (Perkin Elmer, Lambda 25,  
112 Beaconsfield, Bucks, U.K.) and TP were expressed as grams of (+)-catechin equivalents (CE) per kg of  
113 leaf blade/vein fresh weight (FW).

114 Measurement of total proanthocyanidins (PA) in leaves was performed spectrophotometrically  
115 by the improved protein precipitation method of Harbertson et al.<sup>13</sup> Briefly, 1 mL of BSA protein  
116 solution was added to 500 µL of sample extract for PA-protein precipitation. Buffer containing 5% of  
117 triethanol amine (TEA, v/v) and 5% of urea (w/v) was used for dissolving PA-protein pellet after

118 centrifugation and to support the colorimetric reaction with ferric chloride. Background and final  
119 absorbances were measured at 510 nm and sample absorbance was determined by subtracting the  
120 background absorbance from the final reading. The results were expressed as grams of (+)-catechin  
121 equivalents (CE) per kg of leaf blades/veins FW.

## 122 **Analyses of anthocyanins**

### 123 *Sample preparation*

124 Anthocyanin leaf extracts were retained on a Sep-Pak C18 silica-based bonded phase cartridge  
125 (Waters Corp., WAT051910, Milford, USA) and eluted with methanol. The methanolic solution was  
126 evaporated to dryness in a rotary evaporator (Laborata 4000, Heidolph Instruments GmbH & Co. KG,  
127 Schwabach, Germany). The extracts were re-suspended with solvent B and passed through 0.20 µm  
128 membrane filter GHP Acrodisc® (PALL Italia, Buccinasco, Milano, Italy).

### 129 *Qualitative analyses of anthocyanins by HPLC-DAD-ESI-MS/MS*

130 Samples were analyzed by liquid chromatography (1200 HPLC, Agilent Technologies, USA)  
131 equipped with a Luna reverse phase C-18 column (3.00 µm, 150 mm × 3.0 mm, Phenomenex, USA).  
132 The instrument was equipped with a binary solvent pump with the following solvents: (A) MilliQ water  
133 (Millipore, U.S.A.) with 10% v/v of formic acid and (B), methanol/water/formic acid 50/40/10 v/v. The  
134 chromatographic separation was carried out at a constant flow rate (200 µl min<sup>-1</sup>) and to a stepwise  
135 gradient: from 15% to 45% of B in 15 min, to 70% of B at 35 min, to 90% of B at 45 min, then 99% of  
136 B at 55 min, hold for 4 min. The initial mobile phase was re-established for 11 min before the next  
137 injection. DAD detector was set at 520 nm. The mass spectrometry analyses were performed with a 6330  
138 Series Ion Trap LC-MS System (Agilent Technologies, U.S.A.) equipped with an electrospray  
139 ionization source (ESI) operating in positive mode. Qualitative analyses were performed in scan mode  
140 (100–850 m/z) and N<sub>2</sub> dry gas temperature was set at 325°C. Mass spectra were processed and analyzed  
141 by the DataAnalysis for 6330 Series Ion Trap LC/MS 4.0 software (Bruker Daltonik, Bremen, Germany).



142 Identification of spectra was done by analysis of fragmentation pattern and by comparison with literature  
143 data.

#### 144 *Quantitative analyses of anthocyanins by HPLC-DAD*

145 HPLC-DAD analysis was carried out by an Agilent 1200 Series system (Agilent, Waldbronn,  
146 Germany), equipped with a DAD detector (G1316A). Twenty  $\mu\text{L}$  of samples were injected on a reverse-  
147 phase column Purospher® STAR RP-18 endcapped (5  $\mu\text{m}$ ) packed into LiChroCART 250-4 HPLC-  
148 Cartridge (25  $\times$  0.4 cm ID; Merck KGaA, Germany) with a guard column LiChroCART 4-4 of the same  
149 packing material. Solvent A was 10% of formic acid and solvent B was water/methanol/formic acid  
150 (40:50:10, v/v/v), the flow rate was 1 mL/min with a gradient from 28% to 72% of B in 63 minutes.  
151 Individual anthocyanins were detected at 520 nm. Results were expressed as milligrams of malvidin 3-  
152 *O*-glucoside chloride equivalent per kg of leaf blade/vein fresh weight.

#### 153 **Analyses of individual phenolic compounds**

##### 154 *Sample preparation*

155 Leaf extracts were diluted with 1 M phosphoric acid (1.1 fold) and filtered (0.20  $\mu\text{m}$ ) into the  
156 vials.<sup>14</sup>

##### 157 *Qualitative analyses phenolic compounds by HPLC-DAD-ESI-MS/MS*

158 A Bruker Daltonics esquire 3000<sup>plus</sup> ion trap spectrometer (Bruker Daltonics, HB, Germany)  
159 equipped with an Agilent 1100 HPLC-DAD system (Agilent Tech. Inc., CA, USA) was used for  
160 individual phenolic compound identification. Component separation was done with column Luna C-18  
161 150  $\times$  2 mm (Phenomex Aschaffenburg, Germany). For mobile phase solvent A was water/0.1% formic  
162 acid and solvent B was methanol/0.1% formic acid; gradient program was as follows: 0-30 min 0-50%  
163 B, 30-35 min 50-100% B, 35-50 min 100% B, 50-55 min 100% B, 55-65 min 0% with a flow rate 0.2  
164 mL/min. The phenolic compounds were detected at 280, 320 and 360 nm and injection volume was 5  
165  $\mu\text{L}$ . The MS detector operated in positive and negative mode, ionization voltage of the capillary was  
166 4000 V, and the end plate was set to -500 V. The drying gas ( $\text{N}_2$ ) temperature was set at 330  $^\circ\text{C}$  with a

167 flow rate of 9 L/min and full scan mode was between  $m/z$  100 to 800 with a scan resolution of 13,000  
168  $m/z/s$  until the ICC target reached either 20,000 or 200 ms. Tandem MS was carried out using helium as  
169 the collision gas ( $4.21 \times 10^{-6}$  mbar) with 1 V collision voltage. Metabolite identification was based on  
170 mass spectra, product ion spectra, retention time and by comparing mass spectra with those of pure  
171 reference material. Previously published data from literature were used as reference for metabolite  
172 identification, as well.

### 173 *Quantitative analysis of phenolic compounds by HPLC-DAD*

174 Individual phenolic compounds of leaf extracts were separated by a reverse-phase column  
175 Licrosphere 100 RP-18 (5  $\mu$ m particle size) packed with LiChroCART 250-4 (25  $\times$  0.4 cm ID) HPLC-  
176 Cartridge (Merck KGaA, Germany) with a guard column (LiChroCART 4-4); the column was  
177 thermostated at 25 °C. Solvent A was phosphoric acid  $10^{-3}$  M and solvent B was pure methanol.  
178 Chromatographic condition was established according to previously published methods by Di Stefano  
179 and Cravero<sup>14</sup> and by Ferrandino and Guidoni<sup>8</sup> with some modifications. Chromatograms were acquired  
180 at 280 nm, 320 nm and 360 nm simultaneously and run time was 50 minutes. Compounds were identified  
181 based on spectrum correspondence with authentic standards and quantified by the external standard  
182 method through calibration curves.

### 183 **Statistical analysis**

184 All data were analysed by SPSS 32.0 software program version 24.0 for Windows (SPSS Inc.,  
185 Chicago, USA). Analysis of variance was performed by one-way ANOVA and followed by Tukey-b  
186 post-hoc test at  $P \leq 0.05$ . All measurements were performed in triplicate and results were expressed as  
187 means  $\pm$  standard errors (SE). Heatmaps were generated by Rstudio software version 1.0.44, using the  
188 ggplot2 and Complex Heatmap R packages. The compounds used for heatmap realization were those  
189 quantified by HPLC-DAD. For each individual compound concentration, Z-scores were calculated by  
190 subtracting to each average value (variety and date) the general average of the entire population divided  
191 by the standard deviation.

## 192 RESULTS

193 We analyzed polyphenol accumulation in leaves of six *Vitis vinifera* varieties during the  
194 vegetative season expressing data on the basis of fresh weight to limit some negative effects of freeze-  
195 drying and lyophilisation on polyphenols. However, dry weight measures allowed highlighting  
196 differences in water content between leaf blades and veins over the season. It clearly emerged that the  
197 average dry matter content in veins was 10% lower with respect to that of blades for all the tested  
198 varieties; the range of variation among varieties at the same date of sampling was not higher than 6% in  
199 blades and 4% in veins, resulting in no significant differences at two dates out of three (Table 1).  
200 Before sample preparation, several solid-liquid extractions were performed to assess the best solvent to  
201 be used for polyphenol leaf extraction, knowing that different solvents can specifically favor the  
202 extraction of specific group of molecules and that increasing content of ethanol favoured polyphenolic  
203 extraction from grape seeds.<sup>15</sup> We tried seven different solvents, evaluating their efficiency by measuring  
204 total polyphenols (TP) and total flavonoids (TF) in blades and veins, separately. Although  
205 hydroalcoholic buffer with 12% ethanol (one of the most largely used solvent in berry polyphenol  
206 measurements) allowed to measure slightly higher TP in blades, the hydroalcoholic buffer with 40%  
207 ethanol was the optimal solvent for both tissues (Table 2). Moreover, because of the known significant  
208 influence of solvent to sample volume ratio (SSR) onto polyphenol extraction yield,<sup>16</sup> we tested different  
209 SSR and finally we adopted the SSR 25:1 for both tissues.

### 210 **Total Polyphenols (TP) and Total Proanthocyanidins (PA)**

211 The concentration of TP in grapevine blades varied from 30.1 to 50.7 g CE/kg, in line with  
212 previously published data on whole leaves.<sup>17,18</sup> In Barbera leaf blades, TP concentration increased during  
213 the vegetative season; in Shiraz at the last sampling TP concentration was higher respect to that at the  
214 first sampling (Fig. 1A). Grenache showed opposite trend as TP concentration slightly decreased,  
215 similarly to what was observed by Rusjan et al.<sup>17</sup> in Chardonnay healthy leaves. Cabernet Sauvignon,  
216 Nebbiolo and Pinot noir showed an identical TP accumulation trend during the season, displaying a peak

217 of maximum concentration at DOY 183. The concentration of TP in grapevine veins ranged from 14.8  
218 g CE/kg to 24.6 g/kg of fresh weight, which was twice less than in blades. Considering the dilution effect  
219 due to the higher water amount displayed in veins with respect to blades, differences between the two  
220 tissues were slightly less evident, but they were still high. In leaf veins, differently from leaf blades, no  
221 major differences were found among varieties, nor in concentrations or in trends.

222 In blades total proanthocyanidins (PA) increased during the season in all the examined varieties  
223 without any exception (Fig. 1C). Pinot noir and, particularly, Barbera accumulated lower amounts of PA  
224 with respect to the other biotypes. The concentration of PA in veins (Fig. 1D) was twice lower than in  
225 blades and similarly to TP results no major differences were found among the six varieties over the  
226 studied period. However, also in veins and notably at the beginning of the vegetative season, Barbera  
227 displayed a reduced PA concentration. Differences between the two Italian genotypes, Nebbiolo and  
228 Barbera were quite evident as to this parameter (Figure 1C, 1D).

### 229 **Identification, Quantification and Seasonal Accumulation of Anthocyanins in Grapevine Leaves**

230 In the present study, analysis of anthocyanins demonstrated that in healthy grapevine blades,  
231 anthocyanins were absent and their concentration was very low in veins (Fig. 2A, Supplementary Table  
232 6). Available data on anthocyanin accumulation and profiles in healthy leaves are scarce, however the  
233 absence or low concentration of anthocyanins in healthy leaves was previously assessed in  
234 grapevine.<sup>19,20</sup> In veins, the highest concentration of anthocyanins was detected in Cabernet Sauvignon  
235 at DOY 210 (end of July) (Fig. 2A). Nebbiolo, Barbera and Shiraz anthocyanin accumulation trend was  
236 similar: from DOY 142 to 183 (May to mid-July) the concentration increased, afterwards it declined  
237 slightly. Instead, in Pinot noir and Grenache the anthocyanin concentration increased slightly during the  
238 vegetative season, with Grenache displaying particularly low concentrations. In our study, eight  
239 anthocyanins were identified by HPLC-DAD-ESI-MS/MS in grapevine veins (Fig. 3; Table 3). Malvidin  
240 3-*O*-(6-*p*-coumaroyl)-glucoside was the prevalent anthocyanin in healthy leaf veins (accounting for 50%  
241 over total concentration) in all varieties with the only exception of Pinot noir (Fig. 2B), where the

242 prevalent anthocyanin was malvidin-3-*O*-glucoside. (Fig. 2B). In Nebbiolo veins malvidin 3-*O*-  
243 glucoside, malvidin 3-*O*-(6-*p*-coumaroyl)-glucoside, malvidin 3-*O*-(6-*p*-caffeoyl)-glucoside and  
244 peonidin 3-*O*-(6-*p*-coumaroyl) glucoside were detected. Malvidin and its derivatives accounted for 60-  
245 70% of total anthocyanins. Barbera leaf veins showed the most complex profile with seven anthocyanins:  
246 the four detected in Nebbiolo, and, additionally petunidin 3-*O*-(*p*-coumaroyl)-glucoside in all samplings,  
247 cyanidin and delphinidin *p*-coumaroyl glucosides, in the first two samplings. Cabernet Sauvignon  
248 anthocyanin profile was similar to that of Nebbiolo with the exception of one acylated anthocyanin,  
249 whose structure remained unknown (Table 3). The concentration of anthocyanins in veins of Shiraz and  
250 Grenache was particularly low (Supplementary Table 6) and these two varieties displayed the simplest  
251 profiles (Fig. 2).

## 252 **Identification, Quantification and Seasonal Accumulation of Non-Anthocyanin Phenolics in** 253 **Grapevine Leaves**

254 Twenty-four phenolic compounds were identified in veins and twenty in blades (Table 4;  
255 Supplementary Fig. 1). The analysed phenolic compounds belonged to flavonols, hydroxycinnamic  
256 acids, flavan-3-ols, proanthocyanidins, dihydroxybenzoic acid (exclusively protocatechuic acid-  
257 glucoside) and flavanonols.

### 258 *Flavonols*

259 Flavonol glycosides were quantitatively the most abundant phenolic compounds in leaves. The  
260 total flavonol glycoside content ranged from 2596.7 to 5530.9 mg/kg in blades (Fig. 4A, Supplementary  
261 Table 3A) and from 852.4 to 1607.4 mg/kg in veins where the trend of flavonol concentration was similar  
262 among biotypes, even though Barbera showed slightly higher concentrations, particularly at the first two  
263 sampling dates (Fig. 4B, Supplementary Table 3B). In Barbera leaf blades at DOY 210 (end of July) the  
264 highest concentration of total flavonols was measured and, except for Cabernet Sauvignon, which  
265 displayed an increasing trend, in the other varieties flavonols were stable or tended to slight decrease  
266 during the season. Oppositely, in Grenache, Pinot noir and Shiraz, considering that no significant

267 variation of dry matter was detected during the entire season, this reduction in flavonol concentration  
268 could be ascribed to degradation tied to the beginning of senescence, being known that these varieties  
269 display a shorter vegetative cycle with respect to Nebbiolo and Barbera (Fig. 4A). In *Vitis vinifera* blades  
270 and veins, flavonol glycoside qualitative composition was similar (Fig. 4C, 4D). Six flavonols were  
271 identified and quantified: myricetin 3-*O*-glucoside, myricetin 3-*O*-glucuronide, quercetin 3-*O*-  
272 glucoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*-glucoside and kaempferol 3-*O*-glucuronide  
273 (Table 4). Additionally, based on mass spectra, a quercetin-pentoside was tentatively identified in  
274 Nebbiolo and Grenache veins: it showed a molecular ion  $[M]^-$  at  $m/z$  433 and gave a product ion at 301  
275 in  $MS^2$ , which indicated that this compound is a quercetin derivative. Quercetin 3-*O*-glucuronide was  
276 the main component followed by quercetin 3-*O*-glucoside; together, they accounted for up to 94% of all  
277 flavonols (Fig. 4). During the vegetative season, the flavonol profile changed due to the percentage  
278 increase of quercetin 3-*O*-glucoside respect to quercetin 3-*O*-glucuronide over flavonol total  
279 concentration.

#### 280 *Hydroxycinnamic acids*

281 Hydroxycinnamic acids (HCA) were the most abundant non-flavonoid phenolics in *Vitis vinifera*  
282 leaves. Amounts ranged from 1.3 to 4.1 g kg<sup>-1</sup> in blades (Fig. 5A, Supplementary Table 2A) and from  
283 0.8 to 1.8 g kg<sup>-1</sup> in veins (Fig. 5B, Supplementary Table 2B). Barbera leaves were able to accumulate  
284 the highest concentration of HCA compared to the other varieties. Generally, a decreasing trend of HCA  
285 concentration was detected during the vegetative period, particularly in blades. The prevalent HCA was  
286 *trans*-caftaric acid (77-89%), followed by *trans*-coutaric acid, *cis*-caftaric acid, *cis*-coutaric acid and  
287 trace amounts of *trans*-fertaric acid (Fig. 5C, 5D; Table 4). Moreover, in Cabernet Sauvignon veins and  
288 blades a caffeoyl hexoside was tentatively identified: it showed maximum absorbance at 325 nm and a  
289 pseudomolecular ion  $[M-H]^-$  at  $m/z$  341 with two product ions at  $m/z$  179  $[M-H-hexose]^-$  and  $m/z$  135  
290  $[M-H-hexose-CO_2]^-$  in  $MS^2$ , consistent with the data of a metabolite found by Chen et al.<sup>21</sup> in *Taraxacum*  
291 *formosanum*.

292 *Protocatechuic acid-glucoside*

293 Although present in small amount in leaves protocatechuic acid-glucoside was detected in both  
294 blades and veins of all the analysed varieties (Table 4). Its characteristic mass spectra showed a  
295 pseudomolecular ion  $[M-H]^-$  at  $m/z$  315 and a product ion at  $m/z$  153  $[M-H-glucose]^-$ . The tentative  
296 identification was in accordance with previously reported MS<sup>2</sup> profiles of protocatechuic acid-glucoside  
297 in grapes.<sup>22</sup>

298 *Flavan-3-ols*

299 The concentration of flavan-3-ols in leaf blades was quite stable or decreased during the  
300 examined period with the only exception of Shiraz (Fig. 6A). In Grenache an important increase of  
301 flavan-3-ol concentration characterized the period between the first two pickings (Fig. 6B). The  
302 concentrations of flavan-3-ols ranged from 177.8 to 486.3 mg kg<sup>-1</sup> in blades and from 153.7 to 416.7 mg  
303 kg<sup>-1</sup> in veins. Differently from the other classes of compounds whose concentrations were two/three  
304 times higher in blades than in veins, the concentration of flavan-3-ols was similar in the two tissues.  
305 However, considering the dilution effect due to the vein higher water content with respect to blades, it  
306 emerges that flavan-3-ols are more concentrated in veins than in blades. In leaf veins of Cabernet  
307 Sauvignon, Shiraz and Pinot noir, flavan-3-ol concentration increased until DOY 197/210 (middle/end  
308 July) and then it decreased. In Nebbiolo, Barbera, Grenache, from DOY 142 to 183 (end of May until  
309 beginning of July) there was a significant increase of flavan-3-ol concentration, followed by a decreasing  
310 trend.

311 The main flavan-3-ol found in leaves was (+)-catechin, representing up to 75% of total flavan-  
312 3-ols (Fig. 6C, 6D) which is consistent with Topalovic et al.<sup>23</sup> Besides, also (-)-epicatechin was  
313 accumulated in important concentrations in leaf blades, particularly in Barbera where it accounted for  
314 34.5 up to 52.4% of the total flavan-3-ols. Generally, in blades the concentration of (+)-catechin  
315 decreased during the examined period, which implied a profile change characterized by the reduction of  
316 (+)-catechin incidence and a general increase of that of (-)-epicatechin (except in Pinot noir) (Fig. 6A;

317 Supplementary Table 4A, 4B). (-)-Epigallocatechin gallate and low amount of (-)-epicatechin gallate  
318 were detected in *Vitis vinifera* blades and veins. (-)-Epigallocatechin gallate relative abundance ranged  
319 from 2.6% to 18.8% in blades and from 9.8% to 30.8% in veins, in lines with data from Peng et al.<sup>24</sup>  
320 Particularly high percentages of (-)-epigallocatechin were detected in Barbera blades (Fig. 6C) and in  
321 Nebbiolo, Pinot noir and Cabernet Sauvignon veins (Fig. 6D). Pinot noir and Cabernet Sauvignon did  
322 not show any capability to accumulate (-)-epicatechin gallate (except in Cabernet Sauvignon veins at  
323 three sampling dates).

324 Additionally, by LC-ESI-MS/MS proanthocyanidin dimers were tentatively identified. Three  
325 (epi)-gallocatechin-(epi)catechin isomers in blades and two isomers in veins with pseudomolecular ion  
326  $[M-H]^-$  at  $m/z$  593 and three product ions at  $m/z$  425, 407, 289 in MS<sup>2</sup> and two B-type procyanidins  
327 (Table 4), possibly identified as B<sub>1</sub> and B<sub>3</sub>. The identification is supported by previously published  
328 identification of the dimeric flavan-3-ols compounds in grapevine leaves<sup>23</sup> and berries.<sup>25</sup>

### 329 *Flavanonols*

330 During the examined period, the concentration of flavanonols in leaves was stable or slightly  
331 increased. In Pinot noir and Nebbiolo flavanonol concentration was much higher in veins than in blades  
332 (Fig. 8A, 8B). In Nebbiolo veins, flavanonol concentration was the highest, ranging from 139.3 to 251.9  
333 mg kg<sup>-1</sup> during all the examined period compared to the other varieties and it was up to 50 times higher  
334 in veins respect to blades. Taking into account the average higher water content of veins compared to  
335 blades, the higher content of flavanonol in veins was even more remarkable. Oppositely, in Grenache no  
336 major differences were found in the flavanonol concentration of the two leaf tissues. Leaf flavanonols  
337 were a group of four glycosides, sometimes reported in grapes and wines.<sup>26,27</sup> Based on their  
338 characteristic UV maximum absorbance at 290 nm and mass spectra, they were identified as  
339 dihydroquercetin-hexoside (taxifolin-hexoside), dihydroquercetin-rhamnoside (astilbin),  
340 dihydrokaempferol-hexoside and dihydromyricetin-rhamnoside (Table 4, Fig. 7). Dihydroquercetin-  
341 hexoside was identified by its pseudomolecular ion  $[M-H]^-$  at  $m/z$  465; product ions at  $m/z$  303  $[M-H]$



342 hexose; dihydroquercetin]<sup>-</sup>, 285 [M-H-hexose-H<sub>2</sub>O]<sup>-</sup>, 151 ([<sup>1,3</sup>A<sub>0</sub>]<sup>-</sup>; retro Diels–Alder fission), in line  
343 with the fragmentation study of dihydroquercetin by Abad-Garcia et al.<sup>28</sup> Detected peak with  
344 pseudomolecular ion [M-H]<sup>-</sup> at *m/z* 449 and product ions at *m/z* 303, 285, 151 was tentatively identified  
345 as dihydroquercetin-rhamnoside (astilbin), in line with other reports.<sup>25,29</sup> Dihydrokaempferol-hexoside  
346 was identified by its pseudomolecular ion [M-H]<sup>-</sup> at *m/z* 449 and product ions at *m/z* 287, 269, 151 and  
347 dihydromyricetin-rhamnoside was identified by its pseudomolecular ion [M-H]<sup>-</sup> at *m/z* 465 and product  
348 ions at *m/z* 339, 319, 301, 151, as previously described.<sup>30</sup> The flavanonol profile of Nebbiolo veins was  
349 different comparing to the other studied varieties (Fig. 8C). Dihydroquercetin-hexoside (taxifolin-  
350 hexoside) and dihydrokaempferol-hexoside were detected exclusively in Nebbiolo veins, where they  
351 comprised up to 51% of total flavanonols. Pinot noir, Cabernet Sauvignon and Barbera accumulated  
352 dihydromyricetin-rhamnoside and dihydroquercetin-rhamnoside whereas Shiraz and Grenache veins  
353 exclusively accumulated dihydroquercetin-rhamnoside. Dihydroquercetin-rhamnoside accumulated  
354 both in blades and veins and the highest concentration was found in Pinot noir veins where it ranged  
355 from 94.6 to 150.8 mg kg<sup>-1</sup> (Fig. 8C, Supplementary Table 5).

## 356 DISCUSSION

### 357 **Genotypic and tissue-specific differences**

358 Among the analysed genotypes, Barbera showed some peculiar features: the lowest  
359 concentration of proanthocyanidins in blades was the prevalent trait. It is long-time known by  
360 viticulturists and it has recently been demonstrated that Barbera must is characterized by low  
361 proanthocyanidin amounts.<sup>31</sup> Vice versa, Nebbiolo musts and wines owe their aging capability, among  
362 other factors, to the high proanthocyanidin content in berry skins. Apparently, this trait is evident also  
363 in leaves as Nebbiolo showed a higher capability of accumulating PA with respect to Barbera, both in  
364 blades and in veins early in the season, in line with what was previously reported by Margaria et al.<sup>18</sup>  
365 comparing entire healthy leaves of the two varieties. At flowering the constitutive proanthocyanidin  
366 concentration of Barbera healthy leaves was half compared to that of Nebbiolo. When Merlot vines were

367 treated with benzothiadiazole, a plant activator, to induce resistance against gray mold caused by *Botrytis*  
368 *cinerea*, the resistance was associated with an increase of total polyphenols in berry skins, in particular  
369 of the proanthocyanidin fraction that increased up to 36%.<sup>32</sup> All these information taken together allow  
370 speculating that constitutive higher amounts of polymeric proanthocyanidin could limit the diffusion of  
371 specific pathogens and contribute to explain the different levels of susceptibility of *V. vinifera* varieties  
372 to pathogens.

373 Veins were the exclusive leaf sector where anthocyanin accumulated as no anthocyanins  
374 accumulated in blades of healthy leaves (Fig. 9). The absence and/or traces of anthocyanins in healthy  
375 entire leaves were previously but rarely assessed in grapevine.<sup>19,20</sup> Tri-hydroxylated anthocyanins and  
376 malvidin 3-*O*-glucoside were the prevalent anthocyanins in entire leaves of Cabernet Sauvignon and  
377 Sangiovese, with acyl-derivatives being around 61% of total concentrations.<sup>33</sup> Although to our  
378 knowledge, no specific studies focused on leaf veins, previous work on petioles showed the sum of  
379 anthocyanin acyl-derivatives ranged from about 40 to 80% from end of July to leaf senescence in  
380 Barbera,<sup>34</sup> in line with our data where they ranged from 50 to 90% (Fig. 2). Pinot noir represented the  
381 only exception as, similarly to berries, no acylated anthocyanins were found, underlying that in this  
382 cultivar acylation is inactive, regardless the organ. The complexity of the leaf anthocyanin profile was  
383 cultivar-related, Barbera being the genotype displaying the highest complexity, Pinot noir and Grenache,  
384 the lowest (Fig. 9).

385 Barbera and Grenache blades showed the highest flavonol concentration (as average values in  
386 the season), Nebbiolo the lowest. Flavonols identified in blades did not differ among genotypes with the  
387 exception of Nebbiolo and Grenache that accumulated a specific quercetin pentoside in veins. In vegetal  
388 tissues, flavonols play a role in thermal and excess energy dissipation and in photoprotection. Flavonols  
389 have been indicated as dampers of the abscisic acid dependent reactive oxygen species accumulation  
390 that drives stomatal closure and as molecules able to facilitate stomatal opening, modulating plant leaf  
391 gas exchange.<sup>35</sup> This, together with the higher concentration of flavonols in specific genotypes could

392 contribute to explain the cultivar-specific stomata opening mechanism. It is of particular interest in i)  
393 spreading further light on the possible relations between isohydric or anisohydric behaviour of  
394 grapevines and leaf flavonol accumulation and ii) studying the relation of grapevine varieties with fungus  
395 penetrating through stomata (such as *Plasmopara viticola*). Latouche and co-workers<sup>36</sup> stated that  
396 constitutive higher amounts of flavonols (no information about specific molecules as they were  
397 estimated spectrophotometrically) slowed down the accumulation of stilbenoids in grapevine leaves,  
398 thus the phytoalexin-mediated response of leaves to *Plasmopara* attack was delayed. This opens the  
399 hypothesis that constitutive higher amounts of quercetin could at least limit the diffusion of specific  
400 pathogens.

401         Barbera leaves showed a peculiar trait as to hydroxycinnamic acids which concentration was  
402 much higher respect to the other examined biotypes, very clearly in blades (Fig. 9). Main leaf  
403 hydroxycinnamic acids did not differ among varieties and they were the same as in berries,<sup>9</sup> with the  
404 only exception of Cabernet Sauvignon that accumulated one further type of hydroxycinnamic acid,  
405 tentatively identified as a caffeoyl hexoside, both in blades and in veins. *Trans*-caftaric acid was found  
406 to be the main non-flavonoid polyphenol in leaves and *trans*-form of HCA were always prevalent over  
407 *cis*-forms, as reported.<sup>2</sup> Plant hydroxycinnamic acids are involved in defence mechanism and known to  
408 possess antimicrobial and antioxidative effects.<sup>37</sup> In *Arabidopsis* it was shown that hydroxycinnamate  
409 accumulation increased following *Botrytis cinerea* infection<sup>38</sup> and in *Vitis vinifera* cv Chardonnay a  
410 slight higher accumulation was detected after *Oidium* infection.<sup>39</sup> Grapevine leaves of the present study  
411 were considered healthy (no signals of any kind of pathogens were found in collected leaves, total  
412 absence of eye-detectable spots, reddening in leaf blades and no trace of anthocyanins analytically  
413 detectable in blades). However, Barbera leaves displayed a much higher hydroxycinnamic acid  
414 concentration with respect to the other genotypes (Fig. 9) in line with what we previously and  
415 concomitantly found in other studies (data not shown). This suggests that there is a strong genotype-  
416 related influence of hydroxycinnamic acid accumulation in leaves of *Vitis vinifera* varieties or that

417 Barbera leaves of the present study were already reacting to a pathogen. In this second circumstance,  
418 further investigations would be necessary to understand if the accumulation of this class of non-flavonoid  
419 polyphenols could become an early indicator of grapevine sanitary status, well before the appearance of  
420 symptoms.

421 Little is known about flavan-3-ol profile variation in leaves of different *Vitis vinifera* varieties  
422 and accumulation during the vegetative season. The concentration of (+)-catechin and (-)-epicatechin  
423 was similar in leaf blades and veins, regardless of the variety. The concentration of epicatechin slightly  
424 increased in pear leaves after inoculation of *Erwinia amylovora*,<sup>40</sup> a specific reaction to the disease  
425 implemented by the leaf tissue to limit its diffusion, exploiting the high activity of epicatechin as  
426 antioxidant. Moreover, epicatechin can also act as a modulator of cell-signalling, by inhibiting pro-  
427 oxidant enzymes, such as NADPH oxidases and lipoxygenases, by altering the phosphorylation state of  
428 specific molecules or by chelating metals that mask prooxidant actions of reactive nitrogen and oxygen  
429 species.<sup>41</sup> A specific action of epicatechin against the fungus *Venturia inaequalis* infection was also  
430 demonstrated in apples.<sup>42</sup> Varieties which distinguished for a high epicatechin concentration both in  
431 blades and in veins were Nebbiolo, Barbera and Grenache, this last being known for its low susceptibility  
432 to the bacterium *Xylella fastidiosa*.<sup>43</sup> Even though Nebbiolo, Barbera and Grenache displayed the highest  
433 capacity to accumulate flavan-3-ols, exclusively Nebbiolo showed specific peculiarities as to organ  
434 localization: Nebbiolo veins represented the exclusive site where epigallocatechin gallate and  
435 epicatechin gallate were accumulated early in the season and in significant concentrations.

436 The flavan-3-ol profile differed in Pinot noir leaves where epicatechin gallate was totally lacking and,  
437 partially, in Cabernet Sauvignon where it was absent in blades and only sporadically present in veins  
438 (Fig. 9). Epigallocatechin gallate, the only molecule among flavan-3-ols listed by the Italian Health  
439 Ministry document among “Other nutrients and molecules with nutritional and physiological effects”,  
440 was well represented in all the analysed biotypes, notably in veins. Nebbiolo, Pinot noir and Cabernet  
441 Sauvignon displayed the highest concentration in veins, particularly early in the season. Beneficial effect

442 of epigallocatechin gallate, due to its high antioxidant capacity, are known (anti-carcinogenic, among  
443 others, through its capacity to limit cancer cell induction and proliferation, cardio- and neuroprotective,  
444 reviewed by Karas et al.<sup>44</sup>). Constitutive high amounts of this monomeric proanthocyanidin could  
445 represent an important cultivar trait representing an element of protection against reactive oxygen  
446 species induced by stressors, including those of biotic origin.

447 To our knowledge, this is the first time that flavanonols are found in grapevine leaves, although  
448 astilbin was previously detected in *Vitis vinifera* stems.<sup>45,46</sup> Astilbin was the exclusive flavanonol found  
449 in blades of all the examined varieties and in Shiraz and Grenache veins. The other studied genotypes  
450 also accumulated dihydromyricetin-rhamnoside and Nebbiolo was the cultivar that, besides displaying  
451 the highest flavanonol concentration, presented the widest profile differentiation, accumulating  
452 dihydroquercetin-hexoside (taxifolin-hexoside) and dihydrokaempferol-hexoside (Fig. 9), as well.  
453 These last two molecules are probably glucosides even though with our analytical tools we were not able  
454 to distinguish them from galactosides; however, we consider this unlikely, due to the wide presence of  
455 glucoside derivatives in *Vitis vinifera*, rather than galactosides. Flavanonols in plants have different  
456 ecological roles such as phytoalexins in roots<sup>47</sup> or participants to plant anti-herbivore defence  
457 strategies.<sup>48</sup> Astilbin, at relatively low concentration, was proved to be involved in the systemic induction  
458 response to fungal pathogens in Austrian pine.<sup>49</sup> Moreover, dihydroquercetin (taxifolin) has promising  
459 therapeutic potential due to its effect on some anti-cancer mechanism, cholesterol biosynthesis and  
460 antiviral activity.<sup>50</sup> Interestingly, flavanonol concentration was not negligible in the leaves of the six  
461 studied *Vitis vinifera* varieties and it was generally higher in veins respect to blades. Nebbiolo and to a  
462 lesser extent Pinot noir, displayed the highest flavanonol concentrations and, in the case of Nebbiolo  
463 also the widest profile complexity (Fig. 7 and Fig. 8). Nebbiolo, in particular, should be further  
464 investigated in the light of understanding its limited susceptibility to vein-located pathogens, such as  
465 Flavescence dorée<sup>18</sup> and of studying grapevine interaction with herbivore insects as high concentration

466 of flavanonols or of specific molecules among them, could be natural repellents for insects (including  
467 the vector of Flavescence dorée, *Scaphoideus titanus*).

468 Knowledge derived from the present work is a contribution to dissect leaf polyphenol potential  
469 as a part of grapevine defense mechanisms and of genotype-related susceptibility to pathogens.  
470 Moreover, current knowledge represents a starting point for future deepening about grapevine and  
471 vineyard by-products as source of bioactive phenolic compounds.

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#### 475 **Supporting Information.**

476 Supplement Table 1. The weather condition in the vineyard.

477 Supplement Table 2A. Average concentration of individual hydroxycinnamic acids in *Vitis vinifera* leaf  
478 blades.

479 Supplement Table 2B. Average concentration of individual hydroxycinnamic acids in *Vitis vinifera* leaf  
480 veins.

481 Supplement Table 3A. Average concentration of individual flavonols in *Vitis vinifera* blades.

482 Supplement Table 3B. Average concentration of individual flavonols in *Vitis vinifera* veins.

483 Supplement Table 4A. Average concentration of individual flavan-3-ols and flavanonols in *Vitis vinifera*  
484 leaf blades.

485 Supplement Table 4B. Average concentration of individual flavan-3-ols in *Vitis vinifera* veins.

486 Supplement Table 5. Average concentration of individual flavanonols in *Vitis vinifera* veins.

487 Supplement Table 6. Average concentration of individual anthocyanins in *Vitis vinifera* veins.

488 Supplement Figure 1. HPLC-UV-ESI-MS/MS non-anthocyanin polyphenols of Nebbiolo leaf vein  
489 extract at 280 nm (A), 320 nm (B) and 370 nm (C).

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629 Figure Captions

630 **Figure 1.** Changes in the total polyphenol (TP) and total proanthocyanidin (PA) concentration in *Vitis*  
631 *vinifera* leaves during the season. A and C = blades; B and D = veins. Means  $\pm$  standard errors (n=3).

632 **Figure 2.** Accumulation of anthocyanins in *Vitis vinifera* leaf veins during the season. A = evolution of  
633 total anthocyanins (TA); results (means  $\pm$  standard errors, n=3) are expressed as mg of malvidin 3-O-  
634 glucoside equivalent per kg of leaf vein fresh weight (FW). B = anthocyanin profile of leaf veins during  
635 the vegetative season. NE = Nebbiolo; BR = Barbera; PN = Pinot noir; CS = Cabernet Sauvignon; GR  
636 = Grenache; SR = Shiraz. Numbers after the variety acronyms refer to: 1 = DOY 142 (22<sup>nd</sup> of May); 2  
637 = DOY 183 (2<sup>nd</sup> of July); 3 = DOY 197 (16<sup>th</sup> of July); 4 = DOY 210 (29<sup>th</sup> of July) and 5 = DOY 238  
638 (26<sup>th</sup> of August).

639

640 **Figure 3.** HPLC-UV-MS/MS chromatogram of Barbera leaf vein anthocyanins; sampling date at DOY  
641 142 (22<sup>nd</sup> of May). See Table 3 for peak identification.

642 **Figure 4.** Accumulation of flavonols in *Vitis vinifera* leaves during the season. Evolution of total  
643 flavonols in blades (A) and in veins (B); means of the sum of detected flavonols  $\pm$  standard errors (n=3).  
644 Flavonol profile of leaf blades (C) and veins (D) during the season; Q – quercetin, K – kaempferol, Myr  
645 – myricetin, Gluc – glucuronide, Glc – glucoside; see Fig. 2 for variety acronym identification.

646 **Figure 5.** Accumulation of hydroxycinnamic acids (HCA) in *Vitis vinifera* leaves during the season.  
647 Evolution of total HCA in blades (A) and veins (B); means of the sum of all detected HCA  $\pm$  standard  
648 errors (n=3). Profile of HCA of leaf blades (C) and veins (D) during the season; see Fig. 2 for variety  
649 acronym identification.

650 **Figure 6.** Accumulation of flavan-3-ols in *Vitis vinifera* leaves during the season. Evolution of total  
651 flavan-3-ols in blades (A) and veins (B); means of the sum of all detected flavan-3-ols  $\pm$  standard errors  
652 (n=3). Flavan-3-ol profile of leaf blades (C) and veins (D) during the season; see Fig. 2 for variety  
653 acronym identification.

654 **Figure 7.** The extracted ion chromatogram (EIC) at m/z 465 (A) and m/z 449 (B) in negative mode; UV  
655 maximum and product ion spectra at negative mode of dihydroquercetin-hexoside (1); dihydromyricetin-  
656 rhamnoside (2); dihydrokaempferol-hexoside (3) and dihydroquercetin-rhamnoside (4, astilbin) detected  
657 in Nebbiolo leaf vein extracts.

658 **Figure 8.** Accumulation of flavanonols in *Vitis vinifera* leaves during the season. Evolution of total  
659 flavanonols in blades (A; exclusively dihydroquercetin-rhamnoside, astilbin) and veins (B); means of  
660 the sum of all detected flavanonols  $\pm$  standard errors (n=3). Flavanonol profile of leaf veins (C) during  
661 the season; see Fig. 2 for variety acronym identification.

662 **Figure 9.** Evolution of polyphenol concentration in the leaves of six *Vitis vinifera* varieties. Heatmap of  
663 blades and veins represent Z-scores of each compound calculated by subtracting to each average value  
664 (variety and date) the general average of the entire population divided by the standard deviation. See  
665 Fig. 2 for variety acronym identification.

666

**Table 1. Dry matter (%) in *Vitis vinifera* leaves during the vegetative season <sup>a</sup>.**

|               |         | <i>DOY 186</i> | <i>DOY 200</i> | <i>DOY 241</i> | <i>date</i> |
|---------------|---------|----------------|----------------|----------------|-------------|
| <i>blades</i> | NE      | 30.87 ± 0.16   | 33.61 ± 0.04   | 35.90 ± 0.26   | **          |
|               | CS      | 30.77 ± 0.18   | 30.92 ± 0.39   | 34.11 ± 0.57   | **          |
|               | BR      | 31.60 ± 0.35   | 33.42 ± 0.83   | 35.28 ± 0.92   | *           |
|               | GR      | 28.66 ± 0.62   | 27.58 ± 0.82   | 30.76 ± 1.23   | ns          |
|               | PN      | 30.44 ± 0.80   | 32.69 ± 1.10   | 31.19 ± 4.37   | ns          |
|               | SH      | 31.30 ± 1.97   | 32.29 ± 1.37   | 36.59 ± 1.68   | ns          |
|               | average | 30.61 ± 0.39   | 31.75 ± 0.58   | 33.97 ± 0.88   |             |
| variety       | ns      | **             | ns             |                |             |
| <i>veins</i>  | NE      | 22.09 ± 1.13   | 24.12 ± 0.36   | 26.10 ± 0.63   | *           |
|               | CS      | 18.92 ± 0.32   | 21.57 ± 0.47   | 23.28 ± 0.80   | **          |
|               | BR      | 20.92 ± 1.35   | 23.76 ± 0.79   | 25.67 ± 1.33   | ns          |
|               | GR      | 19.39 ± 0.61   | 20.39 ± 0.37   | 23.42 ± 0.77   | **          |
|               | PN      | 20.72 ± 0.97   | 21.26 ± 0.90   | 27.57 ± 1.81   | *           |
|               | SH      | 21.67 ± 1.25   | 22.19 ± 0.74   | 25.39 ± 0.94   | ns          |
|               | average | 20.62 ± 0.44   | 22.22 ± 0.39   | 25.24 ± 0.53   |             |
| variety       | ns      | **             | ns             |                |             |

<sup>a</sup>Means ± standard errors (n=3). Means were separated by ANOVA and significant differences among dates (rows) or varieties (columns) were evaluated by the Tukey-b test,  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*); ns – not significant. Day of year (DOY) refers to 186 – 5<sup>th</sup> of July, 200 – 19<sup>th</sup> of July and 241 – 29<sup>th</sup> of July.

**Table 2. Solvent and sample/volume ratio influence on *Vitis vinifera* leaf total polyphenols and total flavonoids.**

| <i>solvent</i>                              | <i>blades</i> |      |                                |                               | <i>veins</i> |                                |                               |
|---|---------------|------|--------------------------------|-------------------------------|--------------|--------------------------------|-------------------------------|
|   | pH            | SSR  | total polyphenols <sup>a</sup> | total flavonoids <sup>b</sup> | SSR          | total polyphenols <sup>a</sup> | total flavonoids <sup>b</sup> |
| <i>hydroalcoholic buffer ethanol 12%</i>    | 3.2           | 10:1 | 28.60 ± 3.41 b                 | 39.14 ± 4.42 ab               | 20:1         | 16.98 ± 2.27 b                 | 27.13 ± 3.30 ab               |
| <i>methanol-water (80/20)</i>               | 4.6           | 10:1 | 17.05 ± 1.13 a                 | 31.79 ± 2.07 a                | 20:1         | 8.50 ± 1.25 a                  | 18.39 ± 1.93 a                |
| <i>methanol-water (80/20) with HCl 0.1%</i> | 3.5           | 10:1 | 16.97 ± 1.74 a                 | 29.54 ± 1.85 a                | 20:1         | 9.06 ± 1.62 a                  | nd                            |
| <i>acetone-water (50/50)</i>                | 5.6           | 10:1 | 21.99 ± 0.43 ab                | 26.06 ± 2.57 a                | 20:1         | 10.98 ± 0.92 a                 | 17.54 ± 1.41 a                |
| <i>acetone-water (50/50) with HCl 0.1%</i>  | 3.3           | 10:1 | 21.65 ± 1.03 ab                | 26.11 ± 2.70 a                | 20:1         | 11.57 ± 0.71 a                 | 19.90 ± 2.01 a                |
| <i>phosphate-citrate buffer</i>             | 3.6           | 10:1 | 13.72 ± 2.37 a                 | 30.45 ± 1.90 a                | 20:1         | 5.42 ± 0.77 a                  | nd                            |
| <i>hydroalcoholic buffer ethanol 40%</i>    | 3.9           | 10:1 | 27.90 ± 1.17 b                 | 48.17 ± 3.32 ab               | 20:1         | 18.03 ± 1.05 b                 | 33.64 ± 0.96 b                |
| <i>hydroalcoholic buffer ethanol 40%</i>    | 3.9           | 25:1 | 46.11 ± 2.07                   | 59.17 ± 2.87                  | 25:1         | 25.08 ± 2.76                   | 24.18 ± 2.44                  |

<sup>a,b</sup> Means ± standard errors (n=3) as grams of catechin (CE)/kg of Nebbiolo leaf fresh weight. Means were separated by ANOVA and significant differences among solvents were evaluated by Tukey-b test,  $p \leq 0.05$ ; nd – not detected; SSR – sample volume ratio.

**Table 3. Identified anthocyanins in *Vitis vinifera* leaf veins by HPLC-ESI-MS/MS**

| <b>ID<sup>a</sup></b> | <b>Rt<br/>(±0.2 min)</b> | <b>[M]<sup>+</sup><br/>(m/z)</b> | <b>[MS<sup>2</sup>]<sup>+</sup><br/>(m/z)</b> | <b>identification<sup>b</sup></b> |
|-----------------------|--------------------------|----------------------------------|---|-----------------------------------|
| <b>1</b>              | 21.9                     | 463                              | 301   | peonidin 3- <i>O</i> -glucoside   |
| <b>2</b>              | 23.1                     | 493                              | 331   | malvidin 3- <i>O</i> -glucoside   |
| <b>3</b>              | 35.2                     | 657                              | 303   | unknown                           |
| <b>4</b>              | 37.0                     | 611                              | 303   | delphinidin 3- <i>O</i> -(p-coum) |
| <b>5</b>              | 39.2                     | 655                              | 331   | malvidin 3- <i>O</i> -(caff)      |
| <b>6</b>              | 40.5                     | 595                              | 287   | cyanidin 3- <i>O</i> -(p-coum)    |
| <b>7</b>              | 42.0                     | 625                              | 317   | petunidin 3- <i>O</i> -(p-coum)   |
| <b>8</b>              | 45.1                     | 609                              | 301   | peonidin 3- <i>O</i> -(p-coum)    |
| <b>9</b>              | 45.8                     | 639                              | 331   | malvidin 3- <i>O</i> -(p-coum)    |

<sup>a</sup>ID identification numbers corresponding to peaks reported in Figure 3.

<sup>b</sup>p-coum – p-coumaroyl derivatives; caff – caffeoyl derivatives.



**Table 4. Identified non-anthocyanin phenolic compounds in *Vitis vinifera* leaf blades and veins by HPLC-ESI-MS/MS**

| ID <sup>a</sup> | Rt<br>(±0.2 min) | [M] <sup>-</sup><br>(m/z) | [MS <sup>2</sup> ] <sup>-</sup><br>(m/z) | [M] <sup>+</sup><br>(m/z) | [MS <sup>2</sup> ] <sup>+</sup><br>(m/z) | compound identification                                |
|-----------------|------------------|---------------------------|--|---------------------------|--|--|
| 1               | 16.9             | 593                       | 425, 407, 289                            | 595                       | 291                                      | (epi)gallocatechin- (epi)catechin<br>(isomer I)        |
| 2               | 18.0             | 593                       | 425, 407, 289                            |                           |  | (epi)gallocatechin- (epi)catechin<br>(isomer II)       |
| 3               | 18.7             | 315                       | 153                                      |                           |  | protocatechuic acid-glucoside                          |
| 4               | 19.9             | 593                       | 425, 407, 289                            | 595                       | 291                                      | (epi)gallocatechin- (epi)catechin<br>(isomer III)      |
| 5               | 20.7             | 311                       | 179                                      |                           |  | <i>cis</i> -caftaric acid                              |
| 6               | 21.2             | 577                       | 451, 425, 289                            |                           |  | procyanidin B <sub>3</sub>                             |
| 7               | 22.0             | 311                       | 179                                      |                           |  | <i>trans</i> -caftaric acid                            |
| 8               | 23.3             | 289                       | 245, 205, 179                            | 291                       | 273, 165, 123                            | (+)-catechin   |
| 9               | 24.0             | 577                       | 451, 425, 289                            | 579                       | 561, 427                                 | procyanidin B <sub>1</sub>                             |
| 10              | 24.7             | 295                       | 163                                      |                           |  | <i>cis</i> -coumaric acid                              |
| 11              | 25.0             | 341                       | 179                                      |                           |  | caffeoyl-hexoside                                      |
| 12              | 25.7             | 295                       | 163                                      |                           |  | <i>trans</i> -coumaric acid                            |
| 13              | 26.4             | 325                       | 193                                      |                           |  | fertaric acid  |
| 14              | 27.6             | 289                       | 245, 205, 177                            | 291                       | 273, 165, 139                            | (-)-epicatechin  |
| 15              | 29.6             | 465                       | 303, 285, 151                            |                           |  | dihydroquercetin-hexoside<br>(taxifolin-hexoside)      |
| 16              | 31.2             | 465                       | 339, 319, 151                            |                           |  | dihydromyricetin-rhamnoside<br>(ampelopsin-rhamnoside) |
| 17              | 32.9             | 449                       | 287, 269, 151                            |                           |  | dihydrokaempferol-hexoside<br>(aromadendrin-hexoside)  |
| 18              | 33.8             | 479                       | 317                                      | 481                       | 319                                      | myricetin 3- <i>O</i> -glucoside                       |
| 19              | 34.6             | 493                       | 317                                      | 495                       | 319                                      | myricetin 3- <i>O</i> -glucuronide                     |
| 20              | 35.5             | 449                       | 303, 285, 151                            |                           |  | dihydroquercetin-rhamnoside<br>(astilbin)              |
| 21              | 36.3             | 433                       | 301                                      | 435                       | 303                                      | quercetin-pentoside                                    |
| 22              | 36.7             | 463                       | 301                                      | 465                       | 303                                      | quercetin 3- <i>O</i> -glucoside                       |
| 23              | 37.5             | 477                       | 301                                      | 479                       | 303                                      | quercetin 3-glucuronide                                |
| 24              | 38.8             | 447                       | 285                                      | 449                       | 287                                      | kaempferol 3- <i>O</i> -glucoside                      |
| 25              | 39.2             | 461                       | 285                                      | 463                       | 287                                      | kaempferol 3- <i>O</i> -glucuronide                    |

<sup>a</sup>ID numbers correspond to peaks reported in Supplement Figure 1.

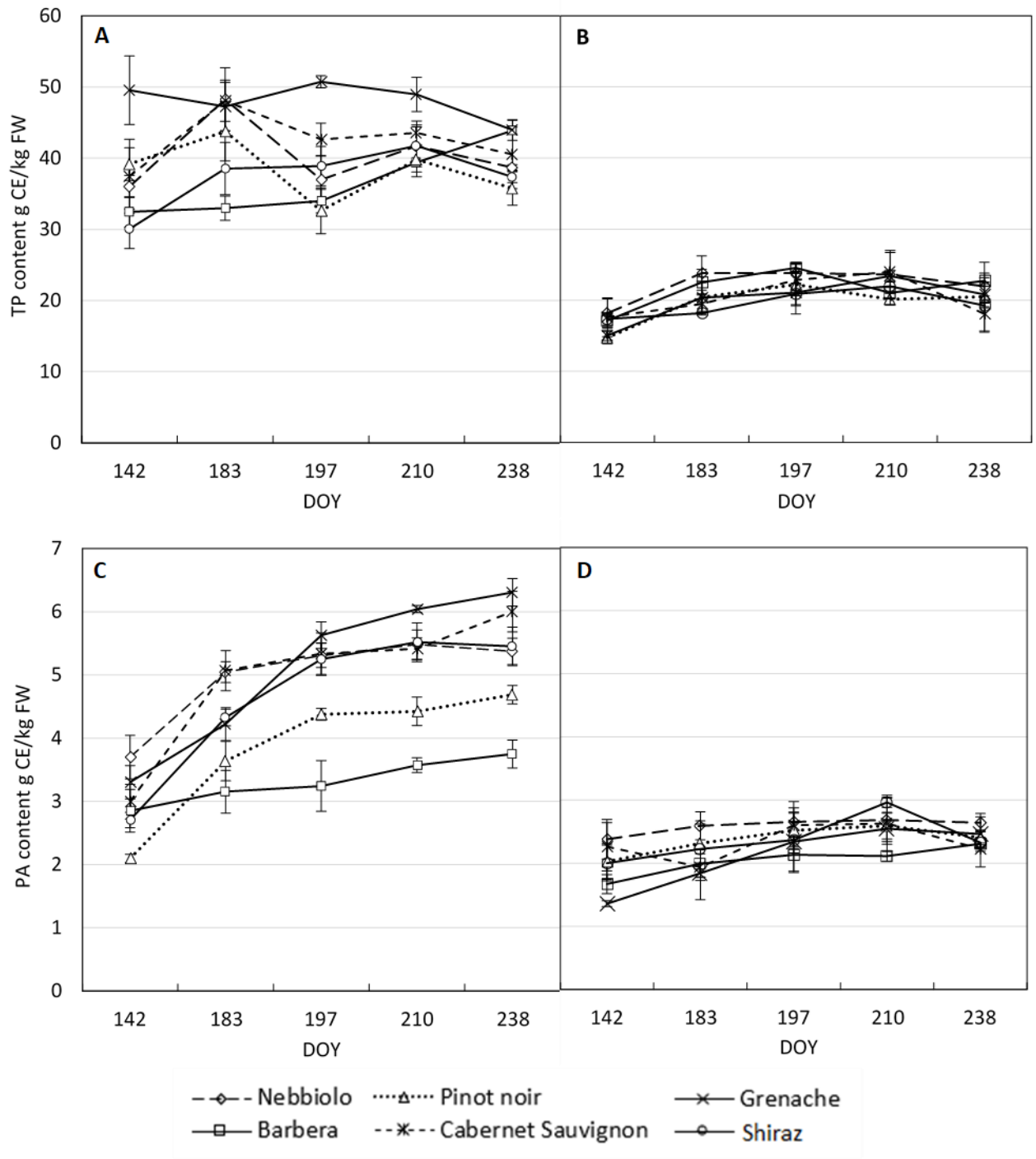


Figure 1

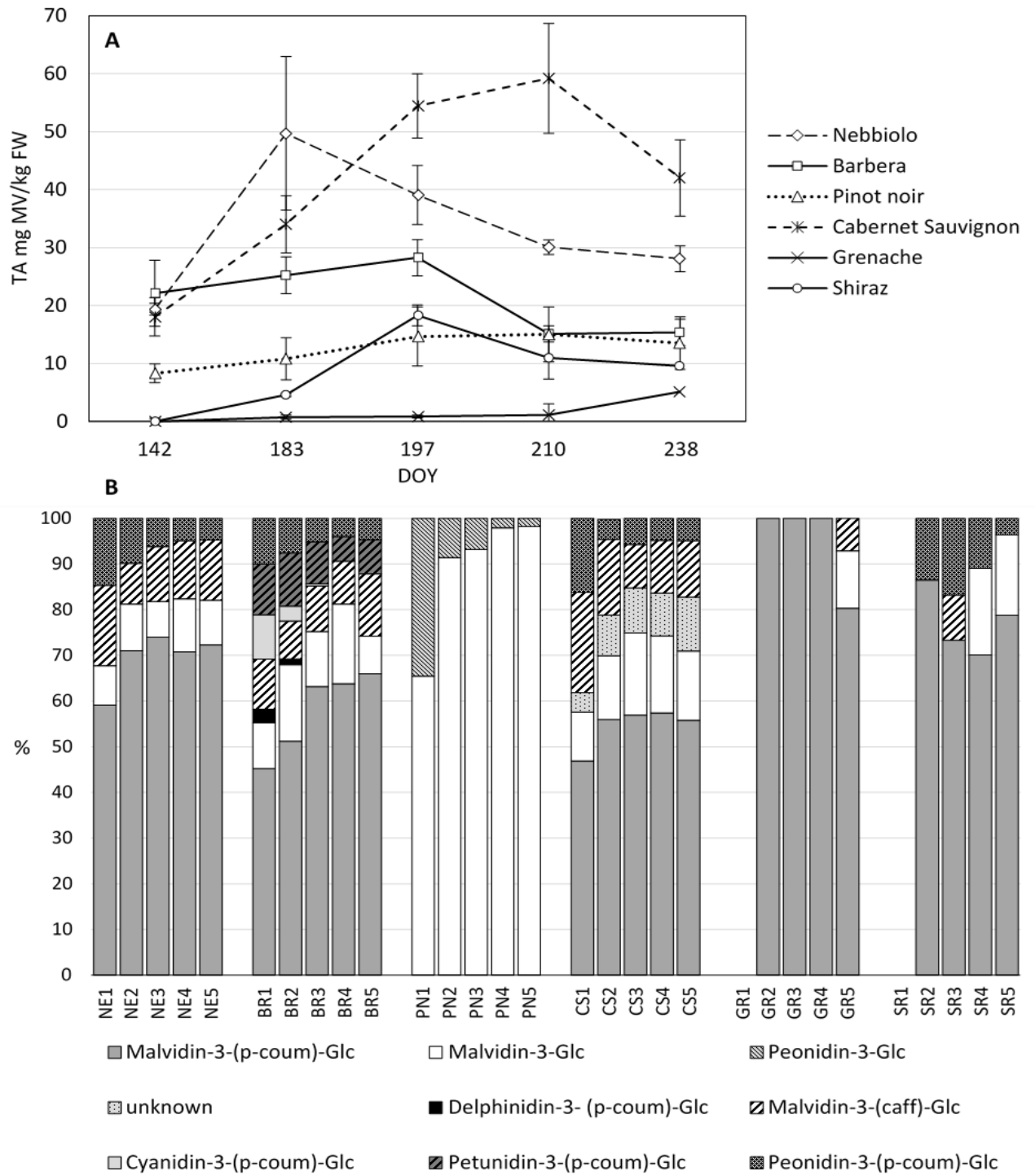


Figure 2

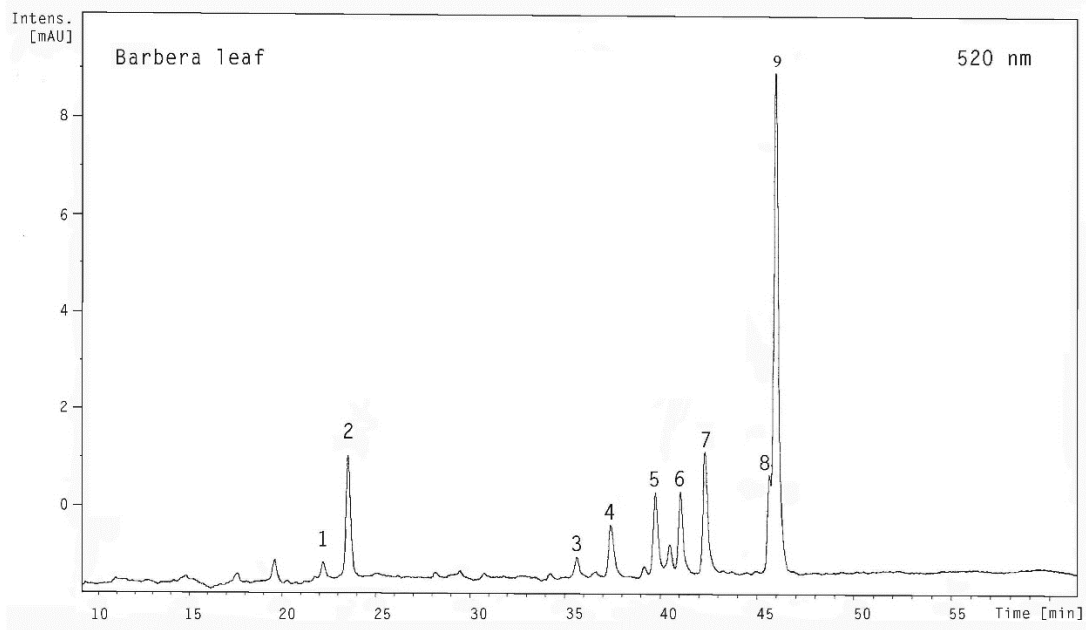


Figure 3

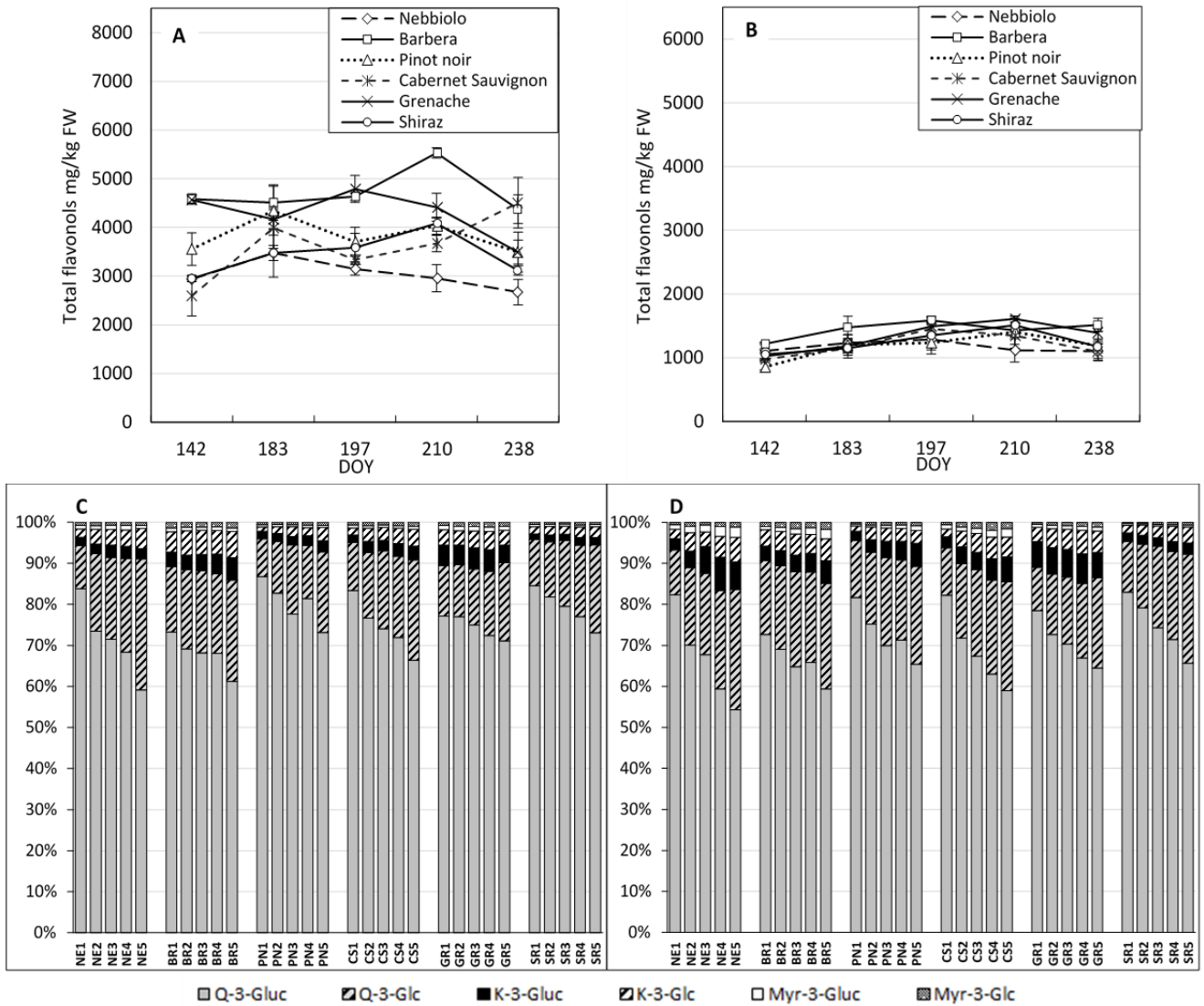


Figure 4

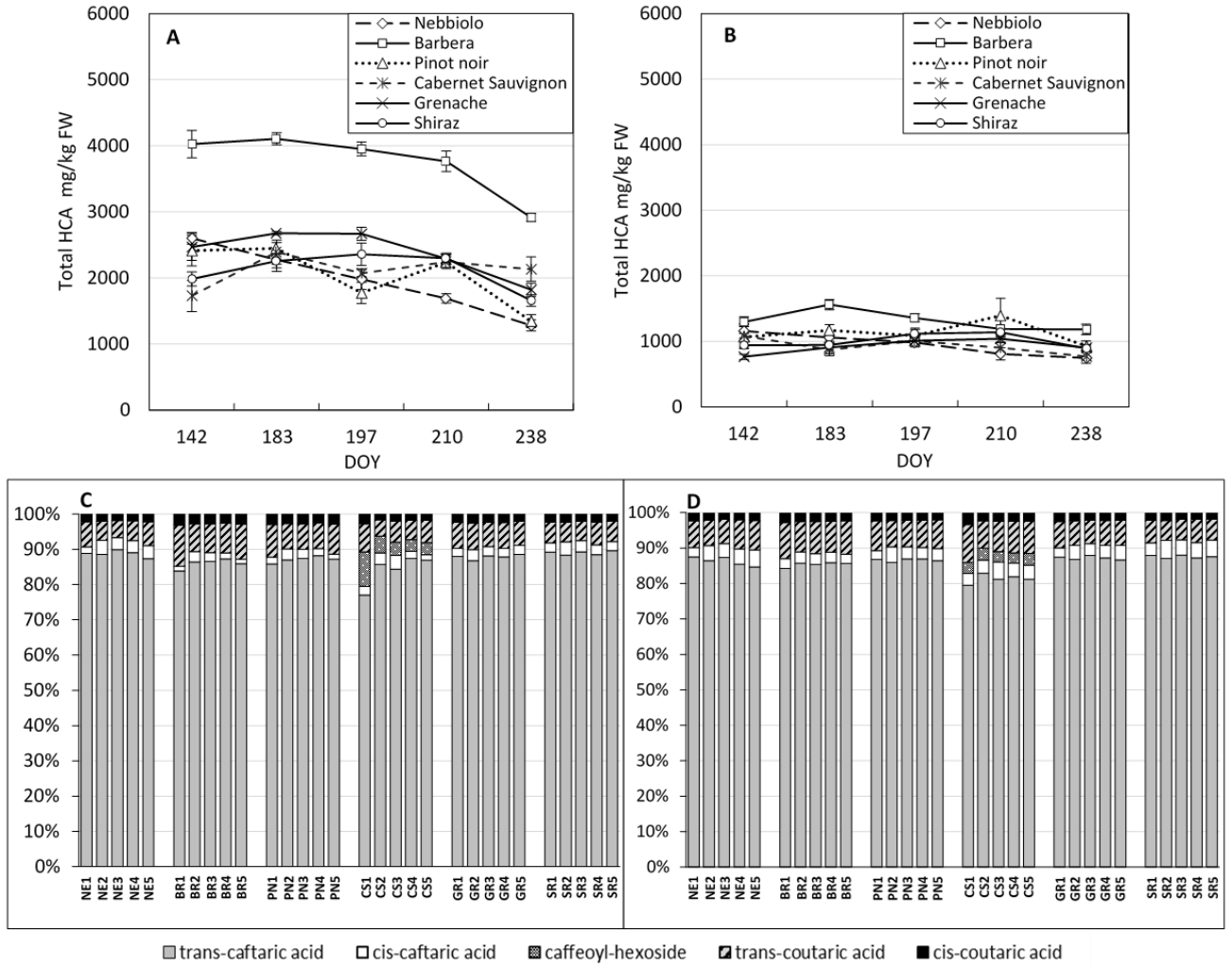


Figure 5

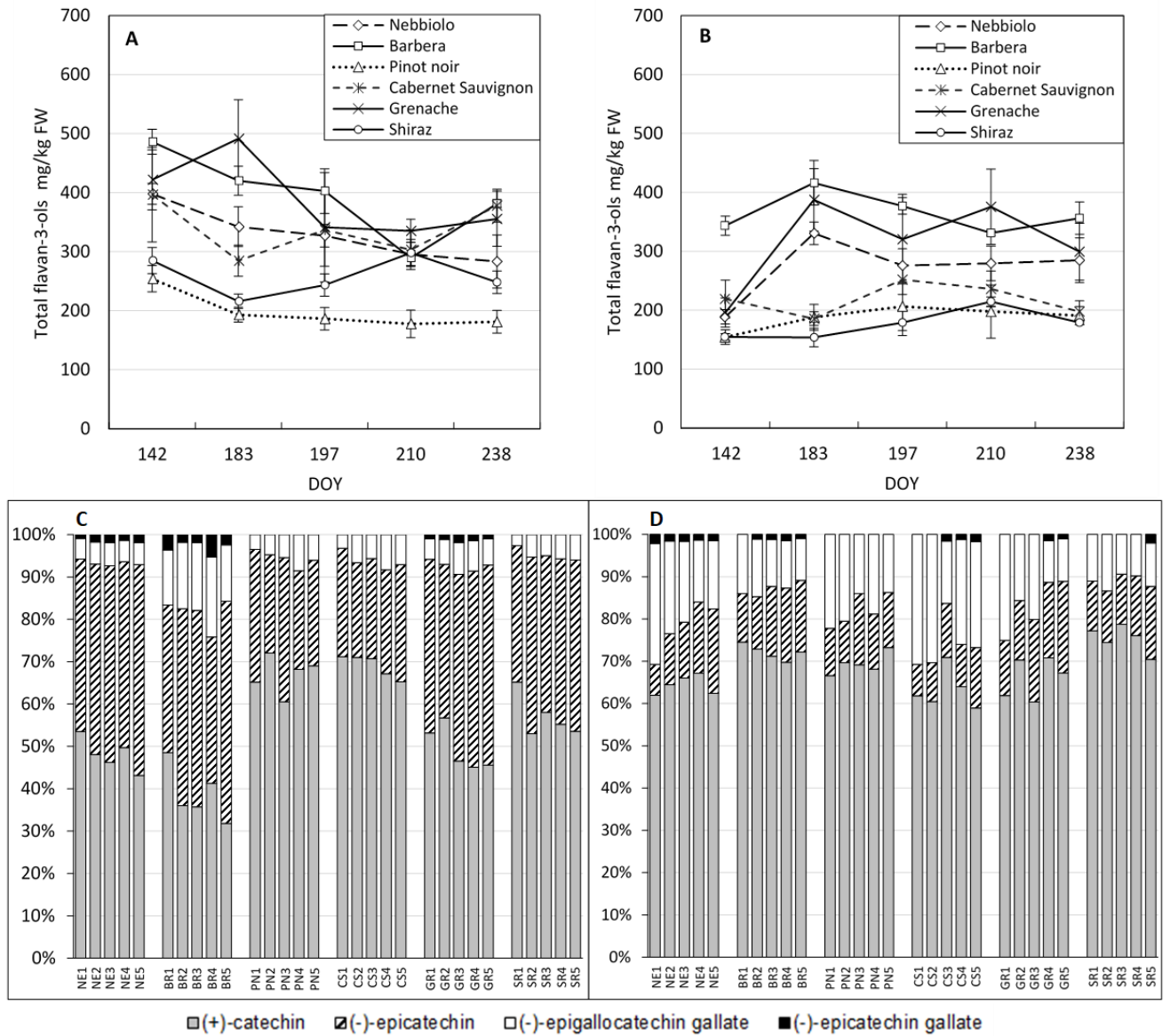


Figure 6

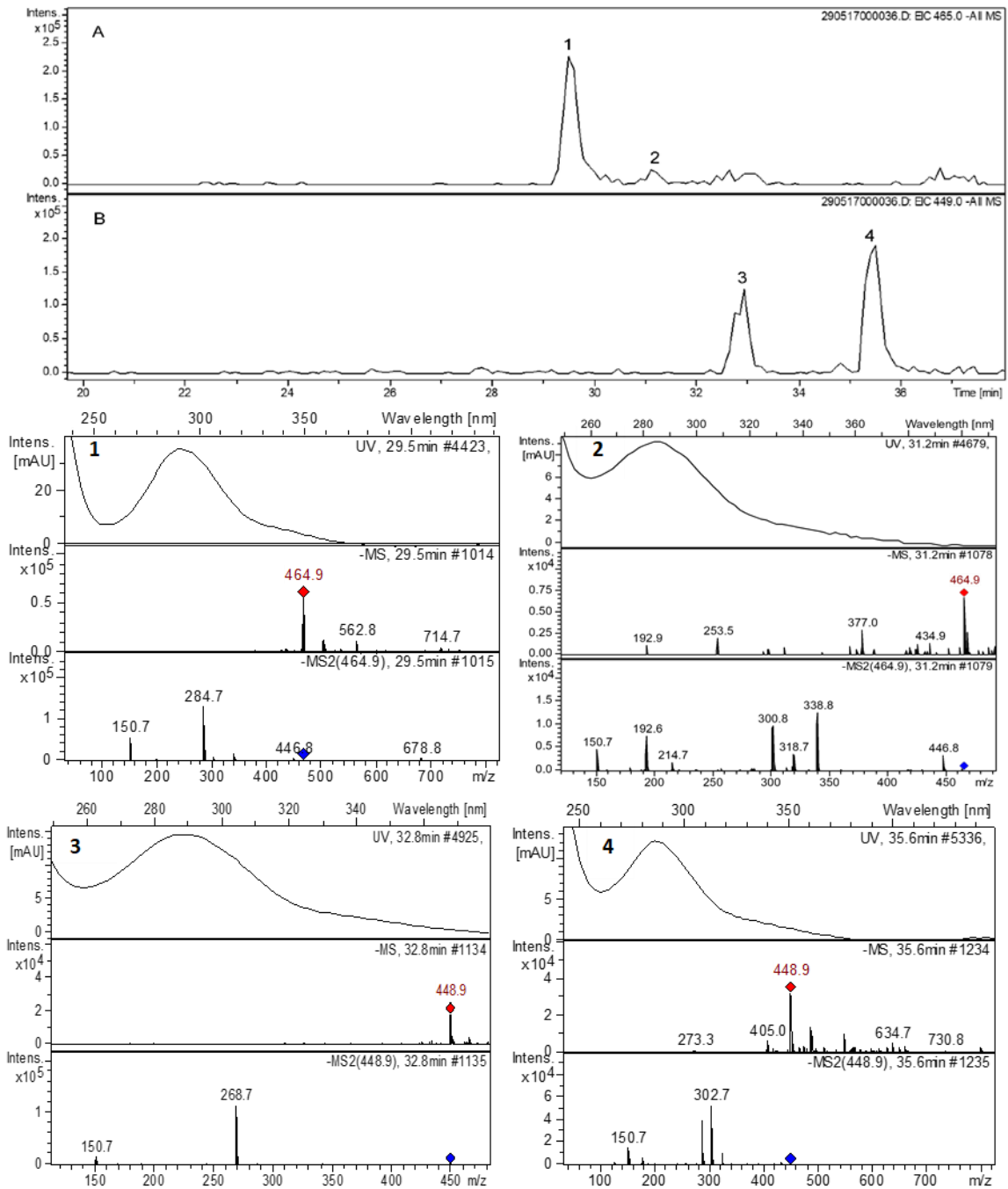


Figure 7



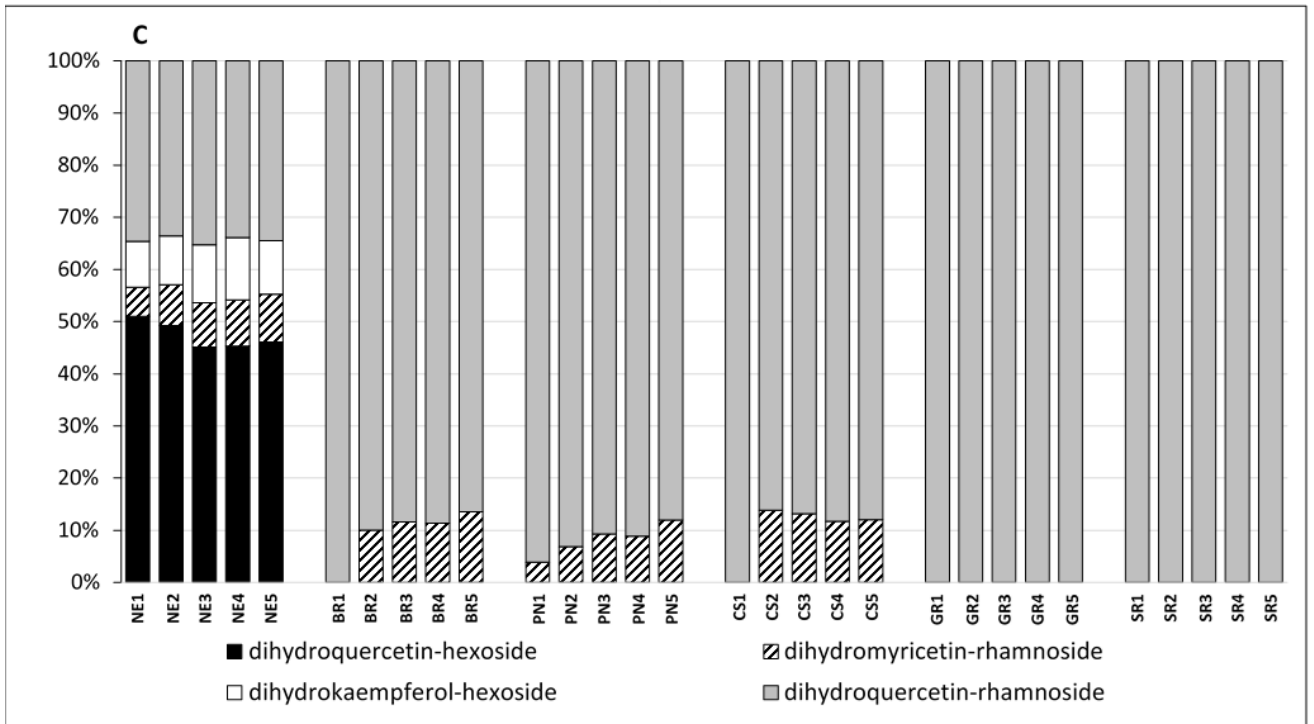
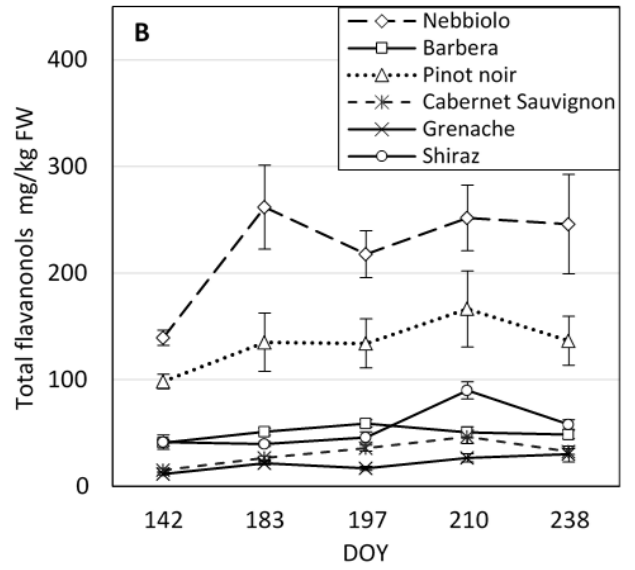
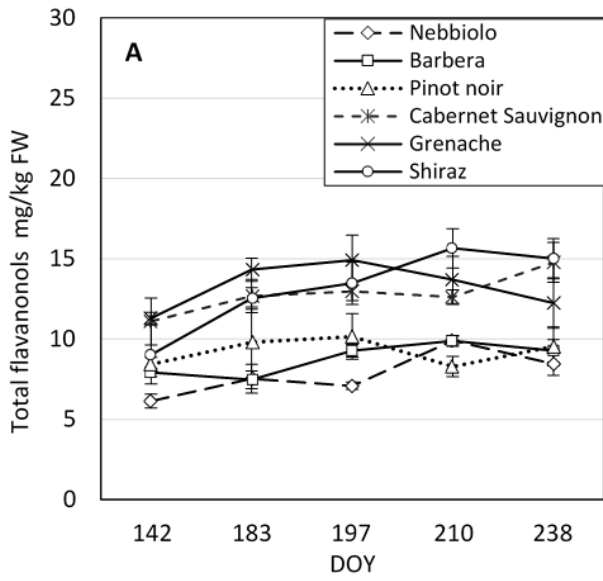


Figure 8

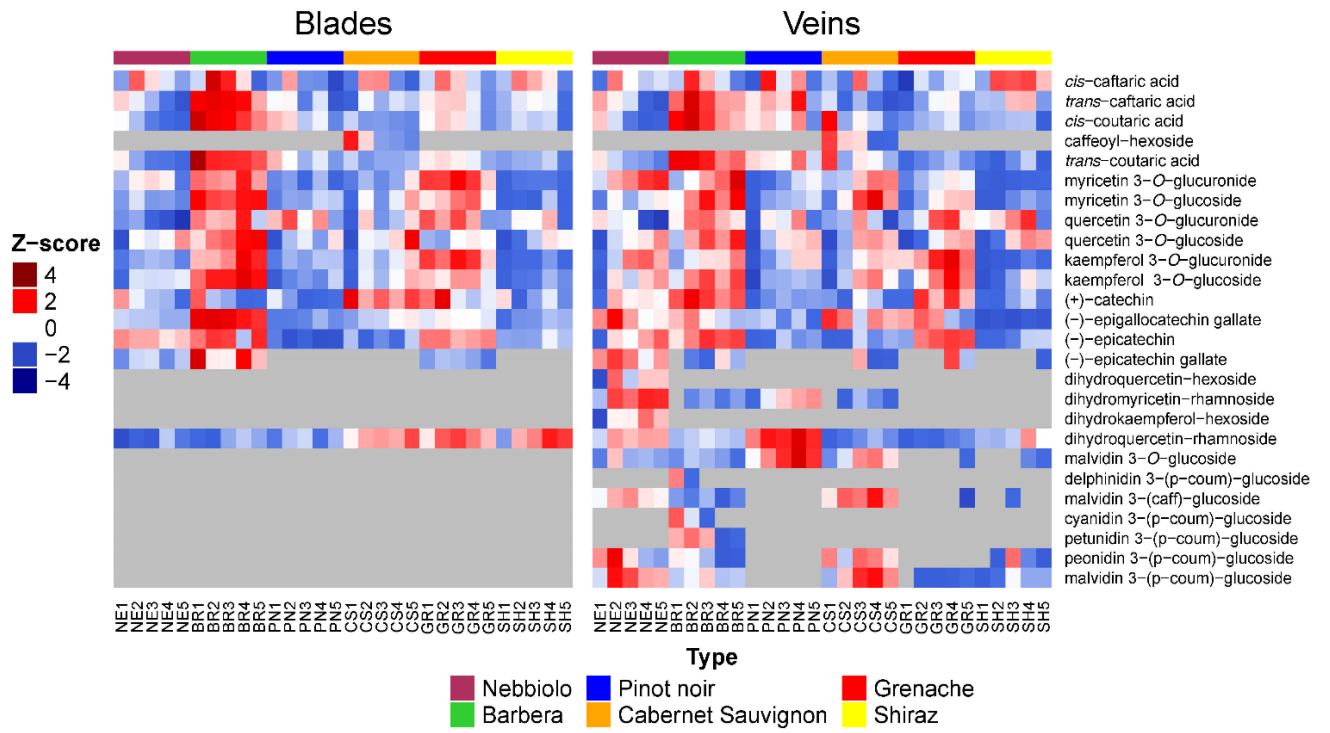


Figure 9

TOC graphic

