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# Effect of leaf removal and ripening stage on the content of quercetin glycosides in Sangiovese grapes

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**ABSTRACT**

Quercetin haze has been observed over the last few years in some aged Sangiovese wines. This problem could be due to an excess of the quercetin in the wine. Leaf removal increases the exposition of clusters to sunlight, which may enhance flavonol synthesis in the grapes. In this study, we evaluated the dynamics related to extractable flavonols in grapes grown in three usually defoliated *Vitis vinifera* (L.) cv. Sangiovese vineyards, whose wines showed quercetin precipitates. The particular structure of the vineyards in which the leaf removal experiments were carried out allowed the influence of vineyard, biotype and rootstock on grape flavonol contents at mid-maturation and technological maturity to be evaluated. The leaves were removed at pre-flowering (early) and at veraison (late). Leaf removal increased the content of extractable glycosidic flavonols in grapes at the two tested ripening stages. In addition, vineyard, biotype and rootstock affected the content of glycosidic flavonols and the interaction between the studied variables was significant. Even though leaf removal induced an increase in extractable quercetin glycosides which can increase the risk of quercetin haze in wine, an examination of the scientific literature on this topic showed that this risk does not depend on the absolute content of these compounds alone.

**KEYWORDS**

leaf removal, level of maturity, rootstock, quercetin glycosides, myricetin glycosides, kaempferol glycosides, Sangiovese grapes

**Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4708**

## INTRODUCTION

Quercetin is a phenolic compound which belongs to the class of flavonols whose glycosides are located in the skins of white and red grapes (Cheynier and Rigaud, 1986; Makris *et al.*, 2006, Mattivi *et al.*, 2006; Castillo-Muñoz *et al.*, 2007). In terms of glycosilated derivatives, white grapes contain small amounts of kaempferol and myricetin (Mattivi *et al.*, 2006), while red grapes contain kaempferol, isorhamentin, myricentin, laricitrin and syringetin (Mattivi *et al.*, 2006; Castillo-Muñoz *et al.*, 2007). On the whole, quercetin is the quantitatively most important flavonol in grape skins; nevertheless, in some red varieties, the content of myricetin–3–glucoside can exceed that of quercetin glucoside (Mattivi *et al.*, 2006; Castillo-Muñoz *et al.*, 2007; Squadrito *et al.*, 2007). Glycosylated derivatives of quercetin have even been found in grapevine leaves (Jeong *et al.*, 2006; Di Stefano and Maggiorotto, 1995) and stems (Souquet *et al.*, 2000; Di Stefano and Maggiorotto, 1995). Quercetin glycosides are present in flower buds and flowers, but they do not seem to be synthesised during the green phase (Cortell and Kennedy, 2006, Downey *et al.*, 2004). Real flavonol synthesis begins shortly before veraison and can continue until the end of maturation (Castellarin *et al.*, 2007; Downey *et al.*, 2004; Keller and Hrazdina, 1998). The genes involved in the synthesis of flavonols have been found to be expressed only in grapes exposed to sunlight (Azuma *et al.*, 2012). In areas where the temperature reaches and exceeds 30 °C for long periods of time, the glycosilated flavonol content of grape usually decreases after reaching a maximum value (Keller and Hrazdina, 1998). It has not been determined whether this decrease is due to flavonol biosynthesis being blocked or to the fact that the degradation reactions exceeded the synthesis reactions. Spayd *et al.* (2002) did not observe any influence of temperature on the synthesis of flavonols. Under normal climatic and cultivation conditions however, the synthesis of glycosidic flavonols occurs during grape maturation (Spayd *et al.*, 2002), except in the case of glucuronides (Castillo-Muñoz *et al.*, 2007). The influence of temperature on the flavonol profile is still to be confirmed (Ferrandino and Lovisolo, 2013). Flavonol synthesis is conditioned by the level of exposure of clusters to direct sunlight: it occurs in limited extent in shaded bunches (Price *et al.*, 1995; Haselgrove *et al.*, 2000; Downey *et al.*, 2004; Cortell and Kennedy, 2006); meanwhile, it is promoted in bunches directly exposed to sunlight (Downey *et al.*, 2004). Flavonol content is also conditioned by grape cultivar (Castillo-Muñoz *et al.*, 2007). Flavonols and their derivatives are considered important for their biomedical and antioxidant properties. In wine, they can contribute to colour intensity and stability through the formation of copigmentation complexes with flavylium, or with chinonoidal forms of anthocyanins (Boulton, 2001). In the latter case, the subtraction of the flavylium ion from the anthocyanin equilibria by flavonol leads to a decrease in the colorless carbinol fraction and an increase in anthocyanin stability. Despite these positive properties, Somers and Ziemelis (1985) and Ziemelis and Pickering (1969) reported a deposit of quercetin in white and red wines respectively. In recent years, a quercetin precipitate has been more frequently observed in bottled wines made from Sangiovese (Gambuti *et al.*, 2020; Lanati *et al.*, 2014) and other grapes in both hemispheres (Marchi *et al.*, 2019). In some aged Sangiovese wines, a quercetin haze can appear after bottling and increase over time. The precipitation of quercetin has been observed to end when it reaches a content lower than 3 mg/L in solution (Gambuti *et al.*, 2020). Under an optic microscope, this precipitate appears as fine needle-shaped crystals (Gambuti *et al.*, 2020; supplementary Figure 1) which do not settle easily. The solubility of quercetin in hydroalcoholic solution and in wine, along with the formation of quercetin deposits, has been extensively studied by Gambuti *et al.* (2020). They found that this solubility depends on several variables, including the content of quercetin and its glycosides in wine, as well as wine composition in terms of other substances; they also revealed that Sangiovese grapes and wines can be rich in quercetin and its glycosides. It seems that quercetin haze can be prevented by microxygenation and wine maturation in barrels (Gambuti *et al.*, 2020; Lanati *et al.*, 2014; Castellari *et al.*, 2001; Castellari *et al.*, 2000), but an examination of the scientific literature on this topic has shown that it would require further research. In previous work we found quercetin deposits in wines from Sangiovese grapes grown in vineyards where leaf removal is applied in the province of Siena in Tuscany. Since leaf removal increases the exposition of clusters to solar radiation, the aim of the present study was to determine the effect of this viticultural practice on flavonol synthesis in berries, which could be related to quercetin haze in wine. The particular structure of the vineyard in which the experiment was carried out also allowed us to evaluate the influence of vineyard, biotype and rootstock on the

  

synthesis of quercetin derivatives at mid ripening and technological maturation.

## MATERIALS AND METHODS

### 1. Standards, reagents and solvents

Quercetin, myricetin and β-glucosidase were purchased from Sigma-Aldrich (Milan, Italy). L(+)-tartaric acid, citric acid, sodium hydroxide, sodium chloride, ortho-phosphoric acid, ethyl acetate, methanol, ethanol 96 %, were purchased from Merck (Milan, Italy).

### 2. Leaf removal

The effect of leaf removal on flavonol biosynthesis was tested on Sangiovese grapevines in three different vineyards (V1, V2 and V3) located in the Siena province (Southern Tuscany, Italy) in 2016. V1, V2 and V3 contained 5000, 6000 and 6500 plants per hectare respectively. Row orientation was north-east/south-west in V1, east/west in V2 and north-west/south-east in V3. The following biotypes were cultivated: CH20 and Tebano 19 both on 420A rootstock in V1, Massale selection on 110R and 420A in V2 and Massale selection on 1103P, 420A and 101-14 in V3. The soil composition of the three vineyards is reported in Supplementary Table 1. V1 was fertilised with auto-produced compost, organic granulate fertiliser (Green Sprint and Choncimer) and field bean green manure; V2 was fertilised with auto-produced compost, organic granulate fertilizer and vetch/oat green manure; and V3 was fertilised with organic granulates only. Irrigation was not carried out on any of the vineyards and all the grapevines were trained on a spurred cordon. Early leaf removal was carried out during the pre-flowering phase in mid May (early) and at veraison (late) on 1 August. Sampling was carried out at mid-ripening (9-12 September ) and at technological maturity (29 September to 3 October). Leaf removal was carried out on 8-10 shoots per plant, and 3-5 leaves were removed from the sprout insertion up to the height of the second bunch in order to fully expose the bunches. The grapevines from which leaves were not removed were the control.

### 3. Flavonol extraction from grape berries

Samples of 200 berries with their pedicels were randomly collected. Each sample of 200 berrieswas placed in a plastic bag. The samples were then transported in a cooler to the laboratory where they were processed for HPLC analysis. The sample preparation for the determination of extractable glycosidic flavonols was similar to the one reported by Ribéreau-Gayon *et al.* (2012) for the determination of phenolic maturity. In brief: 200 berries were homogenized in a blender and the homogenate was centrifuged at 4000 rpm. Then 10 mL of the resulting juice, added with 0.5 g NaCl, were extracted twice with 20 mL of ethyl acetate. After solvent evaporation by rotavapor (35 °C), the precipitate was dissolved in 2 mL of solution composed of $H_3PO_4$ $10^{-3}$ M in 40:60 v:v methanol:$H_2O$, filtered using a 0.45/mm membrane filter and analysed by HPLC.

### 4. HPLC conditions

HPLC-DAD model Series 200 (Perkin Elmer, Waltham, Massachusetts) was used with the following solvents: $H_3PO_4$ $10^{-3}$ M in water (Solvent A), and methanol LiChrosolv (Solvent B) for which the linear gradient was 0 min 0 %, 5 min 5 %, 10 min 10 %, 25 min 30 %, 40 min 60 %, 50 min 100 %, 60 min 100 %, 65 min 5 %. Flow was 0.8 mL/min and λ was 360 nm. Injection volume was 20 µL and column temperature was 40 °C.

The column was a LiChrospher® 100 RP-18 (5 µm) - LiChroCart® 250-4, Merck (Milan, Italy) and the guard column was a LiChrospher® 100 RP-18 (5 µm) - LiChroCart® 4-4, Merck (Milan, Italy)

### 5. Identification of flavonols

Quercetin glucoside (QGs) and myricetin glucoside (MyGs) were identified from the disappearance of a single HPLC peak due the formation of the corresponding aglycon after 1 h incubation at 35 °C with β-glucosidase (Vrhovsek *et al.*, 2004). Quercetin and myricetin were identified by comparing their chromatographic retention time and UV spectra with those of authentic standards. Myricetin-3-glucuronide (MyGr), quercetin-3-glucuronide (QGr), kaempferol glucuronide (KGr) and kaempferol-3-glucoside (Kgs) were tentatively identified from their UV spectra and the sequence of HPLC elution reported by Castillo-Muñoz *et al.* (2007). An HPLC chromatogram of Sangiovese grape flavonols obtained with the method described in sections 3 and 4 is reported in Supplementary Figure 2. All the analyses were performed in duplicate and the data were expressed as mg of quercetin equivalent per kg of grape berries.

### 6. Statistical treatment of data

The obtained data were subjected to a five-way analysis of variance (ANOVA). Where possible,

**TABLE 1.** Flavonol contents of Sangiovese grapes subjected to early (mid May) and veraison (1 August) leaf removal in 2016. Berries sampled at mid-ripening (9-12 September ) and at technological maturity (29 September to 3 October) (as mg of quercetin equivalents per kg of berries).

| | | | Mid-ripening | | | Technological Maturity | | |
|---|---|---|---|---|---|---|---|---|
| | | | Early Leaf Rem. | Veraison Leaf Rem. | Control | Early Leaf Rem. | Veraison Leaf Rem. | Control |
| Vineyard 1 | Biotype CH20, Rootstock 420A | Myricetin Gr | 0.47 ± 0.04 | 0.41 ± 0.03 | 0.25 ± 0.00 | 0.41 ± 0.04 | 0.35 ± 0.02 | 0.30 ± 0.02 |
| | | Myricetin Gs | 7.07 ± 0.07 | 7.05 ± 0.16 | 5.82 ± 0.22 | 6.44 ± 0.21 | 5.55 ± 0.11 | 5.35 ± 0.05 |
| | | Quercetin Gr | 6.96 ± 0.25 | 5.10 ± 0.07 | 3.81 ± 0.22 | 5.29 ± 0.13 | 3.98 ± 0.23 | 3.74 ± 0.04 |
| | | Quercetin Gs | 17.96 ± 0.52 | 17.64 ± 0.82 | 15.22 ± 0.15 | 17.58 ± 0.59 | 15.12 ± 0.39 | 15.98 ± 0.61 |
| | | Kaempferol Gr | 0.66 ± 0.11 | 0.71 0.07 | 0.74 ± 0.01 | 0.68 ± 0.03 | 0.58 ± 0.02 | 0.59 ± 0.01 |
| | | Kaempferol Gs | 2.63 ± 0.08 | 2.79 ± 0.17 | 1.86 ± 0.04 | 2.61 ± 0.04 | 2.29 ± 0.06 | 2.08 ± 0.04 |
| | Biotype Tebano 19, Rootstock 420° | Myricetin Gr | 0.63 ± 0.06 | 0.74 ± 0.03 | 0.35 ± 0.00 | 0.54 ± 0.03 | 0.59 ± 0.03 | 0.32 ± 0.02 |
| | | Myricetin Gs | 5.37 ± 0.10 | 5.52 ± 0.20 | 4.16 ± 0.25 | 6.18 ± 0.14 | 5.91 ± 0.11 | 4.37 ± 0.20 |
| | | Quercetin Gr | 8.45 ± 0.30 | 7.85 ± 0.17 | 5.21 ± 0.14 | 5.74 ± 0.18 | 5.70 ± 0.17 | 3.82 ± 0.27 |
| | | Quercetin Gs | 15.18 ± 0.35 | 15.86 ± 0.10 | 10.79 ± 0.36 | 16.18 ± 0.33 | 16.89 ± 0.34 | 11.16 ± 0.56 |
| | | Kaempferol Gr | 0.70 ± 0.01 | 0.76 ± 0.03 | 0.44 ± 0.01 | 0.74 ± 0.08 | 0.83 ± 0.10 | 0.36 ± 0.06 |
| | | Kaempferol Gs | 2.32 ± 0.02 | 2.21 ± 0.00 | 1.01 ± 0.03 | 2.04 ± 0.15 | 2.31 ± 0.19 | 0.89 ± 0.02 |
| Vineyard 2 | Massale selection, Rootstock 110R | Myricetin Gr | 0.59 ± 0.03 | 0.61 ± 0.03 | 0.42 ± 0.05 | 0.61 ± 0.01 | 0.49 ± 0.03 | 0.42 ± 0.05 |
| | | Myricetin Gs | 5.25 ± 0.14 | 5.15 ± 0.08 | 3.62 ± 0.13 | 6.12 ± 0.19 | 6.82 ± 0.09 | 5.09 ± 0.25 |
| | | Quercetin Gr | 8.47 ± 0.15 | 6.83 ± 0.17 | 5.40 ± 0.17 | 6.89 ± 0.09 | 6.18 ± 0.13 | 4.24 ± 0.25 |
| | | Quercetin Gs | 14.41 ± 0.24 | 14.78 ± 0.34 | 11.99 ± 0.41 | 19.16 ± 0.23 | 20.63 ± 0.61 | 13.98 ± 0.24 |
| | | Kaempferol Gr | 0.76 ± 0.03 | 0.7 ± 0.06 | 0.2 ± 0.11 | 0.84 ± 0.02 | 0.99 ± 0.07 | 0.58 ± 0.02 |
| | | Kaempferol Gs | 1.81 ± 0.12 | 1.73 ± 0.14 | 0.85 ± 0.07 | 2.40 ± 0.07 | 2.92 ± 0.11 | 1.78 ± 0.05 |
| | Massale selection, Rootstock 420° | Myricetin Gr | 0.68 ± 0.02 | 0.48 ± 0.01 | 0.34 ± 0.02 | 0.50 ± 0.04 | 0.34 ± 0.02 | 0.42 ± 0.02 |
| | | Myricetin Gs | 4.61 ± 0.22 | 3.87 ± 0.24 | 2.91 ± 0.08 | 5.75 ± 0.10 | 5.12 ± 0.24 | 4.63 ± 0.09 |
| | | Quercetin Gr | 7.91 ± 0.20 | 6.86 ± 0.23 | 4.77 ± 0.16 | 7.13 ± 0.29 | 4.40 ± 0.19 | 4.21 ± 0.25 |
| | | Quercetin Gs | 16.41 ± 0.15 | 14.89 ± 0.21 | 10.59 ± 0.76 | 18.32 ± 0.59 | 16.65 ± 0.23 | 15.56 ± 0.27 |
| | | Kaempferol Gr | 0.96 ± 0.09 | 0.95 ± 0.03 | 0.17 ± 0.00 | 1.15 ± 0.05 | 0.70 ± 0.02 | 0.42 ± 0.07 |
| | | Kaempferol Gs | 2.78 ± 0.01 | 2.77 ± 0.05 | 0.69 ± 0.05 | 3.34 ± 0.10 | 2.72 ± 0.05 | 1.94 ± 0.05 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Vineyard 3 | Massale selection, Rootstock 110 3P | Myricetin Gr | 0.48 ± 0.03 | 0.40 ± 0.02 | 0.42 ± 0.01 | 0.41 ± 0.03 | 0.28 ± 0.03 | 0.29 ± 0.00 |
| | | Myricetin Gs | 4.25 ± 0.04 | 3.70 ± 0.15 | 4.19 ± 0.10 | 6.26 ± 0.22 | 5.93 ± 0.12 | 6.07 ± 0.05 |
| | | Quercetin Gr | 5.94 ± 0.27 | 5.19 ± 0.07 | 5.32 ± 0.18 | 4.62 ± 0.22 | 3.37 ± 0.19 | 3.09 ± 0.12 |
| | | Quercetin Gs | 15.19 ± 0.58 | 12.57 ± 0.48 | 12.41 ± 0.15 | 17.08 ± 0.21 | 17.15 ± 0.45 | 15.59 ± 0.35 |
| | | Kaempferol Gr | 0.63 ± 0.04 | 0.58 ± 0.04 | 0.56 ± 0.01 | 0.80 ± 0.02 | 0.53 ± 0.08 | 0.58 ± 0.03 |
| | | Kaempferol Gs | 1.81 ± 0.07 | 1.89 ± 0.14 | 1.84 ± 0.08 | 2.33 ± 0.09 | 2.04 ± 0.09 | 1.91 ± 0.10 |
| | Massale selection, Rootstock 420° | Myricetin Gr | 0.61 ± 0.04 | 0.58 ± 0.03 | 0.54 ± 0.02 | 0.49 ± 0.03 | 0.32 ± 0.00 | 0.39 ± 0.02 |
| | | Myricetin Gs | 5.39 ± 0.27 | 4.71 ± 0.17 | 4.61 ± 0.17 | 8.01 ± 0.01 | 6.85 ± 0.14 | 6.58 ± 0.18 |
| | | Quercetin Gr | 5.97 ± 0.33 | 5.55 ± 0.14 | 4.99 ± 0.23 | 5.58 ± 0.11 | 3.41 ± 0.31 | 3.90 ± 0.20 |
| | | Quercetin Gs | 13.83 ± 0.44 | 12.92 ± 0.50 | 12.40 ± 0.33 | 17.78 ± 0.33 | 15.74 ± 0.50 | 14.24 ± 0.38 |
| | | Kaempferol Gr | 0.59 ± 0.11 | 0.58 ± 0.01 | 0.57 ± 0.03 | 1.09 ± 0.06 | 0.77 ± 0.04 | 0.76 ± 0.04 |
| | | Kaempferol Gs | 2.23 ± 0.06 | 1.53 ± 0.05 | 1.32 ± 0.05 | 3.11 ± 0.11 | 2.10 ± 0.06 | 1.89 ± 0.01 |
| | Massale selection, Rootstock 101-14 | Myricetin Gr | 0.49 ± 0.02 | 0.51 ± 0.03 | 0.48 ± 0.02 | 0.34 ± 0.01 | 0.27 ± 0.01 | 0.26 ± 0.01 |
| | | Myricetin Gs | 4.60 ± 0.23 | 5.29 ± 0.28 | 4.39 ± 0.23 | 6.56 ± 0.02 | 6.01 ± 0.12 | 5.79 ± 0.05 |
| | | Quercetin Gr | 5.71 ± 0.23 | 5.30 ± 0.14 | 5.05 ± 0.19 | 4.82 ± 0.18 | 3.79 ± 0.12 | 3.61 ± 0.09 |
| | | Quercetin Gs | 14.66 ± 0.21 | 14.87 ± 0.45 | 14.53 ± 0.49 | 17.46 ± 0.61 | 16.65 ± 0.38 | 15.82 ± 0.37 |
| | | Kaempferol Gr | 0.66 ± 0.01 | 0.76 ± 0.03 | 0.63 ± 0.01 | 0.91 ± 0.07 | 0.68 ± 0.05 | 0.49 ± 0.03 |
| | | Kaempferol Gs | 1.96 ± 0.01 | 2.28 ± 0.08 | 1.74 ± 0.05 | 2.96 ± 0.13 | 2.65 ± 0.10 | 2.40 ± 0.01 |

Gr: glucuronide; Gs: glucoside

the interaction between factors was included in the model (i.e., Vineyard:Sampling time, Biotype:Sampling time, Rootstock:Sampling time, Vineyard:Leaf removal, Biotype:Leaf removal, Rootstock:Leaf removal, Sampling time:Leaf removal, Vineyard:Sampling time:Leaf removal, Biotype:Sampling time:Leaf removal, Rootstock:Sampling time:Leaf removal). In the case of a null hypothesis rejection (F test p-value < 0.05), the levels of the main factors and interactions (where available) were compared using the Tukey's HSD *post hoc* test with an α value of 0.05. The software used was R 3.6.2 (The R Foundation for Statistical Computing, Wien). The R package *agricolae* (De Mendiburu, 2020) was used to perform the *post hoc* test. The interaction *post hoc* results are given in the Supplementary Material section.

## RESULTS AND DISCUSSION

### 1. Extractable flavonols

The contents of extractable flavonols, expressed as mg of quercetin equivalent per kg of grape berries (Tables 1 and 2), may seem low (they fluctuate around 30 mg per kg) for Sangiovese grapes, which are usually considered to be rich in these compounds (Gambuti *et al.*, 2020); however, the content of the flavonols expressed as individual glycosides fluctuating around 40 mg per kg and the fact that only a part of grape flavonols (the extractables) were determined with the method described in Materials and methods, confirm the richness in flavonols of Sangiovese grapes.

Few data about the contents of glycosidic flavonols in grapes are available in the scientific literature. In Sauvignon blanc, Thompson Seedless and Chardonnay extracts, Meyer *et al.* (1997) found 4.8 to 10.4 mg/L of rutin equivalents. Similar amounts were found by Spanos & Wrolstad (1992) and Frankel *et al.* (1998). More recently Mattivi *et al.* (2006), reported the flavonol profile of a vast number of white and red grape varieties. In the latter group, a Sangiovese sample showed a flavonol content of 24.56 mg/kg (probably expressed as aglycons). In terms of flavonols, Sangiovese can thus be considered a medium-rich variety. Higher contents in flavonols (from 129 to 346 µmol/kg) were found by Castillo-Muñoz *et al.* (2007) in three Iberian and four French cultivars. The differences in flavonol concentrations can be attributedto different variables, such as cultivar, environment, viticoltural practices and analytical procedures. For Sangiovese, the differences can widely vary as a result of its sensitivity to environmental and viticoltural conditions, which affect grape skin thickness (Gambuti *et al.*, 2020). It is possible to determine the total concentration of flavonols by extracting them from grape skins using organic solvents (Castillo-Muñoz *et al.,* 2007; Mattivi *et al.*, 2006). In this study, glycosidic flavonols were extracted from grape homogenates (extractable flavonols), which had been processed for the determination of phenolic maturity (Ribéreau-Gayon *et al.*, 2012). This simple method avoids extracting flavonol from grape skins using large volumes of solvents and allows a large number of fresh grape samples to be processed, avoiding grape freezing and complicated manual operations. However, it would be necessary to carry out a study on the extractable and total flavonols based on the level of maturity of different grape varieties. In the present study, the flavonols were concentrated via ethyl acetate extraction from the grape juice obtained from centrifugation of the grape homogenate. The absence of ethanol meant that it was not necessary to de-alcoholise the juice before extraction. The analysis of variance results (Table 2) show that all the main factors studied and all the available interactions between variables (Supplementary Material) influenced the total flavonol concentrations in the grapes. In Table 2 it can be seen that early leaf removal induced an increase in total flavonol concentration compared to leaf removal applyed at veraison. This result suggests that the intensity of the solar radiation that the grapes receive before veraison also influenced glucosydic flavonol biosynthesis. The control (not defoliated) contained less of these molecules. The absolute values for the differences between leaf removal samples and the control were higher than those for the differences between the two sampling times; this is probably due to the two opposite contributions of the flavonol glucuronides and glucosides whose content decreases and increases respectively during maturation. A greater synthesis of flavonols seems to have occured in biotype CH20, vineyards V1 and V2, and rootstock 110R.

### 2. Glucuronide Flavonols (MyGr, QGr, KGr)

For all three glucuronide flavonols (FGr), the analysis of variance showed significant differences induced by all the variables studied (Table 2). Furthermore, the results reported in Tables 1 and 2 show that the content of FGr as quecetin equivalents decreased from mid-ripening to technological maturity, probably because oxidation reactions start near veraison and their synthesis, which begins just before veraison, ends

  

**TABLE 2.** Results of ANOVA and Tukey's test applied to the data in Table 1.

| Factor | Level of factor | QGr | Sign. | QGs | Sign. | MyGr | Sign. | MyGs | Sign. | KGr | Sign. | KGs | Sign. | ∑ flavonols | Sign. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leaf removal | Veraison | 5.25 ± 1.36 b | *** | 15.89 ± 2.02 b | *** | 0.46 ± 0.14 b | *** | 5.53 ± 1.00 b | *** | 0.72 ± 0.14 b | *** | 2.28 ± 0.42 b | *** | 30.14 ± 3.39 b | *** |
| | Early | 6.39 ± 1.25 a | | 16.53 ± 1.64 a | | 0.50 ± 0.09 a | | 5.85 ± 1.02 a | | 0.80 ± 0.17 a | | 2.43 ± 0.48 a | | 32.51 ± 2.87 a | |
| | Control | 4.37 ± 0.74 c | | 13.58 ± 1.97 c | | 0.35 ± 0.07 c | | 4.83 ± 1.00 c | | 0.50 ± 0.18 c | | 1.58 ± 0.51 c | | 25.21 ± 2.96 c | |
| Sampling time | Tech. mat. | 4.64 ± 1.17 b | *** | 16.42 ± 1.97 a | *** | 0.39 ± 0.11 b | *** | 5.97 ± 0.81 a | *** | 0.72 ± 0.21 a | *** | 2.31 ± 0.53 a | *** | 30.45 ± 4.05 a | *** |
| | Mid Rip. | 6.03 ± 1.29 a | | 14.25 ± 1.99 b | | 0.49 ± 0.12 a | | 4.84 ± 1.03 b | | 0.63 ± 0.20 b | | 1.89 ± 0.60 b | | 28.12 ± 4.31 b | |
| Biotype | CH20 | 4.81 ± 1.19 c | *** | 16.58 ± 1.30 a | *** | 0.37 ± 0.08 c | *** | 6.21 ± 0.72 a | *** | 0.66 ± 0.07 ab | * | 2.38 ± 0.35 a | *** | 31.02 ± 3.46 a | *** |
| | Mass. Sel | 5.28 ± 1.35 b | | 15.28 ± 2.25 b | | 0.44 ± 0.11 b | | 5.27 ± 1.13 b | | 0.69 ± 0.22 a | | 2.10 ± 0.61 b | | 29.06 ± 4.21 b | |
| | Tebano 19 | 6.14 ± 1.67 a | | 14.36 ± 2.58 c | | 0.52 ± 0.16 a | | 5.26 ± 0.80 b | | 0.62 ± 0.20 b | | 1.78 ± 0.61 c | | 28.68 ± 5.46 b | |
| Vineyard | V1 | 5.48 ± 1.57 b | *** | 15.47 ± 2.30 a | *** | 0.44 ± 0.15 b | *** | 5.74 ± 0.89 a | *** | 0.64 ± 0.15 b | *** | 2.08 ± 0.57 a | ns | 29.85 ± 4.63 a | *** |
| | V2 | 6.10 ± 1.45 a | | 15.60 ± 2.83 a | | 0.48 ± 0.12 a | | 4.91 ± 1.07 c | | 0.70 ± 0.30 a | | 2.12 ± 0.78 a | | 29.91 ± 5.49 a | |
| | V3 | 4.73 ± 0.95 c | | 15.07 ± 1.78 b | | 0.41 ± 0.10 c | | 5.51 ± 1.13 b | | 0.68 ± 0.15 a | | 2.09 ± 0.48 a | | 28.49 ± 3.03 b | |
| Rootstock | 101-14 | 4.71 ± 0.81 c | *** | 15.68 ± 1.20 a | *** | 0.39 ± 0.11 c | *** | 5.44 ± 0.81 ab | *** | 0.69 ± 0.14 a | *** | 2.33 ± 0.43 a | *** | 29.23 ± 2.17 b | *** |
| | 1103P | 4.59 ± 1.09 c | | 15.04 ± 2.05 b | | 0.37 ± 0.07 c | | 5.07 ± 1.10 c | | 0.62 ± 0.10 b | | 1.94 ± 0.22 c | | 27.62 ± 2.74 c | |
| | 110R | 6.33 ± 1.38 a | | 15.81 ± 3.21 a | | 0.50 ± 0.11 a | | 5.34 ± 1.04 b | | 0.68 ± 0.26 a | | 1.90 ± 0.66 c | | 30.57 ± 5.67 a | |
| | 420° | 5.43 ± 1.46 b | | 15.20 ± 2.26 b | | 0.45 ± 0.13 b | | 5.49 ± 1.16 a | | 0.68 ± 0.22 a | | 2.13 ± 0.67 b | | 29.39 ± 4.63 b | |

Data are reported as concentrations mean ± standard deviation and group according to Tukey's HSD test; all the concentrations are reported as mg of quercetin per kg ofgrape. Qgr = quercetin-3-glucuronide, Qgs = quercetin-3-glucoside, MyGr = myricetin-3-glucoronide MyGs = myricetin-3-glucoside, Tech. Mat. = Technological maturity, Mid Rip. = Mid Ripening ,Sign. = F distribution probability cumulated from right correspondent to the ANOVA F value. ns stands for cumulated probablity ≥ 0.05, * stands for 0.01 ≤ cumulated probability < 0.05, ** stands for 0.001 ≤ cumulated probability < 0.01, *** stands for cumulated probability < 0.001.

with this event (Castellarin *et al.*, 2007; Pilati *et al.*, 2007). This trend is in agreement with Castillo-Muñoz *et al.* (2007). The highest values for FGr were found in the early leaf removal trials (bunches more exposed before veraison) and the lowest in the control trial (less exposed) (Tables 1 and 2). These data clearly show that the lower the exposure of the bunches to sunlight (no leaf removal), the lower the synthesis of FGr, as was also observed by Downey *et al.* (2004). Among vineyards and biotypes, the differences between MyGr and QGr contents were statistically supported (Table 2): for vineyards, the highest value was found in V2 (rows east/west) and the lowest in V3 (rows north-east/south-west); for biotypes, the highest value was found for Tebano 19 and the lowest for CH20; and for rootstocks, 110R showed the highest content in MyGr and QGr. As can be seen in Table 2, the differences in KGr concentration between vineyards, biotypes and rootstocks follows a trend different to those found for MyGr and QGr.

### 3. Glucoside Flavonols (MyGs, QGs, KGs)

The analysis of variance showed that the influence of all the studied variables on the contents of glucoside flavonols (FGs) was significant, with the exception of the vineyard for kaempferol glucoside (KGs) (Table 2). Furthermore, the interactions between pairs of variables (except for KGs biotype:sampling time) were found to be different (Supplementary Material). In contrast to FGr, the content of all glucoside flavonols (i.e., MyGs, Qgs and KGs) as quercetin equivalents increased from mid-ripening to technological maturity, as can be seen in Table 2 (Sampling time). This result suggests that FGs synthesis - which starts at veraison (Castellarin *et al.*, 2007) - continued during the ripening process and may have overcome possible degradation reactions. FGs synthesis was greater in early and veraison leaf removal than in the control, as can be expected given the known effect of reducing the exposure of bunches to sunlight on flavonol synthesis (as in the control) (Downey *et al.*, 2004). However, the fact that FGs content was higher in early leaf removal than leaf removal at veraison (Tables 1 and 2) indicates that the intensity of solar radiation received by grapes before veraison may also influence FGs biosynthesis (see above).

In contrast to the observations for FGr, of the biotypes, CH20 exhibited the highest concentration of FGs, while Tebano 19 showed the lowest. This could be due to CH20 being earlier than Tebano 19 (more time for degradation of FGr and synthesis of FGs). The analysis of the absolute values for the differences in QGs concentration reveals that leaf removal, sampling time and biotype had more influence on the accumulation of this compound than vineyard and rootstock. This effect may be due to the high exposure of the three study vineyards to sunlight. The variability of the grape skin thickness depending on the Sangiovese biotype may also play a role, as there may be a relationship between the accumulation of glycosylated flavonols and skin parameters; however, further studies would be necessary to explore this hypothesis.

### 4. Glycoside quercetin content of grapes and quercetin precipitation in wines

Unlike in grapes, flavonols are present in wines in the form of aglycon. In white wines, the content of flavonol has been found to be from 0.5 to 1.5 mg/L (Hertog *et al.*, 1993), 0.4 to 2.5 mg/L (Simonetti *et al.*, 1997), and either absent or in traces (Soleas *et al.*, 1997). In 47 Spanish sparkling wines, total flavonol content was found to vary between 0.1 and 1.2 mg $L^{-1}$ (Satué-Gracia *et al.*, 1999).

In red wines, glycosidic flavonol content widely varies; for example, from 4.17 to 93.08 mg/L (Makris *et al.*, 2006), 4.6 to 41.6 mg/L McDonald *et al.* (1998), and 5.3 to 54.2 mg/L (Gardner *et al.,* 1999). Meanwhile, in Italian red wines, Gambuti *et al.* (2020) found 2.02 to 33.9 mg/L of glycosidic quercetin and 0.01 to 8.6 mg/L of quercetin. According to the following authors, the quercetin content of wines were generally found to be low: 5 mg/L (Ghiselli *et al.*, 1998), 0.5 to 9.9 mg/L (Gardner *et al.*, 1999), 0.3 to 0.7 mg/L (Buiarelli *et al.,* 2018). In Sangiovese wines, Gambuti *et al.* (2020) found 3.1 to 33.9 mg/L of glycosidic quercetin and 0.4 to 8.6 mg/L of quercetin, while McDonald *et al. (*1998) found 1.2 to 21.8 mg/L of glycosidic quercetin and 0.1 to 15.8 mg/L of quercetin.

Quercetin, which is the main component of the flavonol deposit found in Sangiovese wine, is formed by hydrolysis of its glycosylates, starting from fermentation and during wine storage (Gambuti *et al.*, 2020). The varietal, environmental and technological variables influencing the glycosidic quercetin and quercetin content of wines have been reviewed by Gambuti *et al.* (2020) and Lanati *et al.* (2014). To our knowledge, the only available information on the influence of winemaking techniques on the quercetin content of wine has been documented by Gambuti *et al.* (2020), Lanati *et al.* (2014),

  

Castellari *et al.* (2001) and Castellari *et al.* (2000). The highly variable quantities of quercetin and glycosidic quercetin found in Sangiovese wines indicates that the quercetin precipitation depends on the level of ripeness of the grape and on a considerable number of other variables - some of which (e.g., agricultural practices, rootstock, vineyard and biotype) have been studied here.

Assuming that total or extractable glycoside flavonol content of grapes has an influence on flavonol precipitation, the results of the present study can be considered to contribute to improving knowledge on the subject. They are in agreement with those obtained in previous experiments on the flavonol content of grapes exposed to different intensities of sunlight (Price *et al.*, 1995; Downey *et al.*, 2004).

The leaf removal effect reported in this paper, increased both the exposure of the Sangiovese bunches to light and the glycosidic flavonol concentration; this therefore, indicates that defoliated grapes may produce wines more prone to the formation of quercetin deposits. However, exposure to sunlight is probably not the only factor to have an ultimate affect on quercetin deposits in wine. In fact, as shown by the data in studies by McDonald *et al.* (1998) and Castillo-Muñoz *et al.* (2007), no haze due to the insolubilisation of quercetin was observed in wines from hot and sunny areas with high flavonol content. In addition, the influence of grape maturity, vineyard, biotype and rootstock on the content of extractable flavonols, as shown by the results of the present study, make it difficult to establish general rules for limiting the content of glycosidic quercetin and quercetin in grapes and wines.

The glycosidic quercetin and quercetin contents of Italian wines from different varieties that were found by Gambuti *et al.* (2020) show that also grape variety affect the synthesis of glycosidic flavonols. Sangiovese wines were the richest in flavonols among those produced using autochthonous and international varieties cultivated in Italy (Gambuti *et al.*, 2020) and Cabernet-Sauvignon, Merlot and Syrah were also found to be rich in flavonols in other studies (McDonald *et al.*, 1998; Castillo-Muñoz *et al.*, 2007). However, wines rich in flavonols other than Sangiovese, such as those reported by the latter authors were not found to contain quercetin deposits; other factors must therefore be at play that need to be identified and studied.

## CONCLUSIONS

The results obtained in our field experiment confirmed those of other studies on the influence of the exposure of grape bunches to direct sunlight on the synthesis of flavonols. In fact, the highest content of glycosidic flavonols was observed in grape from defoliated vines, and the lowest in those not defoliated. Leaf removal at veraison also led to a lower increase in flavonol synthesis than defoliation at pre-flowering. It was also observed that QGr content was lower than QGs at harvest. This is probably due to the start of QGs biosynthesis at veraison and to QGr degradation during ripening as a result of oxidation reactions. The results of this study can be considered as a first contribution to understanding the effects of certain variables on QGs synthesis in grapes.

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