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Prevention of quercetin precipitation in red wines: a promising enzymatic solution

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ABSTRACT

Flavonols are known for causing undesirable deposits in both red and white wines. Among flavonols, quercetin is widely considered the principal factor determining this phenomenon. One of the most accredited hypotheses claims that glycosylated derivatives of quercetin undergo hydrolysis of the glycosylic bond during the fermentation and the wine ageing, releasing quercetin aglycone, which is much less soluble in water solution and causes the precipitation. Our work describes the dynamics of quercetin-derived deposition in Chianti wines and purposes a new method, based on the enzymatic quercetin glycoside hydrolysis of the glycosidic bond, to prevent the unpleasant deposit formation during the wine ageing. In our study, forty-four monovarietal wines obtained from 7 different Italian grape varieties were compared in the content of total quercetin-3-glycosides (rutin, quercetin-3-glucuronide, quercetin-3-glucoside) and quercetin aglycone. The data confirmed the literature revealing Sangiovese as the richest in quercetin. We tested then, in a Sangiovese wine, four fining agents (PVPP, PVPP/PVI, bentonite and a vegetal protein) for quercetin removal, showing that only the PVPP had a modest aglycone removal activity. Then, the kinetics of deposit formation was studied in three Chianti wines which differed in the initial content of quercetin aglycone. This investigation highlighted that the chemical equilibrium of quercetin changes over time as the turbidity slowly increases, as previously documented. The comparison of the three dynamics also permitted us to conclude that different wines show a different ability to keep in solution quercetin. Finally, a new approach for deposit prevention was studied by a precocious Chianti wine treatment with a pectolytic enzyme having secondary glycosidase activity. This enzyme significantly accelerated the hydrolysis of glycosylated quercetins into their aglycone, which could enhance the deposition before bottling, without serious wine colour depletion. Our study represents the first evidence of the promising potential of using the pectolytic enzyme with secondary glycosidase activity to prevent quercetin deposit during Chianti ageing, in a way that is compatible with organic wine production.

KEYWORDS: Quercetin aglycone, quercetin-3-glucoside, Chianti, Sangiovese, enzymatic treatment

INTRODUCTION

Flavonoids are natural polyphenolic compounds widely present in plants and foods. They occur as aglycones, glycosides and methylated derivatives in fruits. The basic aglycone flavonoid structure is a fifteen-carbon skeleton consisting of two benzene rings linked via a heterocyclic pyran ring (Figure 1). Flavonoids are often hydroxylated in positions 3, 5, 7, 2 ,3', 4' and 5' and methyl ethers of the alcohol group have been recognised in nature (Kumar and Pandey, 2013). When glycosides are formed, the glycosidic linkage involves usually D-glucose, galactose or L-rhamnose, or more rarely glucorhamnose or arabinose, and it is generally located in positions 3 or 7. These three forms differ for several chemical properties, in particular, aglycones show a reduced solubility in water, high absorbability in the small intestine and antioxidant power when compared to the corresponding glycosides (Kumar and Pandey, 2013).

Among other flavonoids such as anthocyanins and flavanols, red wines contain flavonols (Abraham and Acree, 2014), in particular myricetin and quercetin (Tsanova-Savova and Ribarova, 2002). Quercetin (3,5,7,3′,4′-pentahydroxyflavone, QCT) has gained attention in the past years in light of the impact it could have on wine quality. Flavonols are known to be responsible for unwanted deposit formation and several cases of precipitation have been reported in the literature as early as 1969 in both red wines (Ziemelis and Pickering, 1969) and white wines (Somer and Ziemelis, 1985). The authors reported an unusual form of instability that appeared sporadically, with turbidity and yellowish or yellow-green deposits observed during the storage of musts and wines. These deposits mainly consisted of the flavonol quercetin, with kaempferol and myricetin. These flavonols are derived from the extraction of their corresponding glycosides from grape skins (Gambuti *et al.*, 2004), where they exist mainly as 3-O-glycosides (3-glucuronides, 3-glucosides, etc.). In wine, the free aglycones can be found as a result of acid hydrolysis that occurs during winemaking and ageing (Castillo-Muñoz *et al*., 2009). The aglycones display lower aqueous solubilities, which causes their precipitation (Wang *et al.*, 2016).

Quercetin deposits formation has spread in the Tuscan wine sector, with a dramatic increase in both frequency and intensity. This phenomenon concerns exclusively red wines, in particular those obtained from Sangiovese grapes. This problem is encountered more frequently in recent years, probably because of the viticultural techniques evolution and the new market dynamics, such as the use of machine-harvesting (which increases the presence of contaminant leaves, rich in flavonols), and the greater exposure of berries to the sun (Prince *et al*., 1995; Blancquaert *et al.*, 2018).

The grape variety has been recognised as one of the principal factors affecting deposit formation. Although quercetin content has been extensively studied in grapes and wines, only some studies reported a comparison of its content in different grape varieties (Brossaud *et al.*, 1999; Ojeda *et al*., 2002; Prince *et al.*, 1995; Beslic *et al.*, 2010). Several articles have reported the quercetin accumulation in Sangiovese (Mattivi *et al.*, 2006; Iacopini *et al.,* 2008; Storchi *et al.,* 2008). A study comparing 91 samples of *V. vinifera* berry skins collected at technological maturity from vines cultivated with the same trellis system and in the same environment (ampelographic collection), by Mattivi and colleagues (2006) found high variability in the quercetin content. Sangiovese showed a medium-high content of quercetin, lower than two important international varieties (Cabernet franc, Pinot noir). In another work, Ledda *et al.* (2010) revealed that the Nielluccio variety, genetically similar to Sangiovese, recorded the highest content of quercetin among 7 native grape varieties of Corsica and Sardinia islands. Similarly, Iacopini *et al.* (2008) compared the polyphenol content of different Tuscan native grapes varieties with two international ones (Merlot and Cabernet-Sauvignon) highlighting that the highest concentrations of quercetin were achieved by the four Sangiovese clones, with a mean content of 1.04 mg per 100 g of dry matter, while the average amount of all the other varieties was 0.47 mg per 100 g of dry matter. Furthermore, Storchi *et al.* (2008) compared Sangiovese clone R10 with other 12 red grape varieties (both international and Italian) cultivated in the same experimental vineyard and viticultural conditions and Sangiovese demonstrated again to be the variety with the highest content of quercetin-3-glucoside (34.3 mg/kg). More recently, Gambuti *et al.* (2020)

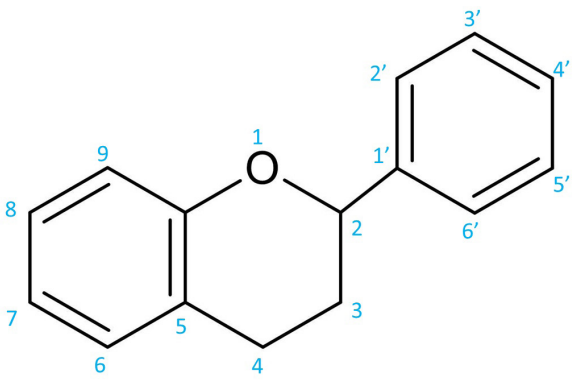

FIGURE 1. Flavonoid aglycone basic structure.

confirmed that this variety is the one with the higher contents of quercetin-3-glucoside comparing different monovarietal Italian wines with Sangiovese.

Even though the concentration of flavonoids in grapes depends primarily on the variety of grapevine, it is also largely influenced by viticultural and environmental factors, which represents an additional complication for their study (Brossaud *et al.*, 1999; Ojeda *et al*., 2002; Prince *et al.*, 1995; Beslic *et al.*, 2010). Regarding the quercetin content in wines, additional variability due to the winemaking process can affect the molecule extraction from the skin and can even mask the varietal effect. Comparing 65 wines from different world regions, McDonald *et al.* (1998) reported that Chianti wines (produced from Sangiovese grapes in a variable percentage between 80 and 100 %) showed the highest quercetin content when compared with other Italian wines. In the same work, authors included wines produced in several countries and evidenced that only those produced in warm regions such as Chile and California were characterised by high QCT content independently from the variety used for production, reaching QCT values even greater than in the Chianti wines.

The absence of defects and the stability of wine are of primary relevance for the industry of the wine sector worldwide. The presence of turbidity or deposits in the bottle are defects that can compromise the marketing of wine, creating non-negligible economic and brand-image damage. Despite the attention displayed by producers, no oenological practice has been demonstrated to successfully prevent deposit formation. Nowadays the most promising treatment is PVPP fining (often in association with other adjuvant products). However, this product is not allowed to produce organic wines (EU regulation. n. 203/2012), whose market is continuously expanding. To our knowledge, no alternative treatments compatible with organic wine production are available on the market.

In this work, a new approach for quercetin deposit control is proposed, which is based on the action of a pectolytic enzyme with a secondary glycosidase activity. In the last decades, the use of pectolytic enzymes in winemaking has spread. In addition to accelerating natural colloids precipitation (clarification), they represent a handy tool for the release of desirable compounds bound to the grape skin pectins (i.e., colour and aroma precursors, phenolics) into the grape juice (Arnous and Meyer, 2010). In the present work, the applicability of an experimental pectolytic enzyme in the winemaking process has been verified in two independent wineries on one of the most unstable products, the Chianti wine.

MATERIALS AND METHODS

1. Wines

The Sangiovese wine samples (22) were provided by different wineries located in Tuscany (Italy). The other 34 wine samples used for the quercetin quantification were varietal wines collected from different regions of Italy within a national project.

2. HPLC analysis

The analysis of quercetin-3-glucoside and quercetin was performed on an HPLC (Waters 1525 Binary Pump) equipped with a column Kinetex (4 × 150 mm, 5 µm, Phenomenex) and a UV detector (Waters 2487 Dual-Band Absorbance Detector) set to 350 nm. The total flow rate was 1 mL/min, the mobile phase A was constituted of water + 0.1 % trifluoroacetic acid, and the elution was performed with phase B (HPLC grade methanol + 0.1 % trifluoroacetic acid) as reported: from 25 to 35 % B in 5 minutes, from 35 to 47 % B in 8 minutes, then washing with 100 % B for 3 minutes and reconditioning of the column with 25 % B. Each sample of wine was filtered at 0.2 µm (cellulose acetate, Sartorius) and 20 µL were injected. Quantification was performed using a calibration curve from 200 to 2.5 µg/mL of commercial standard for both molecules (quercetin with purity ≥95 %, quercetin-3-glucoside with purity ≥98 %). The calibration curves were prepared starting from stock solutions (10 mg/mL in 100 % ethanol) and diluting them in model wine (12 % ethanol, 5 g/L tartaric acid, pH 3.2). The LOD and LOQ were, respectively, 0.38 and 1.03 µg/mL for quercetin and 0.46 and 1.6 µg/mL for quercetin-3-glucoside. All reagents were analytical grade and were purchased from Sigma (Milan, Italy) unless otherwise stated.

3. Fining treatments

After a first screening, a Sangiovese wine with a high content of both quercetin-3-glucoside (160.8 mg/L) and quercetin aglycone (7.7 mg/L) was selected for fining trials. PVPP (Polyex, Oenofrance) was suspended at 1:10 in water and then added to a wine aliquot (50 mL) at 20, 40 and 60 g/hL. PVP/PVI (Diwine 2+/3+, Oenofrance) was prepared in the same way and added at final concentrations of 20 and 60 g/hL. The vegetal protein (potato protein, Vegecoll, Laffort) was dissolved 1:10 in water and used at a concentration of 20 g/hL. The sodic bentonite (Performa, Oenofrance) was suspended 1:20 in water and stirred for 2 hours before addition at a final concentration of 20 g/hL. All the additions were made in triplicate. After 40 hours, the wine samples were centrifuged and filtered before injection in HPLC.

4. Study of the precipitation rate of quercetin

Three different Sangiovese wines with different content and relative proportion of quercetin-3-glucoside and quercetin aglycone were selected for the additional experiments. The wines were previously filtered with GF/A filters (Whatman, 1.6 µm), to have a turbidity value below 2 NTU, then 10 mL aliquots were prepared in glass Pyrex tubes. Commercial quercetin aglycone was prepared at 10 mg/mL in ethanol and was added to the tubes at increasing concentrations, from 5 to 75 mg/L. An aliquot of pure ethanol was added in tubes with lower quercetin addition to compensate for the different volumes added from the quercetin stock solution. Each sample was prepared in triplicate. The turbidity was measured with a nephelometer (Hanna) for each tube at different times for 48–72 hours.

5. Glycosidase treatment

An experimental pectolytic enzyme with secondary glycosidase activity (supplied by Oenofrance) was used for a preliminary trial on a Sangiovese wine with a high content of quercetin-3-glucoside. The test was performed on a 50 mL aliquot of wine, adding the recommended dose (4 g/hL) and a dose ten times higher (40 g/hL) of the enzyme. Each treatment was performed in triplicate. The samples were stored at 25 °C and 1 mL aliquots were taken at the beginning, after 2 and 7 days of incubation, filtered and immediately injected in HPLC for quercetin analysis. The large-scale test at winery conditions was performed in two wineries (Barone Ricasoli and Rocca di Castagnoli) located in Gaiole in Chianti (Siena, Italy). In both wineries, a Chianti classico from the vintage 2018 was divided into 9 barrels: 3 were considered as a control, 3 were treated with bentonite 10 g/hL (Performa, Oenofrance) and 3 were treated with 5 g/hL of the pectolytic enzyme. Samples of 20 mL were taken from each barrel at different times and sent to the laboratory for the analysis of quercetin.

6. Colour reduction of wines

Wine samples were collected from the barrels after one year of barrel storage for the determination of the colour intensities. After centrifugation (14,000 *g*, 5 min), the supernatant was recovered and its absorbance was read in cuvettes (1-cm path length) by a ULTROSPEC 2100 pro spectrophotometer (Amersham Bioscience Europe GmbH, Cologno Monzese, Italy) at 520 nm. The determination was repeated three times for each experimental condition.

7. Statistical analyses

Statistical analysis was performed with XLSTAT-Pro 7.1 Software. Data were analysed using one-way ANOVA and the Tukey multiple comparison test was used to compare the means when significant differences were found in the variance analysis.

RESULTS

1. Quantification of quercetin in different wines

Fifty-six monovarietal wines obtained from 7 different Italian grape varieties were analysed for their content of both quercetin-3-glucoside (3G-QCT) and quercetin aglycone (aQCT). All the production belonged to the same year of vintage (2017). The content of quercetin aglycone was very low, on average below 10 mg/L, with the maximum value of 15.7 mg/L found in a Brunello (made from 100 % Sangiovese) wine (Figure 2A), with

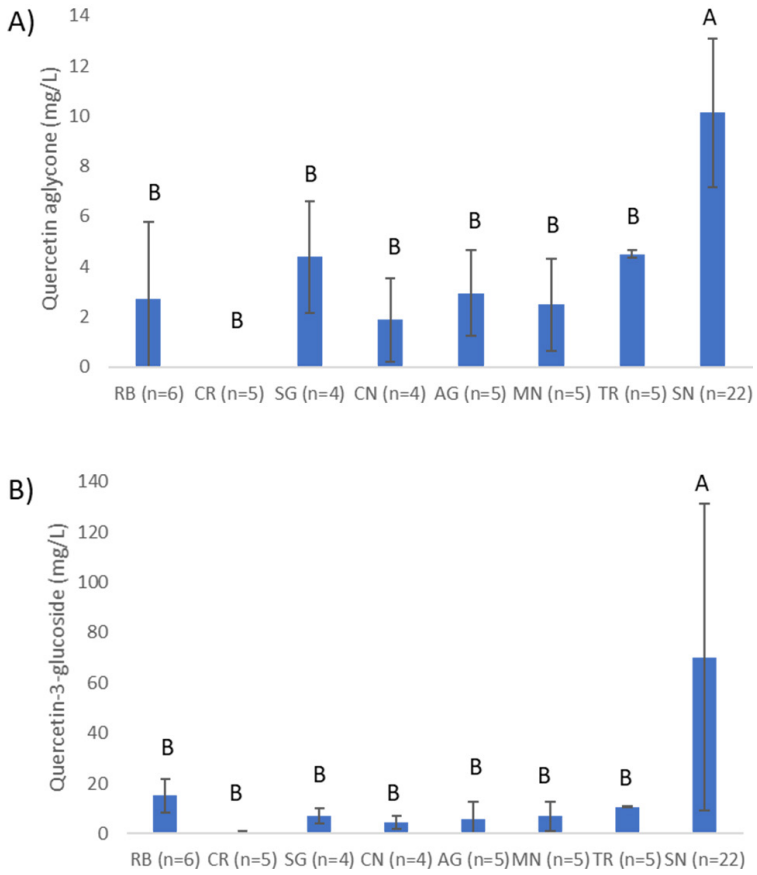

FIGURE 2. Content of quercetin aglycone (A) and quercetin-3-glucoside (B) in Italian monovarietal wines. Capital letters identify statistically significant groups ($p > 0.05$). RB: Raboso, CR: Corvina, SG: Sagrantino, CN: Cannonau, AG: Aglianico, MN: Montepulciano, TR: Teroldego, SN: Sangiovese.

values comparable to literature data for both Sangiovese (Parpinello *et al.,* 2019) and other varieties (Gambuti *et al.,* 2020; Pellegrini *et al*., 2000). As reported in Figure 2B, the mean content of 3G-QCT in Sangiovese wines was significantly higher (about 70 mg/L) than in the other varieties ($p < 0.05$). It should be noted that data demonstrated high variability, even among samples of the same variety (i.e., Sangiovese in the range 7–171 mg/L) similarly to what was previously reported by Gambuti *et al.* (2020). This variability can be the result of the huge differences in the viticultural technics and winemaking process occurring between the producers.

These contents in 3G-QCT could translate into the formation of quercetin precipitates. Although this problem has been extensively described, the mechanism involved is still unknown (McDonald *et al*., 1998). The most accredited hypothesis is that the glycosylated derivatives of quercetin, which are abundant in grapes, are hydrolysed during the fermentation and maturation of wine. This releases the quercetin aglycone (aQCT), which is much less soluble in water solution (Romboli *et al.,* 2015) and this reduction of solubility would cause the precipitation. It has been reported that the solubility of aQCT in water is less than 3 mg/L at 25 °C (Srinivas *et al.*, 2010). Nevertheless, ethanol increases aQCT solubility about 3 times in a 10 % v/v ethanol solution (at 20°) and approx. 5 times when the ethanol concentration reaches 20 % v/v (Razmara *et al*., 2010). Moreover, Lanati *et al.* (2014) suggested that in wine the aQCT concentration could be even higher as it could be involved in copigmentation and thus subtracted from the solubility equilibria. Several studies reported, however, values of aQCT in stable wines that are generally lower than 10 mg/L (Goldberg *et al.*, 1998; Tsanova-Savova and Ribarova, 2002) and only occasionally higher than 15 mg/L (Gambuti *et al.*, 2004), supporting the hypothesis that other phenolic compounds have only a little effect on the stabilization of quercetin. Considering the values of 3G-QCT found in Sangiovese wines (Figure 2), it is reasonable to assume that even partial hydrolysis of the glycosylated form can release a sufficient quantity of quercetin aglycone to overcome the aforementioned solubility limit. To better understand the formation of quercetin deposits in wines, we attempted to study different fining agents, analyse the kinetics of the formation of precipitates, and test a promising alternative based on enzymes to overcome those phenomena.

2. Fining to remove quercetin from wine

Fining agents differ in their specificity towards phenolic compounds given their various mechanisms of chemical binding. Currently, there are no specific adjuvants for the removal of quercetin. One possible wine treatment commonly used in reducing precipitation involves the use of PVPP (Laborde *et al*., 2006). Usually used for white wines to correct colour, this synthetic fining agent is considered the least invasive, as it absorbs fewer aromas and polyphenols when compared to charcoal for example (Lisanti *et al*., 2017). In our work, a Chianti wine rich in quercetin (both aglycone and glucoside) was used for the fining trials, comparing PVPP with other agents, namely a PVI/PVP polymer (chosen for its structural affinity with PVPP), sodic bentonite and vegetal protein fining (VF). As shown in Figure 3a, the effect of PVPP on quercetin-3-glucoside was not statistically significant even with a treatment at 60 g/hL. None of the other products showed either a significant effect on glucoside removal.

However, PVPP demonstrated a clear interaction with quercetin aglycone (Figure 3b), with a direct correspondence between the decrease of the quercetin and the increase in the PVPP dose used. It was previously demonstrated (Laborde *et al*., 2006) that the interaction between quercetin and PVPP depends mainly on H-bond, hydrophobic interaction and van der Waals bonds interactions and that the sugar moiety reduced the interaction strength. The PVI/PVP polymer showed a significant effect only at a dose of 60 g/hL, while bentonite and the vegetal protein did not show any effects at the concentration chosen for this trial. Even though it is known that bentonite interacts with wine

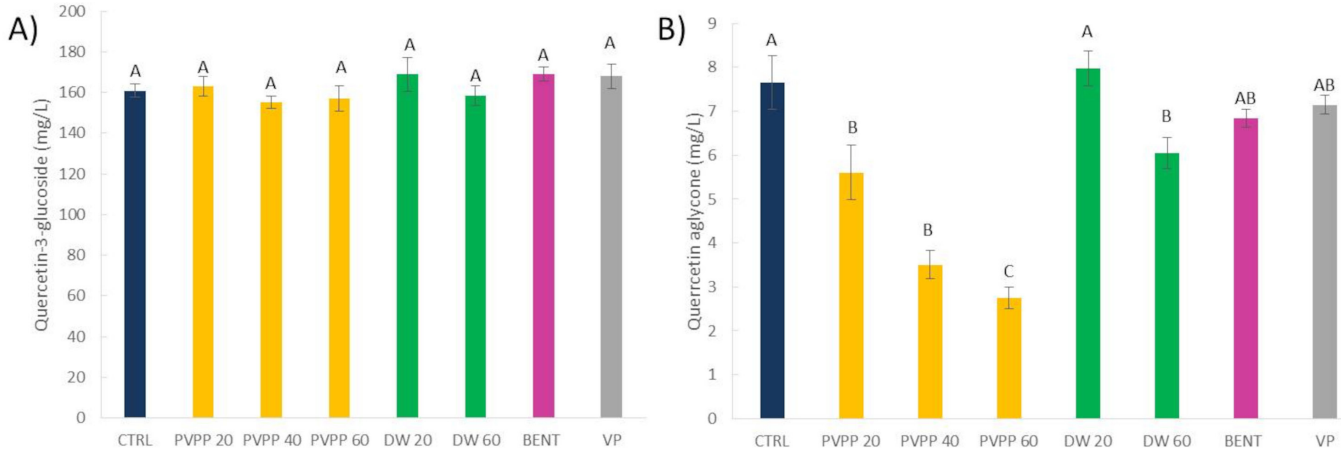

FIGURE 3. Content of A) quercetin-3-glucoside and B) quercetin aglycone in wine after fining. CTRL: no treatment, PVPP 20: PVPP 20 g/hL, PVPP 40: PVPP 40 g/hL, PVPP 60: PVPP 60 g/hL, DW 20: PVI/PVP 20 g/hL, DW 60: PVI/PVP 60 g/hL, BENT: bentonite, VP: vegetal protein.

molecules mainly by ionic interaction, a possible removal of uncharged polyphenols could have been expected using both a direct link, due to the presence of some hydrophobic regions on the bentonite surface (Vincenzi *et al*., 2015) and an indirect link (i.e., removal of proteins, which in turn are linked to polyphenols). A removal of a small fraction of polyphenols has already been demonstrated upon bentonite treatment (Dordoni *et al*., 2015).

These results seem to confirm the PVPP as the adjuvant of choice for quercetin aglycone removal. However, it can be observed that 60 g/hL of PVPP can remove only a few mg of aQCT, which, as reported above, in many cases could not be enough to bring the concentration of aglycone below the quantities capable of triggering precipitation. Considering that 3G-QCT can be a "reservoir" of aQCT because it can release the aQCT slowly into the wine upon acidic hydrolysis at the wine pH, the removal of the quercetin aglycone would only be a temporary remedy. Moreover, the use of PVPP is not free from undesired effects on wine quality, namely on colour, a specific class of polyphenols and some thiols (Gil *et al.*, 2019). Furthermore, the EU regulation. n. 203/2012 did not allow the use of PVPP for organic wine production and, considering the continuous expansion of the organic wine market, it would be important to find an alternative oenological product or treatment.

3. Study on the precipitation rate of quercetin aglycone in three different wines

To describe the precipitation kinetics of quercetin in real conditions, a red wine (Chianti, vintage 2018) containing initially only a low content (2.8 mg/L) of aQCT was added with increasing quantities of aglycone (previously dissolved in ethanol) up to 75 mg/L, by increments of 5 mg/L (Figure 4A).

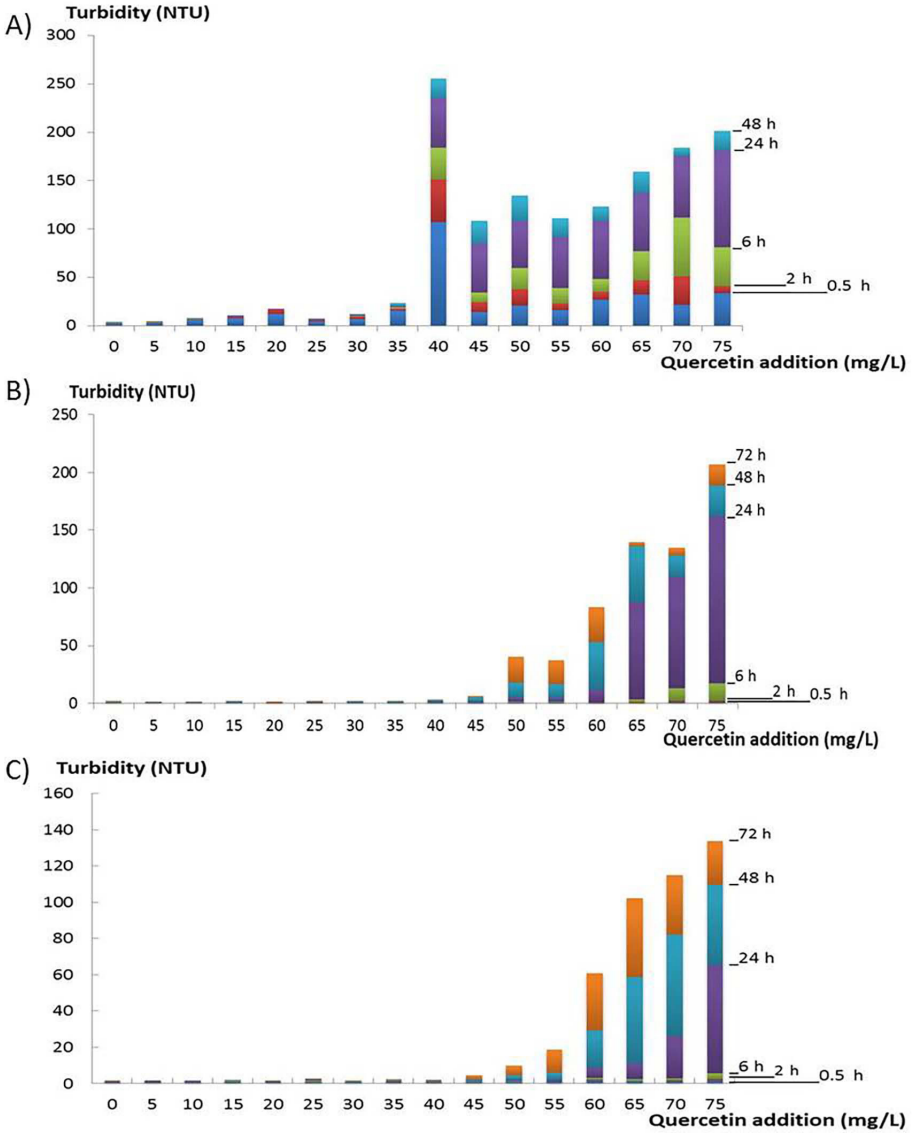

FIGURE 4. Turbidity development in three Chianti wines. A) Low quercetin wine (2018), B) medium quercetin wine (2017), C) high quercetin wine (2018). Different colour areas in the histogram represent successive turbidity increments during the different time intervals (30 minutes, 2 h, 6 h, 24 h, 48 h).

The starting sample was previously filtered to eliminate any suspended particles. The turbidity was measured with a nephelometer at different times during 48 h to establish the precipitation kinetics. In this case, no significant increase in turbidity was observed up to an addition of 35 mg/L (at least in the first 48 hours). With an addition of 40 mg/L or higher, a peak of visible turbidity was observed already 30 minutes after the addition. Oddly, at higher dosages the turbidity decreased and then gradually returned to increase as the dosage rise. This experiment is similar to that proposed by Gambuti *et al.* (2020), who checked for the formation of a visible precipitate in a longer period (until 15 days). Using the visual inspection, those authors were able to detect the precipitate formation only after at least a week of incubation, while in the present experiment the use of a nephelometer allowed us to demonstrate that quercetin addition affects turbidity in a short time.

The same experiment was repeated with a second Chianti wine (vintage 2017) with an initial content of aQCT equal to 21 mg/L. In this case (Figure 4B) no turbidity was observed up to an addition of 45 mg/L. Considering the starting level of quercetin aglycone, an onset of precipitation at lower dosages than the previous wine was expected. The limit of solubility seems to differ between the two analysed wines, as demonstrated by the fact that the latter was able to maintain in solution 20 mg/L of aQCT. A possible explanation is that wines can contain, in different amounts, stabilizing substances affecting the aglycone solubility equilibrium and that these substances were present in the second wine in a quantity able enough to complex even higher amounts of quercetin before triggering the turbidity. It was also noted during the incubation time, that the turbidity became visible (at the highest addition rates) no earlier than 6 hours after the addition, perhaps due to the stabilizing molecules mentioned above. In this case, the measurement was performed up to 72 hours, showing that there is still a further increase in turbidity after 48 hours, even if for the higher concentrations a clear reduction in the increments is visible.

Finally, the test was performed on a third Chianti wine (vintage 2018), characterised by a content of aQCT of 33 mg/L (the highest value found among the 22 wines analysed). Even this wine (Figure 4C) was able to solubilise 45 mg/L of quercetin before observing an onset of haze formation, and also in this case no turbidity was seen before 6 hours.

Overall, from these experiments, it was clear that the precipitation of quercetin is a complex phenomenon that depends not only on the quercetin content but also on the wine matrix. The results were actually different in different wines, and some wines can maintain quantities of quercetin aglycone in solution (even higher than 30 mg/L) without showing undesirable precipitation. The effect of the wine matrix (presumably the presence of protective colloids) seems evident comparing the amount of solubilisable quercetin, in the delay of precipitation onset and the amount of turbidity developed. In fact, the same quantities of quercetin added caused different values of turbidity, gradually lower from wine 1 to wine 3. Further research must be done to fully understand the relationship between the wine matrix and the solubility of quercetin.

4. Enzymatic treatment to hydrolyse quercetin-3-glucoside

To solve the problem of quercetin precipitation definitively and stably over time, it would be important to remove both aglycone and its precursor, for example by a specific enzymatic treatment.

4.1. Effects of enzyme in final wines

A first test was performed on a Sangiovese wine with a high content of 3G-QCT (91 mg/L), using a commercial pectolytic enzyme with secondary glycosidase activity. The test was performed at 25 °C using the recommended enzyme dose (4 g/hL) and a ten times higher dose (40 g/hL).

In a few days, both treatments led to a stark decrease in the 3G-QCT concentration in parallel to an increase in the corresponding aglycone.

These data demonstrated that the enzymatic treatment could increase the 3G-QCT degradation rate and that 3G-QCT degradation depends on the initial enzyme concentration as the increase in enzyme dose affected the reduction of 3G-QCT

TABLE 1. Quercetin content (mg/L) of Sangiovese wine 2017 after the enzymatic treatment at two concentrations (4 g/hL and 40 g/hL). Test: untreated wine.

	Quercetin-3-glucoside			Quercetin aglycone		
Time after treatment (days)	Test	4 g/hL	40 g/hL	Test	4 g/hL	40 g/hL
0	91 ± 4			2.8 ± 0.4		
2		82 ± 6 (–10 %)	65 ±4 (–29 %)		4.2 ± 1.0 (+49 %)	10.4 ±3.1 (+270 %)
7		75 ± 6 (–18.0 %)	46 ± 6 (–49 %)		10.1 ± 2.0 (+261 %)	19.5 ± 3.9 (+597 %)

and the corresponding release of aQCT (Table 1). It should be considered that in this case, it was possible to observe the sharp increase in aQCT because the wine started from a very low content of this compound. However, if we consider longer times of treatment followed by a further release of aQCT from its precursor, we should expect precipitation of the aQCT exceeding its solubility limit.

Using glycosidic enzymes, several points should be addressed. The enzymatic treatment could affect other glycosidic compounds in wine, for example anthocyanins, with a detrimental effect on colour or aroma compounds (Wang *et al.*, 2013). In addition, at winery conditions, i.e., low temperatures, the process could be very slow, which means that it should be necessary to guarantee the activity of the enzymes for enough time to degrade a sufficient amount of quercetin-3-glucoside. Moreover, the tannins present in the red wines could react with the added enzymes precipitating and deactivating them in short times (Claus and Mojsov, 2018).

Regarding the fate of the free quercetin obtained from the glucoside hydrolysis, the addition of glucosidase should be at the beginning of the wine ageing process, to allow the natural precipitation of the released aglycone before the bottling. To study the rate of quercetin aglycone precipitation in real conditions and resolve the applicability of the enzymatic approach, a further experiment was performed.

4.2. Effect of glycosidase enzyme treatment in real condition

To test the feasibility of glycosidase enzyme application for quercetin precipitation prevention, the enzymatic treatment was applied to young Chianti wines in two different wineries at the beginning of the barrel ageing. Then, the evolution of both 3G-QCT and aQCT were measured over 6 months. Two controls were prepared, one without treatment (CNT) and another treated with bentonite (CBT) to remove endogenous glycosidase enzymes eventually remaining after alcoholic fermentation.

Results are shown in Figure 5A. The starting content of 3G-QCT was about 30 mg/L in both wines and their evolution after the enzymatic treatments were very similar.

In all the controls, a slight but constant decrease in 3G-QCT was observed with an overall reduction of glycosylated quercetin of about one third (10 mg/L) in more than 4 months of storage in the barrel (Figure 5A). This natural reduction could be probably due to multiple causes such as the action of endogenous enzymes, chemical hydrolysis caused by the acidic environment and the effect of micro-oxygenation

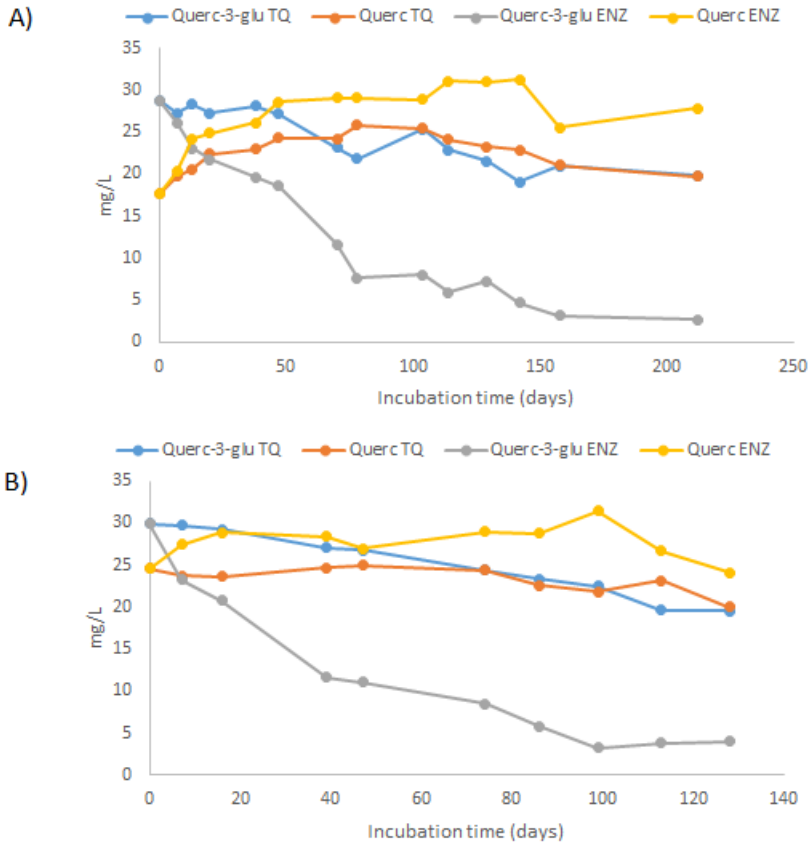

FIGURE 5. Quercetin aglycone and quercetin-3-glucoside evolution over the time in wine 1 (A) and in wine 2 (B). Quercetin 3-glucoside without (Querc-3-glu TQ, blue line) and with enzyme (Querc-3-glu ENZ, grey line) and quercetin aglycon without (Querc TQ, red line) and with enzyme (Querc ENZ, yellow line) are represented.

during barrel ageing (Gambuti *et al.*, 2020). However, the endogenous enzymes contribution should be excluded as in bentonite treated wines, where the amount of residual proteins were severely reduced, the evolution of 3G-QCT was almost identical to that of control CNTs wines (Figure S1 and S2).

Concerning the first wine treated enzymatically, data registered a constant increase in aglycone passing from 17 to 28 mg/L in 100 days, in parallel to a decrease of the 3G-QCT which reached much lower levels (under 5 mg/L) than the control wines (20–21 mg/L). After that, the aQCT value remained almost stable for about 30 days before undergoing a slight decrease, presumably due to the phenomenon of precipitation.

In the second treated wine (Figure 5B), the quercetin aglycone seemed again to slightly increase until 100 days and finally decreased, reaching the initial value. This behaviour was very similar to that of the first wine.

Rapid degradation of glycosylated quercetin was evident in both experiments with enzymes, decreasing from 30 mg/L to less than 5 mg/L in a period ranging from 100 to 140 days. On the other hand, quercetin aglycone had a more evident increase compared to that observed in the controls, but it did not reflect what was expected if all the quercetin released by the enzymatic reaction remained in solution.

Even though the eventual precipitate formation was not checked in the barrels at the end of the ageing period, it can be deduced that with such long times, the excess of aglycone was progressively eliminated from the solution by precipitation, a phenomenon more evident in the second wine, where after the treatment the aQCT value returned practically equal to the initial value.

4.3. Colour stability after enzymatic treatment

As previously suggested, a possible disadvantage of this enzymatic treatment is the possible action of the enzyme on the other glycosylated components of the wine, in particular anthocyanins. Anthocyanins in wine are mainly present in a glycosylated form, which makes them more soluble and more stable against oxidation phenomena. Therefore, enzymatic hydrolysis with the consequent release of the anthocyanin aglycones could have an impact on the wine colour stability. To verify this hypothesis, the colour intensities of the controls and the treated wines were compared after one year from the enzyme addition.

As shown in Figure 6, the red colour intensity slightly decreased in the sample treated with the enzyme, but the statistical analysis demonstrates that the difference was significant only when wine 2 was compared to the untreated control, with a maximum reduction of about 8 %. Thus, in this experimental phase, the supposed side effect on colour demonstrated just a negligible impact.

CONCLUSION

The present study permits us to conclude that PVPP, although a better fining choice over PVI/PVP, bentonite or vegetal proteins for quercetin aglycone removal, has limited effectiveness so that this treatment should not be considered a definitive solution for the precipitation phenomenon.

This work proposes an alternative wine enzymatic treatment, which shows that is possible, in a relatively short time compatible with the wine ageing, to significantly lower the amount of quercetin-3-glucoside, and, consequently, the instability of the wine. Since the onset of quercetin-induced turbidity can happen very fast as shown in our experiments with the addition of quercetin aglycone in

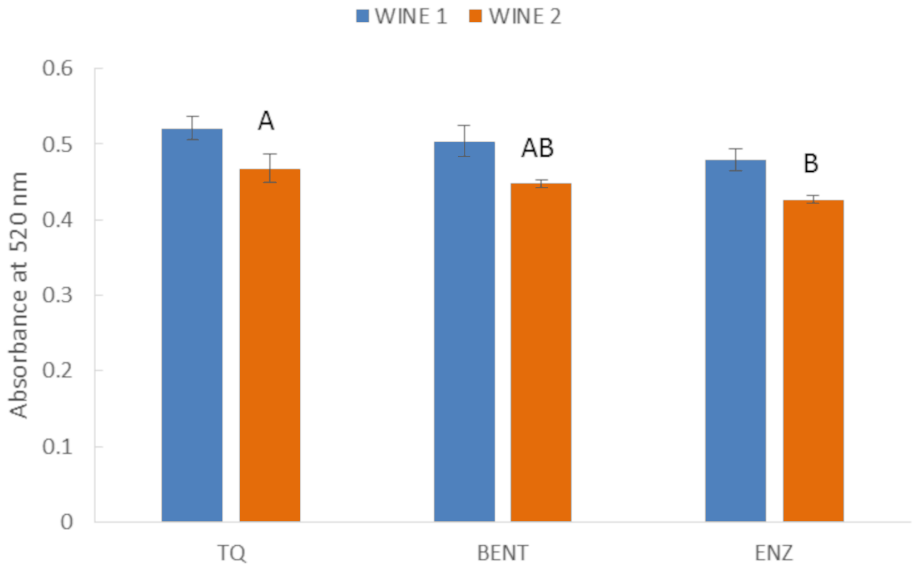

FIGURE 6. Colour intensity (520 nm) of treated wines (BENT: bentonite, ENZ: enzyme) and control (TQ) after long storage. Capital letters identify statistical different groups ($p < 0.05$).

Sangiovese wines, it seems that the excess of aglycone released upon the enzymatic treatment can naturally precipitate before the bottling if the treatment is done in the early stage of wine ageing. This possibility was verified in a large scale experiment performed in two different wineries and the results confirmed the applicability of the enzymatic treatment without several side effects on the treated wine's colour.

Moreover, the quercetin addition experiments showed clearly how the wine composition can influence the quercetin instability. The identification of the wine components involved in the quercetin stabilization could lead to the production of new fining agents specifically targeted for the prevention of quercetin precipitation, allowing the defect prevention even in wines where the production time is not compatible with enzymatic accelerated precipitation.

It is worth noting that in this manuscript we focused on the quercetin-3-glucoside, which was the main quercetin derivative present in our wines. Other minor glycosylated flavanols (rutin, quercetin 3-galactoside, quercetin 3-glucuronide) were also measured but they presented neither significant amounts nor variations during treatments (data not shown). However, in wines where these other quercetin glycosides are present in significant amounts, it is necessary to take them into account as possible aQCT precursors.

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