European Food Research and Technology (2023) 249:1491–1501
https://doi.org/10.1007/s00217-023-04229-6

ORIGINAL PAPER

Biotechnological tools for reducing the use of sulfur dioxide in white grape must and preventing enzymatic browning: glutathione; inactivated dry yeasts rich in glutathione; and bioprotection with *Metschnikowia pulcherrima*

Pol Giménez[1] · Arnau Just-Borras[1] · Pere Pons[1] · Jordi Gombau[1] · José M. Heras[2] · Nathalie Sieczkowski[2] · Joan Miquel Canals[1] · Fernando Zamora[1]

Received: 20 December 2022 / Revised: 13 February 2023 / Accepted: 19 February 2023 / Published online: 23 March 2023

Abstract
Sulfur dioxide is the most used additive today for preventing browning in grape musts and wines. However, since wine consumers are increasingly interested in healthier wines, the wine industry is keen to reduce its use. Some promising alternatives to sulfur dioxide have been proposed in recent years, including glutathione, both pure and in the form of inactivated yeasts, and *Metschnikowia pulcherrima* used as a bioprotective agent. Some information exists about the protective effect against oxidation of glutathione but there is very few about the use of bioprotection for that purpose. Supplementation with glutathione, regardless of the commercial form, reduced oxygen consumption and browning intensity when laccase was not present in the grape juice. *Metschnikowia pulcherrima* also reduced browning intensity in the absence of laccase but increased the total oxygen consumption. However, in the presence of laccase, glutathione and *Metschnikowia pulcherrima* were not effective enough to adequately prevent the grape juice from browning. Glutathione, both pure and in the form of inactivated yeasts, and *Metschnikowia pulcherrima* are interesting tools for protecting grape must against browning, and thus reducing the use of sulfur dioxide.

Keywords Grape must · Browning · Laccase · Bioprotection · Glutathione · *Metschnikowia pulcherrima*

Introduction

One of the problems that most seriously affects oenology today is enzymatic browning [1], especially when the grapes are infected by grey rot [2]. Enzymatic browning is an oxidation process that occurs in many foods that increases the brown color [3], which often leads consumers to reject them. This problem is especially harmful in the wine industry since grape must is highly vulnerable to browning [4, 5].

Enzymatic browning is caused by polyphenol oxidases, a broad family of oxidoreductases (EC. 1 class, according to the International Union of Biochemistry and Molecular Biology—IUBMB [6]). However, in the case of grape juice, browning is caused by just two enzymes: tyrosinase (EC 1.14.18.1, IUBMB [6]), which is naturally present in grapes [4, 5], and laccase (EC 1.10.3.2, IUBMB [6]), which is present only when the grapes are infected by *Botrytis cinerea* [5, 7]. Both enzymes use molecular oxygen to mainly oxidize the diphenols present in grape must and wine such as caftaric and cutaric acids, catechin and other flavanols, anthocyanin, and flavanone, though laccase is able to oxidize a wider range of substrates than tyrosinase [5, 7, 8].

The main consequence of enzymatic browning, irrespective of whether tyrosinase and/or laccase is the enzyme responsible, is that diphenols are oxidized to quinones, which can later polymerize through various reactions to form brown pigments called melanins [9, 10]. These compounds are responsible for increasing the intensity of the brown color in white wines (browning) and for precipitating the coloring matter in red wines (oxidasic haze) [11].

✉ Fernando Zamora
fernando.zamora@urv.cat

[1] Departament de Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, C/ Marcel.Li Domingo 1, 43007 Tarragona, Spain

[2] Lallemand Bio S.L., C/Galileu 303. 1ª Planta., 08028 Barcelona, Spain

Sulfur dioxide is the main and, until recently, only tool used by wineries to protect grape must from browning [12, 13]. This additive (E-220) is widely used in winemaking, thanks to its well-known antioxidant, antioxidasic, and antimicrobial properties [14], which make it practically essential not only in winemaking but also in the manufacture of other foods [15]. However, the current trend in winemaking is to reduce and even eliminate this unfriendly additive owing to its negative effects on the environment [16], the increasing tendency toward minimal intervention [17] and health [18] since it could cause headaches in sensitive people [19].

For all these reasons, the wine sector is extremely keen to find strategies for preventing oxidation and microbiological spoilage and for reducing or even eliminating sulfur dioxide.

Numerous strategies for reducing or replacing sulfur dioxide have been proposed. On one hand, inert gas [20], oenological tannins [21], ascorbic acid [22], glutathione [8], and inactivated dry yeasts that are rich in glutathione [23] or directly consume oxygen [24] have been proposed to protect grape must against browning. On the other hand, chitosan [25], lysozyme [26], bioprotection [27], ultra-high pressure homogenization [28], ozone [29], and, more recently, fumaric acid [30] have been proposed for protecting wine against microbiological spoilage.

Some of the most promising alternative agents for protecting grape juice against browning are glutathione and several non-*Saccharomyces* yeasts such as *Metschnikowia pulcherrima* used as a bioprotective agent.

Glutathione (GSH) limits browning because it reacts with the orthoquinones produced by the enzymatic oxidation of orthodiphenols to form GRP (Grape Reaction product; 2-S-glutathionylcaftaric acid). GSH traps the orthoquinones in a colourless form, and thus restricts the formation of brown polymers [23, 31]. The use of glutathione in winemaking was authorized by the International Organisation of Vine and Wine (OIV) in 2015 at a maximal dose of 20 mg/L [32, 33]. However, the high price of pure glutathione led to the use of certain inactive dry yeasts especially rich in glutathione (IDY-GSH) being proposed as more economical alternatives [34, 35]. The use of these yeasts in winemaking was authorized by the OIV in 2018 [36].

Bioprotection is also nowadays considered a highly promising alternative tool to sulfites in winemaking [27, 37]. In fact, the OIV has authorized the use of non-*Saccharomyces* yeasts in winemaking for various purposes, one of which is bioprotection [38, 39]. Several studies on the use of certain strains of non-*Saccharomyces* yeasts for preventing the development of spoilage microorganisms have been reported in recent years [40–43]. However, few of these studies have proposed their use in protecting the grape must against browning [44, 45]. Specifically, certain strains of *Metschnikowia pulcherrima* have shown interesting results both for preventing the development of spoilage microorganisms [37, 46] and for protecting against browning [27, 47].

In this paper, we compare the protective effect against browning in white grape musts of some of the most promising antioxidant tools—reduced glutathione, both pure and in the form of commercial inactivated dry yeasts rich in glutathione, and a commercial strain of *Metschnikowia pulcherrima*—with the protective effect of classical tools such as sulfur dioxide and ascorbic acid.

Materials and methods

Chemicals and equipment

All samples were handled without exposure to light. Potassium disulfite (CAS No.: 16731-55-8, purity ≥ 98%), polyvinylpolypyrrolidone (PVPP, CAS No.: 9003-39-8, purity ≥ 98%), syringaldazine (purity ≥ 98%), L-ascorbic acid (purity ≥ 99%), L-glutathione reduced (purity ≥ 98%), and $FeSO_4 \cdot 7H_2O$ (purity ≥ 99%) were purchased from Sigma-Aldrich (Madrid, Spain). L-(+)-tartaric acid (purity ≥ 99.5%), sodium hydroxide (purity ≥ 98%), sodium acetate (purity ≥ 99%), and $CuSO_4$ (purity ≥ 99%) were purchased from Panreac (Barcelona, Spain). Ethanol (96% vol.) was supplied by Fisher Scientific (Madrid, Spain). Cellulose membranes of 3.5 KDa (6.4 mL/cm) were supplied by Spectrum Laboratories, Inc (Rancho Dominguez, USA).

The equipment used was as follows: a spectrophotometer UV–Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); and an Entris II Series Analytical Balance (Sartorius, Goettingen, Germany).

Obtaining the samples of grape must

Muscat of Alexandria grapes were handpicked in the vineyard of the Rovira i Virgili University (Mas dels Frares, Constantí, Tarragona: 41°08′44.1″N 1°11′51.0″E) during the 2022 vintage harvest. The grapes were pressed in a nitrogen-saturated hand-press and the must was collected in a bottle also saturated with N_2.

Synthetic buffer

A solution of 4 g/L of L-(+)-tartaric acid, 3 mg of iron/L, in the form of iron (III) chloride hexahydrate, and 0.3 mg of copper/L in the form of copper (II) sulfate pentahydrate adjusted to pH 3.5 with sodium hydroxide was used for all experiments.

Inactivated dry yeasts rich in glutathione and *Metschnikowia pulcherrima* strain

A commercial inactivated dry yeast (IDY-GSH) rich in glutathione (Glutastar™, Lallemand Inc, Montreal, Canada) and a strain of *Metschnikowia pulcherrima* (MP) selected for its high oxygen consumption capacity (Level2 Initia™, Lallemand Inc, Montreal, Canada) were used. Both the IDY-GSH (400 mg/L) and the MP (250 mg/L) were hydrated in ten times their weight of distilled water 10 min before the beginning of the measurements. The water temperature was room temperature for the IDY-GSH and 30 °C for the MP.

Extracellular laccase production and enzymatic activity measurement

Active laccase extracts were obtained from the *Botrytis cinerea* isolate 213 strain following the methodology reported by Vignault et al. [48]. This laccase extract was treated with 0.16 g of PVPP/mL for 10 min and centrifuged at 7500 rpm for 10 min, and the supernatant was subsequently dialysed with 3.5 KDa cellulose membrane for 2 days in a 0.3 M ammonium formate solution and for 2 more days in distilled water. The laccase activity of this extract was determined using an adaptation of the syringaldazine test method [49].

Reaction conditions for measuring the oxygen consumption rate

These assays were performed in 60 clear glass flasks (66 mL) with an oxygen sensor spot (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203- 01_PSt3-0828-01, Regensburg, Germany) for measuring the dissolved oxygen noninvasively by luminescence (Nomasense TM O_2 Trace Oxygen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium).

Thirteen mL of grape must and 52 mL of buffer were added to each flask, to which various antioxidant agents or combinations of them had previously been added. This dilution of the grape must was performed because the pure grape juice consumes oxygen so fast that it is nearly impossible to monitor correctly the oxygen concentration of all the samples. The antioxidants used were: sulfur dioxide (20 mg/L in the form of potassium disulfite), ascorbic acid (100 mg/L), glutathione (20 mg/L), a commercial inactivated dry yeast rich in glutathione (400 mg/L), and a selected commercial strain of *Metschnikowia pulcherrima* (200 mg/L). A control without any addition was also prepared. These assays were also performed by combining sulfur dioxide with each of the other antioxidant agents with and without the addition of 2 UA of laccase/mL. Table 1 shows all the experimental conditions and provides abbreviations for each one. All these assays were performed in triplicate.

The bottles were immediately hand-shaken for a few seconds to saturate the solution in oxygen (around 7–8 mg O_2/L), and the oxygen concentration was measured [50] periodically until reaching an asymptotic behavior (around 5 h) to determine the oxygen consumption kinetics. All measurements were taken in an air-conditioned laboratory at 22 ± 2 °C. The total oxygen consumption capacity (TOCC) was calculated using the mathematic model previously reported by Pons-Mercadé et al. [24]. Once the oxygen concentrations were below 1 mg/L or its consumption reached an asymptotic behaviour, the samples were supplemented with 50 mg of sulfur dioxide/L to stop color evolution.

Table 1 Experimental conditions

Without laccase						Supplemented with 2 UA of laccase/mL					
Experimental conditions	SO_2	AA	GSH	IDY	MP	Experimental conditions	SO_2	AA	GSH	IDY	MP
C	0	0	0	0	0	L	0	0	0	0	0
SO_2	20	0	0	0	0	L + SO_2	20	0	0	0	0
AA	0	100	0	0	0	L + AA	0	100	0	0	0
GSH	0	0	20	0	0	L + GSH	0	0	20	0	0
IDY	0	0	0	400	0	L + IDY	0	0	0	400	0
MP	0	0	0	0	200	L + MP	0	0	0	0	200
AA + SO_2	20	100	0	0	0	L + AA + SO_2	20	100	0	0	0
GSH + SO_2	20	0	20	0	0	L + GSH + SO_2	20	0	20	0	0
IDY + SO_2	20	0	0	400	0	L + IDY + SO_2	20	0	0	400	0
MP + SO_2	20	0	0	0	200	L + MP + SO_2	20	0	0	0	200

All the units are expressed as mg/L

C control, *SO_2* sulfur dioxide, *AA* ascorbic acid, *GSH* glutathione, *IDY* inactivated dry yeast, *MP* *Metschnikowia pulcherrima*, *L* laccase

Color measurements

Measurement of the yellow color (A420nm) and the CieL*a*b* coordinates of the samples were determined according to Ayala et al. (1997) [51]. Data processing was performed with MSCV software [52]. The total color difference (ΔEab*) was calculated as the Euclidian distance between two points in the CieL*a*b* space using the following formula: $\Delta Eab^* = ((L_1\text{-}L_2)^2 + (a^*_1\text{-}a^*_2)^2 + (b^*_1\text{-}b^*_2)^2)^{1/2}$, where L* is the lightness; a* is the color's green–red component, and b* is the color's blue–yellow component. It is generally considered as a criterion that two samples of wine could be distinguished by the human eye through the glass when $\Delta Eab^* \geq 3$ units [21, 57].

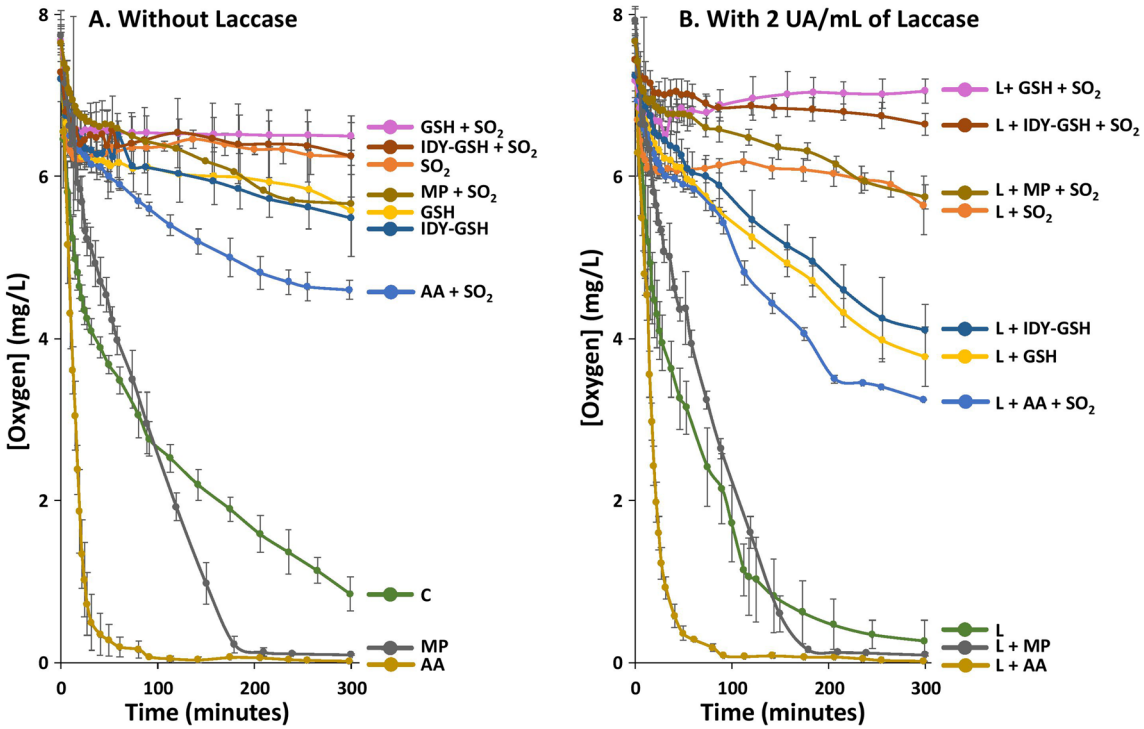
Statistics

All data are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance (ANOVA *F* test) was conducted using SPSS 15.0 software (SPSS Inc., Chicago, IL). Significant differences were considered when *p* value was less than 0.05.

Results and discussion

Oxygen consumption kinetics

Figure 1A and B shows the oxygen consumption kinetics of the diluted grape juice under the experimental conditions with or without supplementation with laccase. Figure 1A clearly shows that the control sample—the diluted grape juice without modification (C)—initially consumed oxygen very quickly. This oxygen consumption rate (OCR) subsequently moderated, probably due to depletion of the substrates, reaching values below 1 mg of O_2/L after 5 h. This figure also shows that when the sample was supplemented with sulfur dioxide (SO_2), the OCR decreased. To statistically compare these curves, a previously reported kinetic model [24, 50] was applied to calculate the total oxygen consumption capacity. This model involves displaying the inverse of consumed oxygen versus the inverse of time. From this mathematical model, the following equation can be established: 1/[O2] = A/t + B. This equation, which describes the relationship between oxygen consumed and time, can be used to determine the total oxygen consumption capacity by calculating the limit when time tends toward infinity.



All the analysis were performed by triplicate. All the units are expressed as mg/L. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY: Inactivated dry yeast; MP: *Metschnikowia pulcherrima*; L: Laccase

Fig. 1 Oxygen consumption kinetics of grape must under different conditions

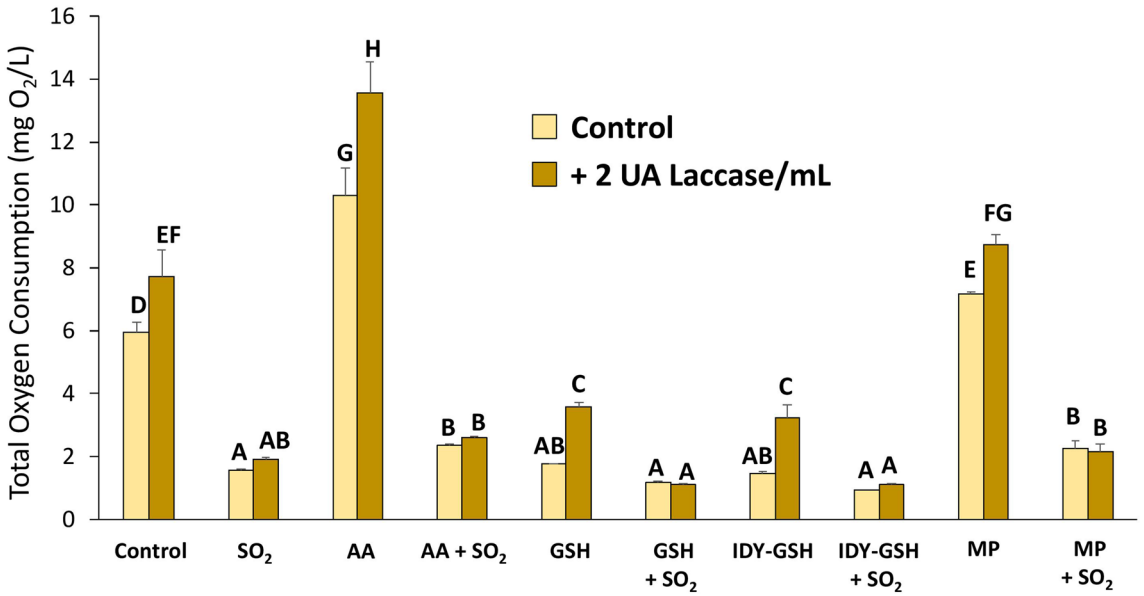
Total oxygen consumption capacity (TOCC)

The results for TOCC are shown in Fig. 2, which confirms what was indicated in Fig. 1A, i.e., that the TOCC of the control sample (C) was significantly higher than that of the sample supplemented with sulfur dioxide (SO_2). These data confirm the well-known inhibitory effect of this additive on tyrosinase activity [8, 12–15].

Supplementation with glutathione (GSH) and with inactivated dry yeasts rich in glutathione (IDY-GSH) also led to a significant decrease in TOCC. This inhibitory effect observed in both experimental conditions may be due to the fact that glutathione reacts with the orthodiquinones produced by the enzymatic oxidation of hydroxycinnamic acids to form the grape reaction product (GRP) [23, 31, 55, 56]. This process can reduce the concentration of orthodiphenols and lead to their depletion, especially in our experimental conditions where the grape must was diluted five times. The lack of substrates for polyphenol oxidases may, therefore, justify the TOCC reduction in our experimental conditions, though in a grape must without dilution, this reduction would probably not be so great. However, these data clearly confirm GSH's protective effect against oxidation, both pure and in the form of IDY rich in GSH.

On the other hand, supplementation with *Metschnikowia pulcherrima* (MP), and especially with ascorbic acid (AA), increased TOCC with respect to the control (C). Ascorbic acid is known to react efficiently with oxygen to produce dehydroascorbic acid and hydrogen peroxide [5, 57]. It can, therefore, compete with polyphenol oxidases [8, 48, 57] for oxygen and prevent grape juice from browning. However, ascorbic acid must be used in combination with sulfur dioxide to eliminate the hydrogen peroxide formed and prevent subsequent oxidation [5, 57]. The highest TOCC observed in the presence of MP probably occurred because this non-*Saccharomyces* yeast directly consumes oxygen very effectively. In fact, use of this yeast has been recommended for that purpose [44, 45]. It should be noted that the used grape must was not sterilized, and therefore the presence of some yeasts, *Saccharomyces* or non-*Saccharomyces* could also have contributed to the observed oxygen consumption albeit presumably to a lesser extent than the inoculated MP.

The combined supplementation of all studied antioxidants with sulfur dioxide ($GSH + SO_2$, $IDY\text{-}GSH + SO_2$, $AA + SO_2$ and $MP + SO_2$) led to a reduction in TOCC with respect to their corresponding references (GSH, IDY-GSH, AA and MP). Note that the TOCC of $GSH + SO_2$ and $IDY\text{-}GSH + SO_2$ were very similar to that of SO_2 alone. These results may be considered logical because in those conditions, tyrosinase is completely inhibited and the possible inhibitory effect of glutathione cannot act because no orthodiquinones are produced. The TOCC of $MP + SO_2$ was also similar to that of SO_2 alone and much lower than that of MP alone. These results may indicate that the presence of sulfur dioxide inhibits this non-*Saccharomyces* yeast.



All the analysis were performed by triplicate. All the units are expressed as mg/L. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*. Different letters indicate the existence of significant differences ($p < 0.05$)

Fig. 2 Influence of laccase, SO_2, ascorbic acid, glutathione, inactivated dry yeast rich on glutathione and *M. pulcherrima* on total oxygen consumption (TOC)

The TOCC of AA + SO_2 was higher than that of SO_2 alone and lower than that of AA alone because in those conditions, oxygen consumption was only due to the direct reaction of this antioxidant with oxygen and not to polyphenol oxidase activity.

As expected, supplementation with laccase significantly increased the TOCC of the control sample. This was probably because total polyphenol oxidase activity was higher and because laccase can oxidize a wider range of substrates than tyrosinase [5, 7]. This trend was also observed in the samples supplemented with laccase and containing AA, GSH, IDY-GSH, and MP in relation to their corresponding samples without laccase.

The samples supplemented with GSH and IDY-GSH in the presence of laccase showed significantly lower values of TOCC than the control sample supplemented with laccase but significantly higher values of TOCC than the corresponding samples without laccase. A possible explanation for these data is that laccase can oxidize more substrates than tyrosinase, especially since this polyphenol oxidase can oxidize GRP [23]. In any case, the reduction in TOCC produced by supplementation with GSH or IDY-GSH suggests that glutathione may perform an antioxidant role even when the grape berries are affected by grey rot, though this protection is not as effective as it is in healthy grapes.

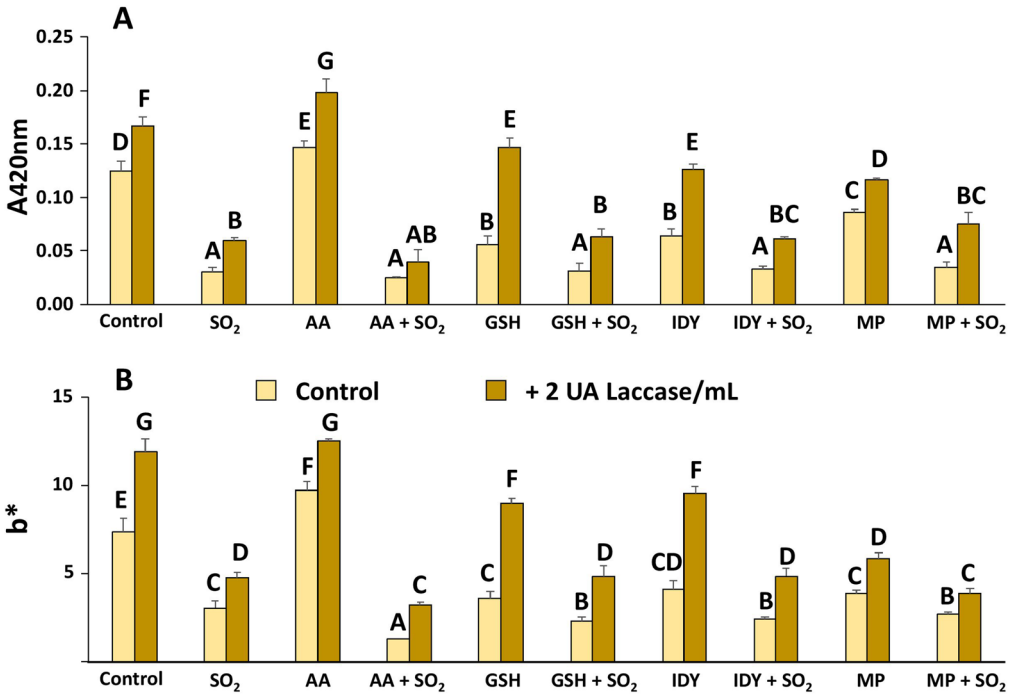
Supplementation with sulfur dioxide inhibited TOCC in all experimental groups in a similar way to the corresponding samples without laccase. These data confirm, as previously described in the literature [4, 5, 8, 48, 57], that sulfur dioxide is also a powerful inhibitor of laccase.

Determining TOCC is necessary to understand which antioxidant agent is the most effective in trapping the oxygen in the grape must and preventing its consumption by polyphenol oxidases. However, since TOCC does not provide direct information about the browning intensity, the final color of the samples was measured by spectrophotometry.

Browning intensity

Figure 3A and B shows the absorbance at 420 nm (A420nm) and the CIEL*a*b* blue–yellow component (b*) of the various samples as indicators of browning intensity.

As expected, supplementation with sulfur dioxide had a clear protective effect against browning since the values of A420 and b* were significantly lower than in the control sample. This effect was also observed when the samples were supplemented with laccase, which confirms that sulfur dioxide not only inhibits the polyphenol oxidase present in healthy grapes (tyrosinase) but also has a powerful inhibitory



All the analysis were performed by triplicate. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*. Different letters indicate the existence of significant differences ($p < 0.05$)

Fig. 3 Influence of laccase, SO_2, ascorbic acid, glutathione, inactivated dry yeast rich on glutathione and *M. pulcherrima* on browning intensity

action on the laccase present in the grapes infected with grey rot [8, 12–15].

In contrast, the sample supplemented with AA showed significantly higher values of A420nm and b* than the control without supplementation although it consumed oxygen faster than the control. This trend, which was also observed when the sample was supplemented with laccase, is probably due to the fact that ascorbic acid produces hydrogen peroxide when it reacts with oxygen [5, 57]. However, when these samples were also supplemented with sulfur dioxide, the A420nm were similar to that of the sample supplemented only with SO_2 and the CIEL*a*b* coordinate b* was even significantly lower. These data confirm that ascorbic acid must be applied together with sulfur dioxide so that it reacts with hydrogen peroxide to nullify its negative effect on browning [22, 57].

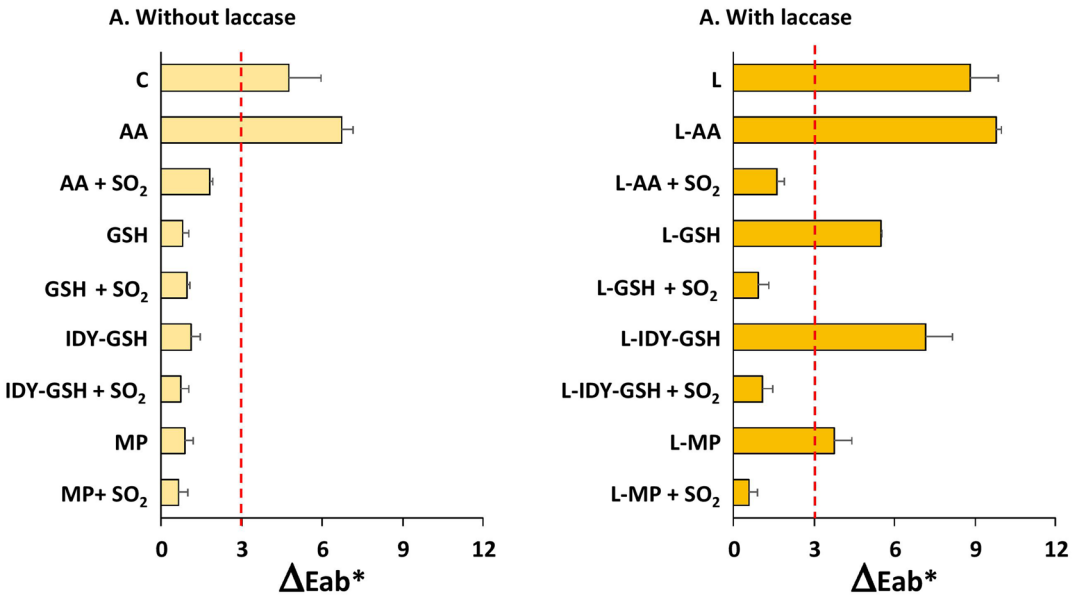
The A420 nm of the samples supplemented with GSH, IDY-GSH, and MP were also significantly lower than those of the control sample. This reduction in A420 nm was highly relevant, though the values were slightly but significantly higher than that of the sample treated with sulfur dioxide. The CIEL*a*b* coordinate b* of the samples supplemented with GSH, IDY-GSH, and MP showed a similar trend to the A420 nm but, in this case, no significant differences were found with the sample treated with sulfur dioxide. These data, therefore, confirm that glutathione, both pure and in the form of inactivated dry yeast, and *Metschnikowia pulcherrima* really do protect grape juice against browning. This protective effect of GSH, IDY-GSH, and MP was also present in the samples supplemented with laccase but the efficiency was much lower. However, when sulfur dioxide was also added, the values of A420 and b* were similar to those of the sample protected only with SO_2. These data indicate that these alternative tools to sulfur dioxide cannot be applied when the grapes are infected with *Botrytis cinerea*.

Note that browning intensity generally has a certain parallelism with the TOCC we observed. In nearly all samples, the higher the TOCC, the higher the browning intensity, with the only exception being the samples supplemented with MP in the presence or absence of laccase. The explanation for this different behavior may be that since *Metschnikowia pulcherrima* consumes oxygen very effectively [27, 44], some of the initially dissolved oxygen is not consumed by the polyphenol oxidases.

Total color difference (ΔEab*)

The total color difference (ΔEab*) between the various samples and the sample supplemented with sulfur dioxide without laccase was calculated to determine whether the browning intensity of the samples can be distinguished by potential consumers.

The results are clear and highly indicative of the protective effect of the various antioxidants. Figure 4A shows the ΔEab* of all samples without supplementation with



All the analysis were performed by triplicate. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*.

Fig. 4 Influence of laccase, SO_2, ascorbic acid, glutathione, inactivated dry yeast rich on glutathione and *M. pulcherrima* on the total color difference (ΔEab*)

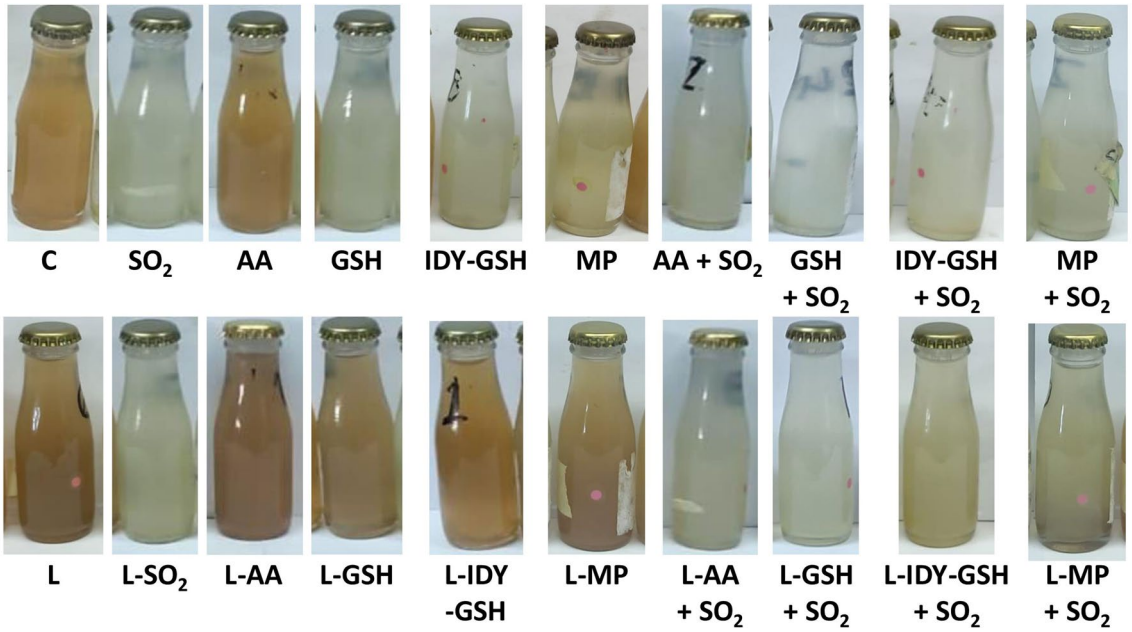


Fig. 5 Influence of laccase, SO_2, ascorbic acid, glutathione, inactivated dry yeast rich on glutathione and *M. pulcherrima* on the final color of the samples

laccase and the corresponding sample with only the addition of sulfur dioxide. As expected, the control sample showed an ΔEab* above 3 units, which indicates that it was much more affected by browning than the sample protected with SO_2. The ΔEab* of the sample supplemented with only AA was even higher than the control, whereas when sulfur dioxide was also present the ΔEab* was below 3 units. These data confirm that using ascorbic acid alone leads to greater oxidation and that it must, therefore, be used in the presence of SO_2 [5, 57].

In contrast, the samples protected with GSH, IDY-GSH, and MP with or without SO_2 showed values below this threshold. However, when the samples were also supplemented with laccase, supplementation with GSH, IDY-GSH, and MP was not effective enough to protect the samples against browning because the values of ΔEab* were above 3 units. These results agree with previously reported data [8, 23, 27, 31, 34, 35, 44, 46] and confirm that using glutathione, pure or in the form of inactivated dry yeasts, and *Metschnikowia pulcherrima* can really protect grape juice against browning at least in healthy grapes.

Since a picture is worth a thousand words, Fig. 5 shows the photographs of the different samples after the experimental process. In them, it can be clearly seen the browning developed in each one of the experimental conditions and, therefore, the protective effect of some of the treatments, which visually corroborates all the experimental data previously shown.

Conclusion

In this study, we analyzed how various antioxidants influence oxygen consumption kinetics and browning intensity in two scenarios. The first scenario was grape must from healthy grapes while the second was grape must enriched with laccase to reproduce what occurs when the grapes are infected with *Botrytis cinerea*. Our experimental design measured oxygen consumption kinetics and browning intensity caused by polyphenol oxidases, tyrosinase, and laccase. As expected, our results confirm that grape must consumes oxygen and browns very quickly and that the presence of laccase accelerates both of these processes. Our results also confirm that sulfur dioxide is highly effective in preventing browning even in grape musts with high levels of laccase activity. On the other hand, using only ascorbic acid leads to higher oxygen consumption and browning, which indicates that this antioxidant must be used in association with sulfur dioxide.

The other alternative antioxidants—glutathione, both pure and in the form of inactivated dry yeasts, and the non-*Saccharomyces* yeast, *Metschnikowia pulcherrima* used as a bioprotective agent—can be interesting tools for protecting

grape juice against browning and perhaps for reducing the use of sulfur dioxide, at least in healthy grapes. Specifically, glutathione and inactivated dry yeast rich in glutathione reduced oxygen consumption and reduced the intensity of browning when no laccase was present in the medium. However, their effectivity was reduced in the presence of laccase. The mechanism by which glutathione protects against enzymatic browning and reduces oxygen consumption is probably its capacity to combine with the orthodiquinones formed by the action of the polyphenol oxidases in stopping the browning process and depleting the medium on substrates for these enzymes.

Metschnikowia pulcherrima also reduced browning intensity but its action mechanism is different from that of glutathione. This non-*Saccharomyces* yeast protects because it consumes oxygen very efficiently, and therefore reduces its availability for the polyphenol oxidases.

More studies are needed to further investigate these promising alternatives to sulfur dioxide since many consumers are searching for healthier wines, and the wine industry is very keen to reduce this unfriendly additive.

Acknowledgements Authors want to thank professors Marc Fermaud and Jean Roudet from INRAE, UMR SAVE, Bordeaux Science Agro, ISVV, France for having provided us with the B. cinerea strain.

Funding Open access funding provided by Universitat Rovira i Virgili. This research was funded by CICYT (Efecto de las lacasas sobre la sensorialidad, calidad y salubridad de los vinos. Evaluación de la influencia de lacasas sobre el color, la astringencia y la calidad del vino—project RTI2018-095658-B-C33).

Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval All authors were compliant and followed the ethical guidelines, according to the requirements of European Food Research and Technology.

References

1. Li H, Guo A, Wang H (2008) Mechanisms of oxidative browning of wine. Food Chem 108:1–13
2. Ky I, Lorrain B, Jourdes M, Pasquier G, Fermaud M, Gény L, Rey P, Donèche B, Teissèdre P-L (2012) Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. Aust J Grape Wine Res 18:215–226
3. Friedman M (1996) Food browning and its prevention: an overview. J Agric Food Chem 44:631–653
4. du Toit WJ, Marais J, Pretorius IS, du Toit M (2006) Oxygen in must and wine: a review. S Afr J Enol Vitic 27:76–94
5. Oliveira CM, Silva-Ferreira AC, De Freitas V, Silva AM (2011) Oxidation mechanisms occurring in wines. Food Res Int 44:1115–1126
6. IUBMB. International union of biochemistry and molecular Biology. https://www.qmul.ac.uk/sbcs/iubmb/. Accessed 19 Dec 2022
7. Steel CC, Blackman JW, Schmidtke LM (2013) Grapevine bunch rots: Impacts on wine composition, quality, and potential procedures for the removal of wine faults. J Agric Food Chem 61:5189–5206
8. Giménez P, Anguela S, Just-Borras A, Pons-Mercadé P, Vignault A, Canals JM, Teissedre PL, Zamora F (2022) Development of a synthetic model to measure browning caused by laccase activity from *Botrytis cinerea*. LWT Food Sci Technol. https://doi.org/10.1016/j.lwt.2021.112871
9. Claus H, Sabel A, König H (2014) Wine phenols and laccase: an ambivalent relationship. Wine: phenolic composition classification and health benefits. Nova publishers, New York, pp 155–185
10. Queiroz C, Mendes-Lopes ML, Fialho E, Valente-Mesquita VL (2008) Polyphenol oxidase: characteristics and mechanisms of browning control. Food Rev Int 24:361–375
11. Ribéreau-Gayon P, Glories Y, Dubourdieu D, Donèche B, Lonvaud A (2006) The microbiology of wine and vinifications, 2nd edn. John Wiley & Sons, Chichester, pp 193–221
12. Fazio T, Warner CR (1990) A review of sulphites in foods: analytical methodology and reported findings. Food Addit Contam 7(4):433–454
13. Wedzicha BL, Bellion I, Goddard SJ (1991) Inhibition of browning by sulfites. Adv Exp Med Biol. https://doi.org/10.1007/978-1-4899-2626-5_16
14. Ough CS, Crowell EA (1987) Use of sulfur dioxide in winemaking. J Food Sci 52:386–389
15. Schroeter LC (1966) Sulfur dioxide application in foods, beverages and pharmaceuticals. New York Pergamon Press, New York
16. D'Amico M, Di Vita G, Monaco L (2016) Exploring environmental consciousness and consumer preferences for organic wines without sulfites. J Clean Prod 120:64–71
17. Massov O. A natural wine glossary: from organic to "minimal intervention" The Washington Post, June 13, 2019. https://www.washingtonpost.com/goingoutguide/a-natural-wine-glossary-from-organic-to-minimal-intervention/2019/06/13/5ade77c4-84b6-11e9-933d-7501070ee669_story.html. Accessed 19 Dec 2022
18. Lester MR (1995) Sulfite sensitivity: significance in human health. J Am Coll Nutr 14:229–232
19. Costanigro M, Appleby C, Menke SD (2014) The wine headache: consumer perceptions of sulfites and willingness to pay for non-sulfited wines. Food Qual Pref 31:81–89
20. Walls J, Sutto S, Coetzee C, du Toit WJ (2022) Sparging of white wine. Aust J Grape Wine Res 28:450–458

21. Vignault A, Pascual O, Jourdes M, Moine V, Fermaud M, Roudet J, Canals JM, Teissedre PL, Zamora F (2019) Impact of enological tannins on laccase activity. Oeno One 1:27–38
22. Peng Z, Duncan B, Pocock KF, Sefton MA (1998) The effect of ascorbic acid on oxidative browning of white wines and model wines. Aust J Grape Wine Res 4:127–135
23. Kritzinger EC, Bauer FF, du Toit WJ (2013) Role of glutathione in winemaking: a review. J Agric Food Chem 61:269–277
24. Pons-Mercadé P, Anguela S, Giménez P, Heras JM, Sieczkowski N, Rozès N, Canals JM, Zamora F (2021) Measuring the oxygen consumption rate of some inactivated dry yeasts: comparison with other common wine antioxidants. Oeno One 2:147–158
25. Petrova B, Cartwright ZM, Edwards CE (2016) Effectiveness of chitosan preparations against *Brettanomyces* grown in culture media and red wines. J Int Sci Vigne Vin 50:49–56
26. Gao YC, Zhang G, Krentz S, Darius S (2002) Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. Aust J Grape Wine Res 8:76–83
27. Simonin S, Roullier-Gall C, Ballester J, Schmitt-Kopplin P, Quintanilla-Casas B, Vichi S, Peyron D, Alexandre H, Tourdot-Maréchal R (2020) Bio-Protection as an alternative to sulphites: impact on chemical and microbial characteristics of red wines. Front Microbiol 11:1308
28. Loira I, Morata A, Bañuelos MA, Puig-Pujol A, Guamis B, González C, Suárez-Lepe JA (2018) Use of ultra-high pressure homogenization processing in winemaking: control of microbial populations in grape musts and effects in sensory quality. Inno Food Sci Emerg Technol 50:50–56
29. Guzzon R, Bernard M, Barnaba C, Bertoldi D, Pixner K, Larcher R (2017) The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine. J Food Sci Technol 54:810–821
30. Morata A, Bañuelos MA, López C, Song C, Vejarano R, Loira I, Palomero F, Suarez-Lepe JA (2020) Use of fumaric acid to control pH and inhibit malolactic fermentation in wines. Food Addit Contam 37:228–238
31. Singleton VL, Salgues M, Zaya J, Trousdale E (1985) Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. Am J Enol Vit 36:50–56
32. OIV, Resolution OIV-OENO 445-2015, Treatment of must with glutathione https://www.oiv.int/public/medias/1686/oiv-oeno-445-2015-en.pdf. Accessed 19 Dec 2022
33. OIV, Resolution OIV-OENO 446–2015, Treatment of wine with glutathione https://www.oiv.int/public/medias/1687/oiv-oeno-446-2015-en.pdf. Accessed 19 Dec 2022
34. Gabrielli M, Aleixandre-Tudo JL, Kilmartin PA, SieczkowskiI N, du Toit WJ (2017) Additions of Glutathione or specific glutathione-rich dry inactivated yeast preparation (DYP) to Sauvignon blanc must: effect on wine chemical and sensory composition. S Afr J Enol Vitic 38:18–28
35. Bahut F, Youzhong L, Romanet R, Coelho C, Sieczkowski N, Alexandre H, Schmitt-Kopplin P, Nikolantonaki M, Gougeon RD (2019) Metabolic diversity conveyed by the process leading to glutathione accumulation in inactivated dry yeast: a synthetic media study. Food Res Int 123:762–770
36. OIV, Resolution OIV-OENO 603-2018, Monograph on inactivated yeasts with guaranteed glutathione levels. https://www.oiv.int/public/medias/6444/oiv-oeno-603-2018-en.pdf. Accessed 19 Dec 2022
37. Windholtz S, Redon P, Lacampagne S, Farris L, Lytra G, Cameleyre M, Barbe JC, Coulon J, Thibon C, Masneuf-Pomarède I (2021) Non-Saccharomyces yeasts as bioprotection in the composition of red wine and in the reduction of sulfur dioxide. LWT Food Sci Technol 149:111781
38. OIV, Resolution OIV-OENO 576B-2017, Monograph Non-Saccharomyces yeasts can be used for the inoculation of grapes, musts and wine https://www.oiv.int/public/medias/5371/oiv-oeno-576b-2017-en.pdf. Accessed 19 Dec 2022
39. OIV, Resolution OIV-OENO 576A-2017, Monograph of saccharomyces yeasts https://www.oiv.int/public/medias/5370/oiv-oeno-576a-2017-en.pdf. Accessed 19 Dec 2022
40. Morata A, Loira I, González C, Escott C (2021) Non-Saccharomyces as biotools to control the production of off-flavors in wines. Molecules 26:4571
41. Peña R, Vílches J, Poblete C, Ganga MA (2020) Effect of Candida intermedia LAMAP1790 antimicrobial peptides against wine-spoilage yeasts Brettanomyces bruxellensis and Pichia guilliermondii. Fermentation 6:65
42. Simonin S, Alexandre H, Nikolantonaki M, Coelho C, Tourdot-Maréchal R (2018) Inoculation of Torulaspora delbrueckii as a bioprotection agent in winemaking. Food Res Int 107:451–461
43. Rubio-Bretón R, Gonzalo-Diago A, Iribarren M, Garde-Cerdán T, Pérez-Álvarez EP (2018) Bioprotection as a tool to free additives winemaking: effect on sensorial, anthocyanic and aromatic profile of young red wines. LWT Food Sci Technol 98:458–464
44. Di Gianvito P, Englezos V, Rantsiou K, Cocolin L (2022) Bioprotection strategies in winemaking. Int J Food Microbiol 364:109532
45. Chacon-Rodriguez L, Joseph CML, Nazaris B, Coulon J, Richardson S, Dycus DA (2020) Innovative use of non-Saccharomyces in bioprotection: T. delbrueckii and M. pulcherrima applied to a machine harvester. Catalyst 4:82–90
46. Windholtz S, Nion C, Seabrook A, Coulon J, Masneuf-Pomerède I (2022) Assessment of O2 consumption, a new tool to select bioprotection yeast strains. IVES Conference Series-WAC 2022 abstracts https://www.ives-openscience.eu/14802/. Accessed 19 Dec 2022
47. Windholtz S, Vinsonneau E, Farris L, Thibon C, Masneuf-Pomarède I (2021) Yeast and filamentous fungi microbial communities in organic red grape juice: effect of vintage, maturity stage, SO2, and bioprotection. Front Microbiol 12:748416
48. Vignault A, Gombau J, Jourdes M, Moine V, Canals JM, Fermaud M, Roudet J, Teissedre PL, Zamora F (2020) Oenological tannins to prevent Botrytis cinerea damages: kinetics and electrophoresis characterization. Food Chem 316:126334
49. Grassin C, Dubourdieu D (1986) Optimisation de la méthode de dosage de l'activité laccase de Botrytis cinerea par la syringaldazine. J Int Sci Vigne Vin 20:125–130
50. Diéval JB, Vidal S, Aagaard O (2011) Measurement of the oxygen transmission rate of co-extruded wine bottle closures using a luminescence-based technique. Package Technol Sci 24:375–385
51. Ayala F, Echavarri JF, Negueruela AI (1997) A new simplified method for measuring the color of wines. ii. white wines and brandies. Am J Enol Vitic 48:364–369
52. MSCVes.zip. (2013) http://www.unirioja.es/color/descargas.shtml. Accessed 19 Dec 2022
53. García-Marino M, Escudero-Gilete ML, Heredia FJ, Escribano-Bailón MT, Rivas-Gonzalo JC (2013) Color-copigmentation study by tristimulus colorimetry (CIELAB) in red wines obtained from Tempranillo and Graciano varieties. Food Res Int 51:123–131
54. Pascual O, Vignault A, Gombau J, Navarro M, Gómez-Alonso S, García-Romero E, Canals JM, Hermosín-Gutíerrez I, Teissedre PL, Zamora F (2017) Oxygen consumption rates by different oenological tannins in a model wine solution. Food Chem 234:26–32
55. Nikolantonaki M, Magiati P, Waterhouse AL (2014) Measuring protection of aromatic wine thiols from oxidation by competitive reactions vs. wine preservatives with ortho-quinones. Food Chem 163:61–67
56. Nikolantonaki M, Julien P, Coelho C, Roullier-Gall C, Ballester J, Schmitt-Kopplin P, Gougeon RD (2018) Impact of glutathione on wines oxidative stability: a combined sensory and metabolomic study. Front Chem 6:182

57. Barril C, Rutledge DN, Scollary GR, Clark AC (2016) Ascorbic acid and white wine production: a review of beneficial versus detrimental impacts. Aust J Grape Wine Res 22:169–181