Evaluation of the use of sulfur dioxide and glutathione to prevent oxidative degradation of malvidin-3-monoglucoside by hydrogen peroxide in the model solution and real wine

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Abstract: In this study the oxidative degradation by hydrogen peroxide of native grape anthocyanin was studied in model solutions and in red wines added with increasing concentration of sulfur dioxide and glutathione (GSH). The presence of hydrogen peroxide and metal ions in traces allowed to investigate the possibility to use GSH to prevent Fenton reaction in wine conditions. Two different pH of wine were considered: 3.20 and 3.80. The protective effect of sulfur dioxide on malvidin 3-monoglucoside degradation was higher at lower pH in model solution. No effect of pH on sulfur dioxide action towards the native anthocyanin in real wine was detected. Surprisingly GSH determined an increase in the degradation of malvidin 3-monoglucoside regardless of pH. Therefore, GSH is not effective in prevent native anthocyanins loss due to the Fenton reaction during red wine aging.

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April, 3th 2017

Dear Editor,

I would like to submit for publication this paper about "**EVALUATION OF THE USE OF SULFUR DIOXIDE AND GLUTATHIONE TO PREVENT OXIDATIVE DEGRADATION OF MALVIDIN-3-MONOGLUCOSIDE BY HYDROGEN PEROXIDE IN MODEL SOLUTION AND REAL WINE.**"

The originality of the work lies into the fact that, for the first time, the role of glutathione as an alternative to sulfur dioxide to prevent oxidative loss of native pigments of red wine in strong oxidative conditions was evaluated.

In last decades concerns over the ability of sulfur dioxide to induce severe allergic reactions have created a great need for its reduction or replacement in wine and glutathione has been proposed as an alternative antioxidant. However only its ability to prevent the loss of some aroma compounds has been showed and its effect on other important compounds such as pigments of wine is not clear.

Data obtained in this study showed that, in presence of hydrogen peroxide, glutathione accelerates pigments degradation. These results could have important implications into the use of glutathione to produce red wines with reduced content of sulfur dioxide. This may help in regulating the use of this compound as additive in wine production.

Instructions for authors have been carefully followed.

I hope that our editing satisfies the standards required and that the manuscript can be submitted to the attention of the referees.

I keep waiting for your answer.

Yours sincerely

Prof. Angelita Gambuti

***Detailed Response to Reviewers**

RESPONSE TO FIRST REVIEW - LIST OF CHANGES

Dear Anderson de Souza Sant'Ana,

in response to each of the comments mentioned in the letter received on Mon, 10 Apr 2017, we changed the manuscript as follow (answers as colored text).

Sincerely yours,

Angelita Gambuti

a) Use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

We named the file Highlights. Each research highlight was represented with bullet points and was maximum of 85 characters, including spaces, per bullet point.

b) Define better the aims of the MS at the end of INTRODUCTION section

We better defined the aim of the MS (lines 75-87).

c) Take care of FRI style in guide for authors. For example:

21. Gambuti, A., Han, G., Peterson, A. L., & Waterhouse, A. L. (2015). Sulfur dioxide and glutathione alter the outcome of microoxygenation. American Journal of Enology and Viticulture,ajev-2015.

Should be: 21. Gambuti, A., Han, G., Peterson, A. L., & Waterhouse, A. L. (2015). Sulfur dioxide and glutathione alter the outcome of microoxygenation. American Journal of Enology and Viticulture, 66, 411-423.

We considered FRI style: lines 334-335, 387-389, 419-420, 460-463.

***Highlights (for review)**

- The possibility to use GSH to prevent Fenton reaction in wine has been evaluated.
- GSH determined an increase in the degradation of malvidin 3-monoglucoside.
- The preventive action of SO_2 in red wine does not depends on pH.
- GSH is not effective in prevent anthocyanins loss during red wine aging.

EVALUATION OF THE USE OF SULFUR DIOXIDE AND GLUTATHIONE TO PREVENT OXIDATIVE DEGRADATION OF MALVIDIN-3-MONOGLUCOSIDE BY HYDROGEN PEROXIDE IN THE MODEL SOLUTION AND REAL WINE.

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Running title: Glutathione and Sulfur Dioxide affect the degradation of wine native pigments by hydrogen peroxide.

ABSTRACT

In this study the oxidative degradation by hydrogen peroxide of native grape anthocyanin was studied in model solutions and in red wines added with increasing concentration of sulfur dioxide and glutathione (GSH). The presence of hydrogen peroxide and metal ions in traces allowed to investigate the possibility to use GSH to prevent Fenton reaction in wine conditions. Two different pH of wine were considered: 3.20 and 3.80. The protective effect of sulfur dioxide on malvidin 3-monoglucoside degradation was higher at lower pH in model solution. No effect of pH on sulfur dioxide action towards the native anthocyanin in real wine was detected. Surprisingly GSH determined an increase in the degradation of malvidin 3-monoglucoside regardless of pH. Therefore, GSH is not effective in prevent native anthocyanins loss due to the Fenton reaction during red wine aging.

Keywords: anthocyanins, wine pigments, oxidation, sulfur dioxide, glutathione.

1. Introduction

During production and aging of red wine a change of wine color is observed due to the involvement of wine pigments, the anthocyanins, in numerous reactions with other compounds present in solution and with compounds deriving from oxygen action. The shift of wine color from red to yellow hue is an indication of aging but also of oxidative spoilage of red wine and it is essentially due to the action of oxygen determining a loss of native anthocyanins not balanced by the formation of more stable red pigments. The chemical oxidation of wine is trigged by the oxidation of polyphenols to quinones while oxygen is reduced to hydrogen peroxide in presence of trace metals such as iron and cupper (Danilewicz, 2011). Wine polyphenols involved in this starting phase contain at least two vicinal hydroxyls. Because wine native red pigments, such as malvidin 3-monoglucoside, contain isolated phenolic hydroxyl groups and need higher potential to be oxidized (Kilmartin, Zou & Waterhouse, 2001), they are not involved in the first steps of wine oxidation. Their loss is due to the action of hydrogen peroxide H_2O_2 that, in the second step of wine oxidation, in presence of ferrous or cuprous species, reacts in the Fenton reaction to give the destructive oxidant radicals (Elias, Andersen, Skibsted & Waterhouse, 2009). A direct reaction of malvidin 3-monoglucoside with H_2O_2 has been reported (Sondheimer & Kertesz, 1952; Ozkan, Yemenicioglu, Asefi & Cemeroglu, 2002) but the main reason of their loss is linked to their involvement in complex reactions trigged by reactive carbonyls produced by the action of radicals produced by Fenton reaction such as acetaldehyde from oxidation of ethanol and glyoxylic acid from oxidation of tartaric acid (Es-Safi, Fulcrand, Cheynier & Moutounet, 1999; Es Safi, Cheynier & Moutounet, 2003; He et al., 2012).

Oxidation is a long-standing problem in wine industry and sulfur dioxide SO_2 is the generally used chemical to control it. SO_2 acts as antioxidant in three ways, scavenging hydrogen peroxide, reacting with ortho-quinones acting as sacrificial nucleophiles and binding carbonyl compounds produced by Fenton reaction (Adachi et al., 1979; Danilewicz & Wallbridge, 2010). However, concerns over its ability to induce severe allergic reactions have created a great need for its reduction or replacement and, as a consequence, regulatory restrictions has been established by World Health Organization (WHO) and International Organization of Vine and Wine (OIV). Moreover, its excessive use in winemaking can determine a distinctive irritating odor in wine.

In last decades, the tripeptide glutathione (GSH) has been proposed in winemaking as alternative antioxidant to decrease the use of SO_2 (Kritzinger, Bauer & Du Toit, 2012). This is

why it has been recently authorized by OIV (International Organization of Vine and Wine) in must (maximum up to 20 mg/L) but it is still not admitted by EC as wine additive. Low concentration (20 mg/L) of GSH protected against loss of esters, terpenes (Roussis, Lambropoulos & Tzimas, 2007) and volatile thiols during bottle storage (Ugliano et al., 2011). At higher concentrations (180 mg/L) it delayed the oxidative browning limiting the formation of yellow xanthylium cation pigments in white wines (Roussis, Lambropoulos & Tzimas, 2007; Sonni, Clark, Prenzler, Riponi, & Scollary, 2011; Bouzanquet, Barril, Clark, Dias & Scollary, 2012;). Recently a moderate protective effect of GSH (30 mg/L) on native anthocyanins during micro-oxygenation (MOx) has been showed (Gambuti, Han, Peterson & Waterhouse, 2015). Its anti-oxidant activity in wine is mainly due to the abilities to reduce back *o*-quinone compounds (Cheynier & Van Hulst, 1988; Nikolantonaky & Waterhouse, 2012) and it can also bind wine reactive aldehydes (Sonni, et al., 2011).

GSH also prevent cellular damage owing to its hydrogen peroxide scavenging activity (Winterbourn & Metodiewa, 1999) but the same activity in wine has not been evaluated. Among wine features, pH is one of main wine parameter influencing wine oxidation because it affects the formation of new stable polymeric pigments from native anthocyanins (Kountoudakis et al., 2011; Pechamat, Zeng, Jourdes, Ghidossi, & Teissedre, 2014;) and SO_2 forms in hydroalcoholic solution. However, no information on the effect pH on the antioxidant activity of GSH in wine conditions has been reported.

The aim of the present study was to investigate the potentiality of GSH as an alternative to SO_2 to scavenge hydrogen peroxide, to inhibit Fenton reaction and to prevent grape native anthocyanins loss during wine aging. With this purpose the oxidative degradation of malvidin 3-monoglucoside was studied in model solutions and in red wines treated with hydrogen peroxide and added with increasing concentration of sulfur dioxide and glutathione. The experiments were carried out at two different wine pH: 3.20 and 3.80.

~~In addition, GSH prevent cellular damage owing to its hydrogen peroxide scavenging activity (Winterbourn & Metodiewa, 1999) but the same activity in wine has not been evaluated.~~

~~In this study, to better understand if GSH can be proposed as an alternative to SO_2 to scavenge hydrogen peroxide, inhibit Fenton reaction and prevent grape native anthocyanins loss during wine aging, the oxidative degradation of malvidin 3-monoglucoside was studied in model solutions and in red wines treated with hydrogen peroxide and added with increasing concentration of sulfur dioxide and glutathione.~~

~~Moreover, because pH is one of main wine feature affecting wine oxidation owing to its role on the formation of new stable polymeric pigments from native anthocyanins (Kountoudakis et al., 2011; Pechamat, Zeng, Jourdes, Ghidossi, & Teissedre, 2014;) and on SO_2 forms in hydroalcoholic solution, the experiments were carried out at two different wine pH: 3.20 and 3.80.~~

2. MATERIALS AND METHODS

2.1 Experimental trial. Oxidation reactions were performed in 10 mL reagent bottles. The bottles were purged with nitrogen and placed in darkness at 20 °C. In the first experiment the effect of antioxidants and pH was evaluated in model solutions. All solutions contained malvidin 3-monoglucoside 100 mg/L (203 mM), ethanol (12% v/V) and tartaric acid (q. s.). The pH was adjusted adding NaOH. The oxidation Ox was performed adding hydrogen peroxide at a concentration of 39.2 mg/L of O_2 eq (1.225 mM) to trigger Fenton reaction (Elias and Waterhouse, 2010). Because this reaction involves hydrogen peroxide and metal ions at concentration (< 0.2 μM) much lower than expected in all commercial water supplies (Clark, Prenzler & Scollary, 2007) the occurrence of Fenton reaction in our experimental conditions is guaranteed. Six oxidized (Ox) samples were obtained: Ox, adding only hydrogen peroxide; Ox+SO_2 low, adding hydrogen peroxide and 37.4 mg/L of SO_2 (0.584 mM); Ox+SO_2 high, adding hydrogen peroxide and 202 mg/L of SO_2 (3.16 mM); Ox+GSH, adding hydrogen peroxide and 30 mg/L of glutathione (0.098 mM); Ox+SO_2 low+GSH, adding hydrogen peroxide, 37.4 mg/L of SO_2 (0.584 mM) and 30 mg/L of glutathione (0.098 mM); Ox+SO_2high+GSH, adding hydrogen peroxide, 202 mg/L of SO_2 (3.16 mM) and glutathione (0.098 mM). Samples were prepared at two pH: 3.20 and 3.80 and monitored after 0, 16 and 72 hours of incubation at 20 °C. Dilution coefficient were considered to compare treated sample with control one. In the second experiment the same treatments were performed on a red wine produced in 2013(*Vitis vinifera* L. Casavecchia). The base parameters were: ethanol content 13.60 ± 0.07 % v/V, pH 3.80 ± 0.03, residual sugars 1.72 ± 0.06 g/L. The pH of wine was adjusted to pH 3.20 by adding tartaric acid. All samples (model solutions and wines) were prepared in duplicate. On each replicate two analyses were performed to have a datum from the mean of four values.

2.2 Reagents and standards. Solvents of HPLC-gradient grade and all other chemicals of analytical reagent grade were purchased from Sigma (Milan, Italy). The solutions were prepared in deionized water produced by a Purelab Classic system (Elga Labwater, Marlow,

UK). About standards for calibration curves, syringic acid was purchase from Sigma-Aldrich (Milan, Italy) whereas malvidin-3-glucoside chloride was supplied by Extrasynthèse (Genay, France). For identification purposes, anthocyanin standards (delphinidin-3-glucoside chloride, malvidin-3-glucoside chloride, petunidin chloride, peonidin-3-glucoside chloride, and cyanidin-3-glucoside chloride) were purchased from Extrasynthèse.

2.3 Methods. HPLC separation of anthocyanins and for the determination of syringic acid were carried out according to the OIV Compendium of International Methods of Analysis of Wine and Musts (2017). Analyses were performed in a HPLC Shimadzu LC10 ADVP apparatus (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA) equipped with a 50 µL loop. A Waters Spherisorb column (250 x 4.6 mm, 4µm particles diameter) with pre-column was used. Twenty µL of wine or calibration standards were injected onto the column. Detection was performed by monitoring the absorbance signals at 518 nm. All the samples were filtered through 0.45 mm, Durapore membrane filters (Sigma Aldrich, Milan, Italy) into glass vials and immediately injected into the HPLC system. The HPLC solvents were: solvent A: water/formic acid/acetonitrile (87:10:3) v/v; solvent B: water/formic acid/acetonitrile (40:10:50) v/v. The gradient used was: zero-time conditions 94 % A and 6 % B, after 15 min the pumps were adjusted to 70 % A and 30 % B, at 30 min to 50 % A and 50 % B, at 35 min to 40 % A and 60 % B, at 41 min, end of analysis, to 94 % A and 6 % B. After 10-min equilibrium period the next sample was injected. The flow rate was 0.80 mL/min. For calibration the external standard method was used: the calibration curve was plotted for the malvidin-3-monoglucoside (Extrasynthese, Lyon, France) on the basis of peak area and the concentration was expressed as mg/L of malvidin-3-monoglucoside. The calibration curve for the identification and determination of syringic acid was prepared starting from a stock solution containing 5 mg/L of syringic acid (Sigma-Aldrich, Milan, Italy). All the analyses were made in duplicate on each experimental replicate.

2.4 Statistical Analyses. Quantitative data of the wines were compared using Fisher's least significant differences (LSDs) procedure. Multifactorial ANOVA with third-order interactions was used to evaluate the relationships among factors. Differences of $p < 0.05$ were considered significant. These analyses were performed using XLSTAT (Addinsoft, XLSTAT Version 2013.6.04). All data are means of four values (2 experimental replicates X 2 analytical replicates).

3. RESULTS AND DISCUSSION

3.1 Model solution experiments

In aqueous solution anthocyanins exist in different forms in equilibrium depending on the pH: the reddish-pink flavylium salt predominates at lower pH values while the carbinol pseudo-base and chalcone are the main species at pH higher that 7 (Brouillard & Dubois, 1977). Concerning the range of pH typical of red wines it has been reported that at pH of 3.4–3.6, 20–25% of anthocyanins are in the colored flavylium forms, whereas at pH of 4.0, only 10% of anthocyanins are in such ionized state (Jackson, 2008). In the first part of this study a model solution containing malvidin 3-monoglucoside was analyzed by detection at 518 nm after chromatographic separation and oxidation by means of H_2O_2 addition. A degradation of malvidin 3-monoglucoside after the addition of hydrogen peroxide occurred. Lopes et al. (2007) showed that under wine pH malvidine 3-O-glucoside was degraded to 2,4,6-trihydroxybenzaldehyde, syringic acid, and the 8-β-D-glucopyranosyl-2,4-dihydroxy-6-oxo-cyclohexa-2,4-dienyl acetic acid (anthocyanone A). These last molecules results from the degradation of malvidin 3-O-glucoside, formed by Baeyer-Villiger-type oxidation trigged by hydrogen peroxide. The degradation peaks (peaks 1-5 in Fig. 1) detected during chromatographic run are the same previously reported by Lopes and colleagues (2007). In this study we evaluated the concentrations of malvidin 3-monoglucoside and that of the main degradation product, the syringic acid (peak 4 in the chromatogram). As expected by the chemistry of anthocyanins in aqueous solution, a lower content of malvidin 3-monoglucoside was observed at higher pH (Fig. 2). After the addition of hydrogen peroxide a dramatic loss of malvidin 3-monoglucoside has been detected at both pH; the loss is slightly enhanced increasing the pH. It is well known that fruit native anthocyanins are susceptible to be destroyed by H_2O_2 (Sondheimer & Kertesz, 1952; Ozkan, Yemenicioglu, Asefi & Cemeroglu, 2002) but the effect of pH seems to be in disagreement with a previous study where flavilum cation, which is dominant at lower pH, has been showed to react 4.3 ± 0.4 times faster with hydrogen peroxide molecules than the neutral pseudo-base (Thompson, Spiro & Griffith, 1996) (Fig. 2). However, Zhang, Duan, Ji and Pang (2000) found, in agreement with our results, a less degradation of litchi anthocyanins by H_2O_2 at lower pH. The discrepancy between Thompson, Spiro and Griffith (1996) results and our study can be related to the fact that in solution the direct chemical degradation of malvidin3-monoglucoside is not the only reaction trigged by hydrogen peroxide. It is likely that part of radicals produced by Fenton

reaction are quenched by tartaric acid (Elias & Waterhouse, 2010) more than from malvidin 3-monoglucoside in solution at pH 3.20.

At pH 3.20 an antioxidant effect of SO_2 is detected regardless the concentration used. In contrast, at higher pH, the protective activity against oxidative degradation was detected only at higher SO_2 concentration. Probably at these concentrations SO_2 not only quenches hydrogen peroxide limiting Fenton reaction but, also binds malvidin 3-monoglucoside. To understand these results the chemistry of SO_2 in aqueous solution has to be considered. As well-known SO_2 exists in different forms, molecular SO_2(g) in equilibrium with bisulfite ion, in turn in equilibrium with bound SO_2. In the model solution under investigation bound SO_2 is related to the reaction of bisulfite ion with carbonyls deriving from oxidation of tartaric acid and ethanol (glyoxylic acid and acetaldehyde) as well as with malvidin 3-O glucoside. The extend of combination and the rate of binding is slower the lower the pH (Amerine & Joslin, 1970). In addition, lower is the pH more the equilibria are shifted towards the molecular SO_2. Therefore, at lower pH both phenomena determine a higher presence of bisulfite ions dissociating from bound SO_2 and capable to react with hydrogen peroxide and act as quenching compounds (Danilewicz, 2007).

Surprisingly at pH 3.80 low concentration of SO_2 determined a loss of malvidin 3-monoglucoside (Fig. 2). It is possible that in these conditions SO_2 did not protect the ethanol but promoted its oxidation, which is in agreement with the fact that a substantial proportion of SO2 is oxidized to produce highly oxidizing SO radicals such as the peroxomonosulfate radical (Danilewicz, 2007). A past study of Connick and Zhang (1986) showing that the reaction of formation of peroxomonosulfate radical is enhanced at higher pH, confirm this hypothesis. The bleaching of anthocyanin solutions due to pH and SO_2 should be also considered (Brouillard, & El Hage Chahine, 1980) but this reaction is secondary with respect to the dominant kinetic of reaction between SO_2 and H_2O_2 in presence of trace metals and organic acids (Breytenbach, van Pareen, Pienaar, & van Eldik, 1994) and with respect to direct degradation of malvidin 3-Oglucoside by hydrogen peroxide when in excess of SO_2.

To understand if GSH may fulfil the antioxidant roles of SO_2 in wine conditions, such as to bind aldehyde compounds and to scavenge hydrogen peroxide, GSH and a combination of both antioxidant (SO_2 and GSH) were used. An increase in the degradation of malvidin 3-monoglucoside was detected when GSH was used alone and in combination with low concentration of SO_2 at both pH. The scavenging effect of SO_2 was instead dominant when its concentration was high and, in this case, no significant acitivity of GSH was detected. These

results seem in disagreement with previous results obtained during micro-oxygenation of wine (Gambuti, Han, Peterson, & Waterhouse, 2015) where a little protective effect of GSH against anthocyanins degradation was detected. The reason of the different behavior can be found in the fact that in the present experiment only the anti-Fenton activity of GSH was determined while in the previous study the oxidation was obtained adding directly oxygen (in a micro-oxygenation experiment). It is well known that mechanism of oxidation of wine can be separated in two parts, the first one regulated by quinones chemistry and leading to the reduction of O_2 and production of H_2O_2, the second one regulated by Fenton reaction and giving the high reactive radicals. The present results suggest that the antioxidant action of GSH at wine pH is mainly due to its action on quinones chemistry and not on hydrogen peroxide scavenging activity. The small loss of malvidin 3-monoglucoside may be due to the formation, in a strong oxidant medium, of oxidized glutathione GSSG that may acts as oxidant even if this activity has been reported only in living systems and in presence of enzymes (Ceballos-Picot et al., 1996). Recently it has been showed that, at higher GSH/SO_2 ratio, in the presence of oxygen, GSH gives glutathione disulfide (GSSG), and GSSG reacts with SO_3H^- to provide S-sulfonated glutathione ($GSSO_3H$) (Arapitsas et al., 2016). This mechanism was favored in wine stored with a larger amount of oxygen. In our experimental condition it could be assumed that a large amount of GSH was transformed into its sulfonated analogue, thus simultaneously depleting the concentration of the two major wine antioxidants, SO_2 and GSH. Further investigation can help to elucidate the reason of the disappear of malvidin 3-monoglucoside in presence of GSH at lower pH.

The possible action of GSH as oxidant is confirmed by data on syringic acid (Table 1). This molecule derive from breakdown of malvidin-3-O-monoglucoside (Lopes et al., 2007). It has been detected after photodegradation (Maccarone, Ferrigno, Longo & Rapisarda, 1987) and enzymatic and thermal degradation of anthocyanins (Piffaut, Kader, Girardin & Metche, 1994). It origins, together with carboxyaldehyde from the ring opening of 2,4,6-trihydroxychalcone and proton transfer and rearrangements in the acidic aqueous medium. Syringic acid concentrations detected after oxidation of model solutions containing GSH are higher than in Ox solution indicating that, under strong oxidative stress, GSH never acts as oxidant. Moreover, these results confirmed the possibility to use syringic acid as marker of malvidin-3-O-glucoside oxidation. Another chemical antioxidant used in winemaking, the ascorbic acid, showed a similar behavior (Iacobucci & Sweeny, 1983). Hence only proper concentration of sulfur dioxide is effective in prevent Fenton reaction and, at the best of our knowledge, no other molecule can exercise the same effect.

3.2 Red wine experiments

The degradation of malvidin 3-monoglucoside by hydrogen peroxide is lower in wine than in model solution (Tab. 2 and Tab. 3). This result can be easily explained considering that in wine very dangerous radicals produced by Fenton reaction react with a greater number of compounds in solution and not only with native anthocyanins, therefore it is likely that the occurrence of other competitive reactions decreased the malvidin degradation in wine. In contrast with results obtained in model solution, in wine the lower the pH was the higher and faster pigment oxidation. This is in agreement with previous results obtained on wine (Pechamat, Zeng, Jourdes, Ghidossi & Teissedre, 2014) and it is probably due to the occurrence of reactions involving malvidin 3-monoglucoside, the glyoxylic acid produced by tartaric acid oxidation and, other flavanols present in wine and not in model solution (Es-Safi, Cheynier & Moutounet, 2003).

Among native anthocyanins, malvidin-3-acetylglucoside and malvidin-3-monoglucoside are less degraded by hydrogen peroxide than delphinidin-3-monoglucoside and peonidin-3-monoglucoside. This results is due to O-methylation of these molecules that results in a higher stability of anthocyanidin molecule while, the existence of hydroxyl groups makes the molecule more sensitive to oxidation (Mazza & Francis 1995). In agreement with previous studies (Bakker & Timberlake, 1997; Morata, Calderón, González, Gómez-Cordovés & Suárez, 2007), a lower degradation of vitisin B has been even observed as expected because pyranoanthocyanidins are more stable. As expected a positive action of SO_2 against malvidin 3-monoglucoside and all anthocyanins degradation at both pH was detected and it resulted highly correlated with concentration. In contrast with model solution never an oxidant activity of SO_2 was detected. Departure from model solution in the activity of SO_2 in real wine has been also recently observed by Danilewicz (2016). These results are not surprising and indicate that in real wine ethanol, anthocyanins and tartaric acid are not the only target of radicals produced by Fenton but other compounds such as other phenolics, malic acid, glyceraldehyde and volatile compounds compete with them (Elias & Waterhouse, 2010). These results showed that, as suggested by Danilewicz (2007), the oxidant activity of SO_2 is prevented by the radical scavenging action of polyphenols in real wine and its ability to scavenge H_2O_2 is dominant (Table 2 and Table 3). About the GSH, as observed in model solution, a slight oxidative action was observed when it was used alone or in combination with high concentration of SO_2 to contrast hydrogen peroxide action. Thus, also in real wine, GSH is not capable to inhibit Fenton reaction and/or to bind efficiently carbonyls as SO_2

Field Code Changed

while it seems that, in presence of hydrogen peroxide it can instead contribute to oxidation and/or reacts with flavonoids. Considering the protective role played by GSH towards varietal thiols (Nikolantonaky & Waterhouse, 2012) and previous results obtained during micro-oxygenation (Gambuti, Han, Peterson & Waterhouse, 2015) this study furnish serious evidences that the action of this tripeptide on wine may be limited only to its effect on quinones chemistry but not on complementary antioxidant actions of SO_2. Future work must be conducted in real wine to understand the reasons of the slight loss of malvidin 3-monoglucoside observed in presence of an excess of hydrogen peroxide and GSH.

ANOVA analysis of Ox samples showed that all of the variables tested in this study had, in model solution, the ability to significantly affect the degradation of malvidin 3-monoglucoside by hydrogen peroxide while, in red wine, pH showed no significant effect (Table 4). This last result seems in contrast with data reported by Pechamat, Zeng, Jourdes, Ghidossi and Teissedre (2014) who observed a higher formation of pyranomalvidin−procyanidin dimers in oxygenated red wines at lower pH. However, we use very strong oxidative conditions and, the effect of pH on the loss of monomeric anthocyanins during MOx changes with time (Kontoudakis et al., 2011). As expected SO_2 has the most significant effect in preventing malvidin 3-monoglucoside loss (significant at $p < 0.001$). A significant effect of GSH was observed and it resulted in a slight loss of anthocyanin in presence of hydrogen peroxide. The interaction between pH and SO_2 was significant only in model solution while interactions between SO_2 and GSH were significant in real wine leading to a slight minor anthocyanin preservation. These results partially agree with a recent study showing no protective activity of GSH to prevent white wines oxidation after one year of aging in bottles (Panero, Motta, Petrozziello, Guaita & Bosso, 2015).

4. CONCLUSIONS

The protective effect of sulfur dioxide on malvidin 3-monoglucoside degradation was, as expected, higher at lower pH in model solution. The use of GSH alone determined an increase in the degradation of malvidin 3-monoglucoside regardless of pH in model solution and in real wine. Results obtained in this study showed that the possibility to use GSH to prevent anthocyanins oxidation is not linked to its capability to quench hydrogen peroxide but only, in the first steps of oxidation, to act on quinones chemistry and limit the reduction of oxygen to hydrogen peroxide. When in wine is present hydrogen peroxide GSH is not able to scavenge it and contrast Fenton reaction nor alone and not in combination with SO_2 at concentration

usually proposed during winemaking. Taking into account these results, the use of this tripeptide as an alternative to SO_2 has to be revised and the chemistry of action of these compounds in wine conditions better understood.

5. REFERENCES

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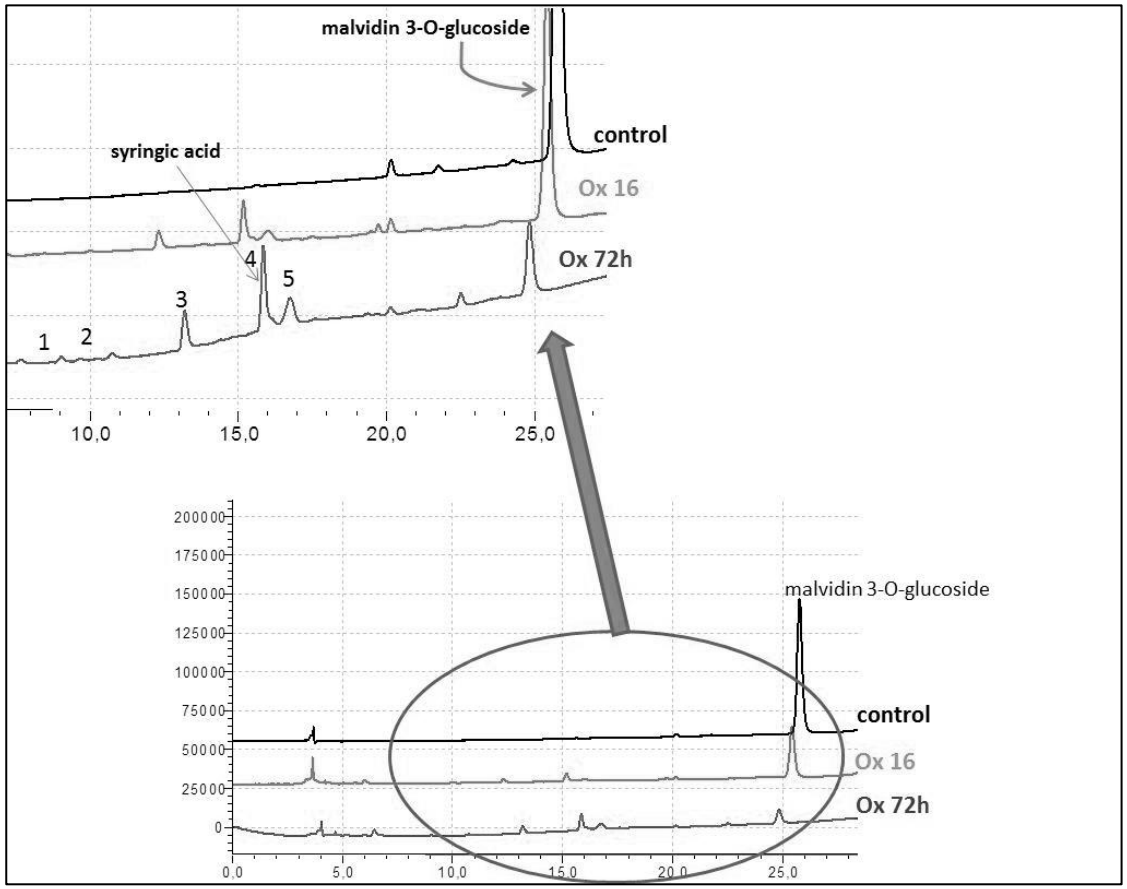
Figure captions

Fig. 1. HPLC chromatogram recorded at 280 nm representing the malvidin 3-O-glucoside degradation over 72 hours of reaction with hydrogen peroxide. Compounds 1−5 correspond to breakdown products.

Fig. 2 Effect of SO_2 and GSH on the degradation of malvidin 3-monoglucoside by hydrogen peroxide in model solution. Ox: sample added with hydrogen peroxide 39.2 mg/L of O2 eq; Ox+SO_2 low: Ox + 37.4 mg/L of SO_2; Ox+SO_2 high: Ox + 202 mg/L of SO_2 (3.16 mM); Ox+GSH: Ox+ 30 mg/L of glutathione (0.098 mM); Ox+SO_2 low+GSH: Ox + 37.4 mg/L of SO_2 and 30 mg/L of glutathione (0.098 mM); Ox+SO_2high+GSH: Ox + 202 mg/L of SO_2 and 30 mg/L of glutathione.



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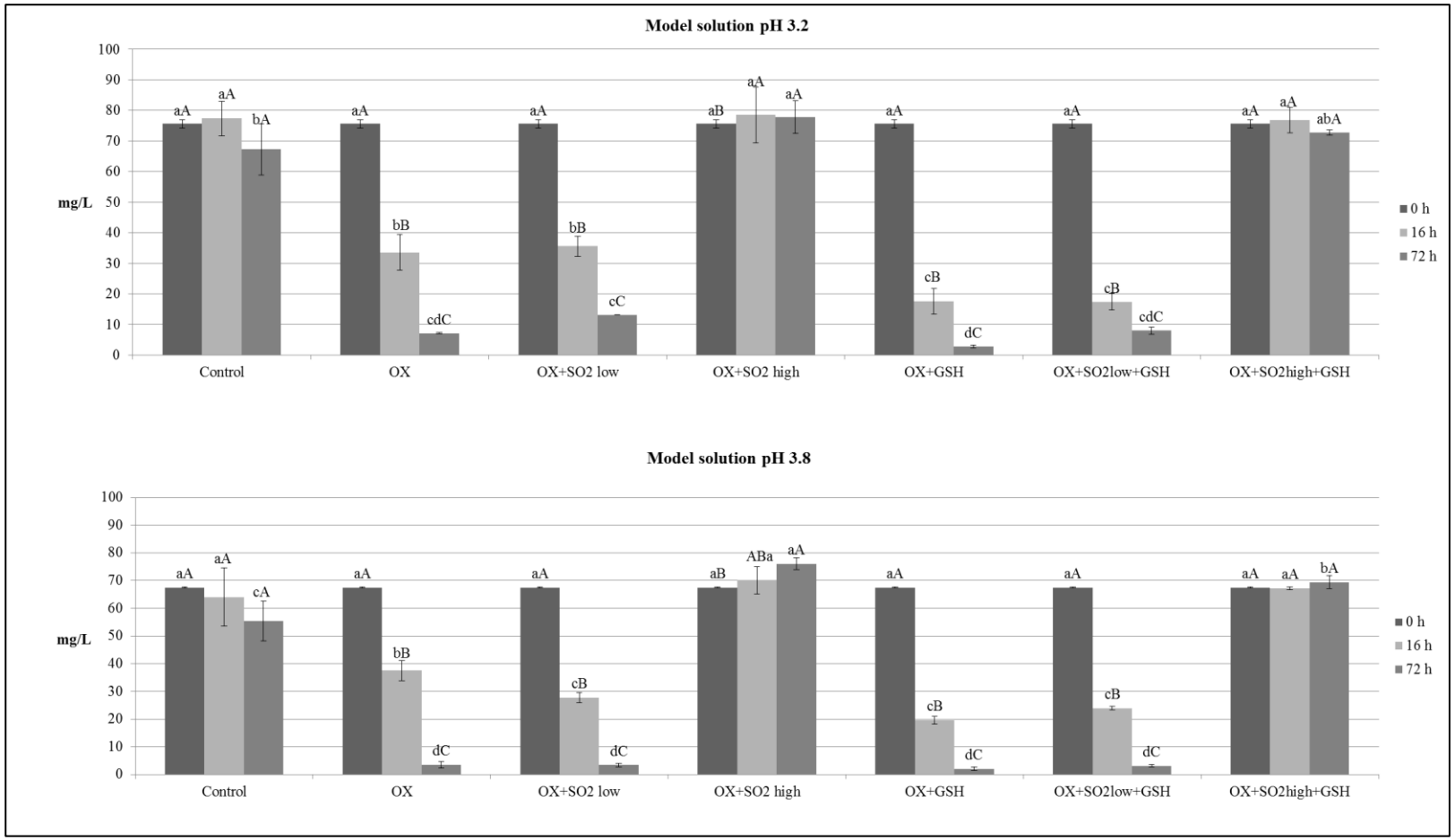

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Table

Table 1. Effect of SO_2 and GSH on the formation of syringic acid (microgram/L), a breakdown compound of malvidin 3-O-glucoside detected at 15.25 min at 280 nm (peack 4) during HPLC run under oxidative conditions over time. Oxidative treatments (a, b, c, ….) and oxidation time (A, B) sharing the same letters are not significantly different.

pH 3.2			
	0 h	16 h	72 h
Control	8 ± 1 C	47 ± 1 eB	107 ± 5 dA
OX		224 ± 14 dB	528 ± 17 aA
OX+SO_2 low		266 ± 16 cB	487 ± 3 cA
OX+SO_2 high		24 ± 0 fA	24 ± 1 eA
OX+GSH		349 ± 11 bB	504 ± 4 bA
OX+SO_2low+GSH		381 ± 8 aB	492 ± 6 bcA
OX+SO_2high+GSH		22 ± 1 fB	30 ± 1 eA
pH 3.8			
Control	9 ± 1 C	43 ± 2 eB	94 ± 1 eA
OX		208 ± 14 dB	461 ± 1 dA
OX+SO_2 low		260 ± 12 cB	526 ± 1 aA
OX+SO_2 high		30 ± 5 efA	27 ± 0 gA
OX+GSH		378 ± 7 aB	519 ± 2 bA
OX+SO_2low+GSH		330 ± 6 dB	503 ± 4 cA
OX+SO_2high+GSH		24 ± 0 fB	36 ± 1 fA

Table 2. Effect of SO_2 and GSH on the degradation of monomeric anthocyanins by hydrogen peroxide in red wine at pH 3.2. Ox: wine added with hydrogen peroxide 39.2 mg/L of O2 eq; Ox+SO_2 low: Ox + 37.4 mg/L of SO_2; Ox+SO_2 high: Ox + 202 mg/L of SO_2 (3.16 mM); Ox+GSH: Ox+ 30 mg/L of glutathione (0.098 mM); Ox+SO_2 low+GSH: Ox + 37.4 mg/L of SO_2 and 30 mg/L of glutathione (0.098 mM); Ox+SO_2high+GSH: Ox + 202 mg/L of SO_2 and 30 mg/L of glutathione.

	Df3glc (mg/L)		Pn3glc (mg/L)		Mv3glc (mg/L)		Vit.B (mg/L)		Mv3acglc (mg/L)		Tot of mon. anth.* (mg/L)	
Control	61.9 ± 3.6	A	39.5 ± 0.3	A	649.5 ± 7.2	A	11.6 ± 0.0	A	139.2 ± 1.8	A	997.1 ± 14.3	A
Control16h	62.7 ± 1.9	aA	38.8 ± 2.9	aA	652.0 ± 14.1	abA	11.7 ± 0.0	aA	136.8 ± 5.2	aA	1000.5 ± 22.2	aA
Control 72h	57.6 ± 0.3	bA	39.2 ± 0.3	abA	640.3 ± 1.4	aA	9.8 ± 2.9	aA	136.4 ± 3.3	aA	977.0 ± 7.8	aA
Ox 16h	28.6 ± 0.1	cA	17.6 ± 0.4	cA	349.6 ± 4.3	dA	9.4 ± 0.0	dB	77.2 ± 2.7	cA	537.4 ± 7.8	cA
Ox 72h	25.5 ± 0.1	eB	16.2 ± 0.1	dB	313.7 ± 5.8	cB	9.5 ± 0.0	aA	69.7 ± 2.4	cB	487.0 ± 8.6	dB
Ox+SO_2 low 16h	37.3 ± 0.1	bA	23.5 ± 0.1	bA	428.8 ± 1.0	aA	9.8 ± 0.0	aB	92.5 ± 0.6	bcA	660.5 ± 0.8	bA
Ox+SO_2 low 72h	34.3 ± 0.3	dB	21.5 ± 0.4	cB	397.5 ± 8.3	cB	9.9 ± 0.0	aA	86.1 ± 3.9	bB	613.2 ± 13.6	aB
Ox+SO_2 high 16h	64.0 ± 3.3	aA	39.0 ± 0.5	aA	665.0 ± 6.9	cA	11.8 ± 0.1	cA	139.5 ± 9.4	aA	1009.0 ± 21.0	aA
Ox+SO_2 high 72h	65.7 ± 0.2	aA	40.6 ± 0.2	aA	660.7 ± 4.4	aA	11.5 ± 0.1	eA	142.2 ± 1.6	aA	1012.6 ± 6.4	cA
Ox+GSH 16h	27.2 ± 0.6	bA	18.8 ± 0.6	cA	354.9 ± 6.5	dA	8.8 ± 0.0	eB	77.7 ± 3.1	cA	546.0 ± 11.0	cA
Ox+GSH 72h	23.5 ± 2.8	eA	15.6 ± 2.6	dB	297.2 ± 37.0	cB	9.6 ± 0.6	aA	64.3 ± 9.1	cB	459.7 ± 55.9	dB
Ox+SO_2low+GSH 16h	35.2 ± 1.0	cA	23.8 ± 0.0	bA	425.0 ± 0.2	cA	9.3 ± 0.1	dB	101.4 ± 13.9	bcA	662.5 ± 12.6	bA
Ox+SO_2low+GSH 72h	34.5 ± 1.4	dA	22.7 ± 1.3	bA	412.0 ± 24.9	bA	9.7 ± 0.0	aA	89.5 ± 4.9	bA	635.8 ± 33.1	cA
Ox+SO_2high+GSH 16h	61.5 ± 1.1	aA	38.3 ± 1.1	aA	636.7 ± 15.1	bA	10.9 ± 0.3	bA	139.5 ± 9.2	aA	979.4 ± 24.0	aA
Ox+SO_2high+GSH 72h	42.1 ± 0.3	cB	37.3 ± 0.4	cA	621.6 ± 2.1	aA	11.1 ± 0.4	aA	144.5 ± 0.8	aA	943.9 ± 1.4	aA

Dp3glc = delphinidin 3-glucoside, Pn3glc = peonidin 3-monoglucoside, Mv3glc = malvidin 3-glucoside, Vit.B=vitisin B, Mv3acglc = malvidin 3-(6^{II}-acetyl)-glucoside, *Sum of monomeric anthocyanins. Oxidative treatments (a, b, c, ….) and oxidation time (A, B) sharing the same letters are not significantly different.

Table 3. Effect of SO_2 and GSH on the degradation of monomeric anthocyanins by hydrogen peroxide in red wine at pH 3.8. Ox: wine added with hydrogen peroxide 39.2 mg/L of O2 eq; Ox+SO_2 low: Ox + 37.4 mg/L of SO_2; Ox+SO_2 high: Ox + 202 mg/L of SO_2 (3.16 mM); Ox+GSH: Ox+ 30 mg/L of glutathione (0.098 mM); Ox+SO_2 low+GSH: Ox + 37.4 mg/L of SO_2 and 30 mg/L of glutathione (0.098 mM); Ox+SO_2high+GSH: Ox + 202 mg/L of SO_2 and 30 mg/L of GSH.

	Dp3glc (mg/L)		Pn3glc (mg/L)		Mv3glc (mg/L)		Vit.B (mg/L)		Mv3acglc (mg/L)		Tot of mon. anth.* (mg/L)	
Control	56.1 ± 1.7	AB	38.4 ± 0.8	A	627.7 ± 22.0	A	12.0 ± 0.5	A	136.5 ± 10.2	A	962.4 ± 37.5	A
Control16h	57.6 ± 0.5	bA	36.0 ± 0.3	bB	629.0 ± 7.6	bA	12.1 ± 0.1	aA	137.6 ± 18.9	aA	967.7 ± 28.6	aA
Control 72h	53.9 ± 0.1	cB	35.7 ± 0.8	bB	609.3 ± 0.4	bA	11.9 ± 0.1	aA	131.1 ± 1.2	bA	940.7 ± 0.5	bA
Ox 16h	33.3 ± 0.5	eB	18.2 ± 0.4	dA	385.3 ± 6.9	eB	9.1 ± 0.1	eA	85.0 ± 4.4	bcA	596.5 ± 14.9	cA
Ox 72h	29.2 ± 1.4	eC	17.0 ± 1.6	eA	344.9 ± 3.0	eC	8.8 ± 0.0	eA	75.2 ± 0.1	dB	533.5 ± 6.5	dB
Ox+SO_2 low 16h	39.3 ± 0.6	cA	23.0 ± 0.8	cA	443.1 ± 9.1	cA	9.8 ± 0.0	dA	96.0 ± 4.1	bA	685.1 ± 14.2	bA
Ox+SO_2 low 72h	36.1 ± 1.6	dA	22.3 ± 0.0	cA	407.2 ± 0.7	dB	9.5 ± 0.0	dB	86.3 ± 0.1	cB	630.1 ± 2.6	cB
Ox+SO_2 high 16h	61.9 ± 0.1	aA	39.6 ± 0.7	aA	651.8 ± 3.8	aA	11.8 ± 0.1	bA	139.9 ± 2.5	aA	992.1 ± 7.1	aA
Ox+SO_2 high 72h	63.7 ± 0.2	aA	39.0 ± 0.1	aB	636.3 ± 4.2	aA	11.3 ± 0.1	bB	137.6 ± 1.7	aA	975.8 ± 6.7	aA
Ox+GSH 16h	29.7 ± 0.5	fA	17.8 ± 1.8	dA	358.2 ± 6.6	cA	8.4 ± 0.1	gA	77.4 ± 1.6	cA	551.6 ± 10.4	dA
Ox+GSH 72h	26.4 ± 1.4	fB	16.7 ± 0.4	eA	323.9 ± 7.4	eB	8.4 ± 0.0	gA	70.9 ± 2.7	eB	500.6 ± 12.7	eB
Ox+SO_2low+GSH 16h	34.5 ± 0.9	dA	19.8 ± 0.3	cA	401.2 ± 5.2	cA	9.2 ± 0.0	dA	87.0 ± 0.4	bcA	616.9 ± 5.5	bA
Ox+SO_2low+GSH 72h	36.2 ± 0.7	dA	23.2 ± 1.1	dB	434.2 ± 5.3	cB	9.9 ± 0.0	dB	94.7 ± 1.4	cB	670.2 ± 5.6	cB
Ox+SO_2high+GSH 16h	60.8 ± 0.1	aA	38.5 ± 0.9	aA	633.0 ± 9.8	bA	11.3 ± 0.2	cA	136.9 ± 4.3	aA	968.2 ± 16.0	aA
Ox+SO_2high+GSH 72h	60.1 ± 0.8	bA	37.4 ± 0.9	abA	607.9 ± 14.2	bA	10.3 ± 0.3	cB	129.8 ± 1.7	bA	932.2 ± 14.6	bA

Dp3glc = delphinidin 3-glucoside, Pn3glc = peonidin 3-monoglucoside, Mv3glc = malvidin 3-glucoside, Vit.B=vitisin B, Mv3acglc = malvidin 3-(6^{II}-acetyl)-glucoside, *Sum of monomeric anthocyanins. Oxidative treatments (a, b, c, ….) and oxidation time (A, B) sharing the same letters are not significantly different.

Table 4. F values and significance of variables SO_2, GSH and pH for malvidin 3-monoglucoside degradation by hydrogen peroxide.

	Model solution		**Red wine**	
	F	Pr > F	F	Pr > F
pH	24.24	0.000	2.887	0.124
SO_2	4558.60	< 0.0001	1548.140	< 0.0001
GSH	18.46	0.000	6.563	0.031
pH x SO_2	5.75	0.028	2.558	0.144
pH x GSH	0.73	0.405	0.178	0.683
SO_2 x GSH	2.44	0.137	10.828	0.009

*Graphical Abstract

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