American Journal of Enology and Viticulture (AJEV). doi: 10.5344/ajev.2015.15005
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Research Article

Sulfur Dioxide and Glutathione Alter the Outcome of Microoxygenation

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Acknowledgments: The authors thank POR CAMPANIA FSE 2007/2013. Sviluppo di Reti di Eccellenza tra Università – Centri di Ricerca – Imprese. PROGETTO CARINA "Sicurezza, sostenibilità e competitività nelle produzioni agroalimentari della Campania" CUP B25B09000080007 for the award of a grant. The authors also thank Michael R. Webb for helpful assistance with the MOx system preparation.

Manuscript submitted Dec 2014, revised May 2015, accepted Jun 2015

Abstract: Cabernet Sauvignon wines with low and high levels of SO_2 and glutathione (GSH) were treated with micro-oxygenation (MOx) in a 23 L pilot experiment. Treatment generally increased O_2, aldehydes and derived products while decreasing anthocyanins, vanillin reactive flavonoids and SO_2. During the treatment, when free SO_2 was depleted in the low GSH wine, dissolved oxygen levels collapsed, with a concomitant increase of acetaldehyde, pyranoanthocyanins, polymeric pigments and acetaldehyde acetals. This outcome indicates a possible acceleration of the Fenton oxidation of ethanol and other oxidation reactions via direct free radical reactions with oxygen. In wines containing high levels of GSH, anthocyanins were protected, revealing a protective effect for GSH for the first time. However the protection was only partial, so, while GSH may be very effective in preventing the loss of volatile thiols, its use does not prevent color stabilization in red wines. Since both SO_2 and GSH are able to modulate the reactions initiated by MOx, but have somewhat dissimilar reactions, it may be possible to manage

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oxidation outcomes by choosing one or the other during wine processing and aging. As SO_2 and O_2 levels are related to large differences in MOx reaction rates, those levels are candidate indicators of the rate of MOx oxidation. On the other hand, the levels of acetaldehyde acetals may be useful indicators of the cumulative extent of oxidation under MOx conditions.

Key words: micro-oxygenation, dissolved oxygen, glutathione, sulfur dioxide, red wine

Introduction

Mild oxygenation processes can impart benefits to a broad range of red wines by contributing to softening of tannin harshness, stabilizing color and decreasing vegetative aromas (Atanasova et al. 2002, Cano-Lopez et al. 2008, Cejudo-Bastante et al. 2011a, Cejudo-Bastante et al. 2011b). Because micro-oxygenation (MOx) can promote these effects in a fairly short time frame, allowing a rapid improvement of red wine quality, this practice has become widely used in winemaking (Gomez-Plaza and Cano-Lopez 2011, Schmidtke et al. 2011). Nevertheless there are several serious issues associated with the application of MOx. Excessive oxygen can result in the appearance of oxidation-spoiled aromas, loss of color, precipitation of colorant matter and development of off-flavours (Cano-Lopez et al. 2008, McCord, 2003). Although numerous studies have been performed on evaluating the effect of MOx on wine composition and sensory characteristics (Arapitsas et al. 2012, Cejudo-Bastante et al. 2011a, Cejudo-Bastante et al. 2011b, Gambuti et al. 2013) measuring the progress of MOx is still difficult due to the lack key chemical indicators of the process.

The general chemistry of wine oxidation is widely accepted. First of all molecular oxygen (triplet state) cannot directly react with wine organic compounds (singlet state) but, in wine, it can accept electrons by interacting with transition metals, ions iron and cupper, in presence of catechol

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(Danilewicz 2011). The catechol is oxidized to a quinone, and oxygen is reduced to hydrogen peroxide. In a subsequent step, ferrous or cuprous species present react with hydrogen peroxide in the Fenton reaction to give the most reactive oxidant, the hydroxyl radical. This reacts with all organic constituents in rough proportion to concentration (Elias et al. 2009), and with a 10-fold excess of ethanol compared to all other compounds acetaldehyde is the major product of oxidation by far.

Acetaldehyde is a reactive molecule and reactions involving acetaldehyde are among the most significant for the evolution of wine phenolics during aging. It reacts with anthocyanins and flavanols to form ethyl-linked oligomers (Es-Safi et al. 1999, Fulcrand et al. 1996, He et al. 2012). The latter compounds can, in turn, react with additional acetaldehyde, anthocyanins, and flavanols to generate polymeric-type structures that can create significant changes in wine sensory attributes (Atanasova et al. 2002, Fulcrand et al. 2004, He et al. 2012). In addition to acetaldehyde, other oxidation products such as pyruvic acid can react with anthocyanins to form stable colored structures named pyranoanthocyanins (Fulcrand et al. 2004). Besides polyphenols, these substances react with other components of wine. One example is the condensation reaction between acetaldehyde and glycerol leading to the formation the dioxolane and dioxane acetals (da Silva Ferreira et al. 2002).

Preservative compounds used in wine production can exert their action by interfering at different steps of wine oxidation, and thereby inhibiting the formation of oxidation products. Sulfur dioxide prevents oxidative spoilage by scavenging hydrogen peroxide (Danilewicz 2011, Danilewicz and Wallbridge 2010) and by reacting with quinones, reducing them back to the catechol form (Saucier and Waterhouse 1999) or yielding sulfonates as addition products

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(Nikolantonaki and Waterhouse2012). Also the sulfur-containing tripeptide glutathione (GSH) found in grapes, has been suggested to provide a protective effect by acting as sacrificial nucleophiles for quinones (Nikolantonaki and Waterhouse 2012). In addition, GSH reacts with H_2O_2 (Finley et al. 1981), so it may also slow the Fenton reaction. A recent review summarizes the impact of GSH in winemaking (Kritzinger et al. 2013), showing specific antioxidant effects in preserving thiols and preventing oxidation, and noting that some inactive yeast preparations usable in winemaking are high in GSH. In the US, GSH is not yet listed on materials available for winemaking (Code of Federal Regulations 2014).

Although some reports suggest that exogenous antioxidants could alter the effect of MOx treatment, only one study directly investigated the role of SO_2 (Tao et al. 2007), and a marked effect of this preservative on the evolution of polyphenols was observed. No data are reported in the literature that investigates the effect of antioxidants on the impact of MOx with regard to the development of the other processes involved in red wine oxidation such as oxygen consumption and acetaldehyde production.

The aim of this study was to evaluate the influence of SO_2 and GSH on the outcome of micro-oxygenation treatments, using a model system in which wines underwent MOx in small tanks (23 L). Two treatment levels, i.e. wines with low and high levels of SO_2 and GSH were compared during the micro-oxygenation of a Cabernet Sauvignon wine. Numerous components were analyzed, including dissolved oxygen, free SO_2, acetaldehyde, color, anthocyanins and derived pigments, tannin, and acetaldehyde acetals were evaluated in order to assess, as broadly as possible, changes to the oxidation treatment.

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Materials and Methods

Wine. The red wine was produced during the 2013 vintage with 91% Cabernet Sauvignon and 9% Petite Sirah. The wine was produced by E.& J. Gallo Winery (Sonoma California). After the end of malolactic fermentation the wine was centrifuged and microfiltered (0.45 micron) to remove residual yeasts before it was used for the experiment to avoid any microbial involvement in the process. The base parameters (mean ± standard deviation) of wine at the start of the experiment were: ethanol content 13.43 ± 0.09 % v/V, pH 3.76 ± 0.03, titratable acidity 5.94 ± 0.08 g/L expressed as tartaric acid, residual sugars 0.40 ± 0.01 g/L, malic acid 0.087 ± 0.008 g/L, acetic acid 0.31 ± 0.01 g/L, free SO_2 16.5 ± 0.7 mg/L, total SO_2 38.5 ± 0.7 mg/L. The pH was measured using an Orion 5 Star (Thermo Scientific, Boston, MA). Ethanol was analyzed using an alcolyzer (Anton-Paar, Ashland, VA). Titratable acidity was determined by titration with a sodium hydroxide solution to pH 7.0 (Iland et al., 2004). A photometric measurement based on the formation or consumption of NAD or NADH was used to determine malic and acetic acids. For these analyses a photometric analyzer Thermo Scientific Gallery (manufactured by Thermo Fisher Scientific Oy, Finland) was used. Free and total SO_2 were determined using the aspiration method (Iland et al. 2004).

Experimental design. The effect of MOx, SO_2 and GSH addition on the acetaldehyde and the main classes of wine phenolics was studied in a 2×2×2 factorial design. Eight experimental wines were prepared in duplicate. Before treatments the wines received an addition of potassium metabisulfite to result in a final concentration of free SO_2 of 25 mg/L (Low addition) or of 65 mg/L (High addition). The same wines received/or no addition of 20 mg/L of GSH (ACROS Organics, New Jersey USA). This protocol gave a total of four matrix-related combinations, coded as follows: LL (Low SO_2 25 mg/L, Low GSH as no addition); LH (Low SO_2 25 mg/L, High GSH

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added 20 mg/L); HL (High SO_2 65 mg/L, Low GSH as no addition) and HH (High SO_2 65 mg/L, High GSH added 20 mg/L). Each wine was divided in two, one half underwent the micro-oxygenation treatment, the other was left for 30 days in a tank in the same condition of temperature as the micro-oxygenated wines and was considered a control. In each case, the treatments were conducted in duplicate, i.e. there were two tanks holding LL wine with MOx treatment. Wines that underwent the micro-oxygenation treatment were coded as: MLL (Micro-oxygenated, Low SO_2 25 mg/L, Low GSH as no addition); MLH (Micro-oxygenated, Low SO_2 25 mg/L, High GSH 20 mg/L); MHL (Micro-oxygenated, High SO_2 65 mg/L, Low GSH as no addition) and MHH (Micro-oxygenated, High SO_2 65 mg/L, High GSH 20 mg/L). Overall sixteen 23 L tanks were used.

MOx system. All tanks used in MOx experiment were made of stainless steel and included lid fittings and three entry points for a) the 46 cm loop of tubing used as oxygen delivery system, b) the sampling device and c) the dissolved oxygen detector (Fig. 1) The three entry points and an additional exit for the oxygen delivery tubing were all located on the tank lid which was purpose-built starting from a conventional tank lid in the lab. Conventional lids were used to hermetically close the control tanks that were not exposed to oxygen treatment. Both tanks and lids were pre-sterilized.

Oxygen delivery system. Oxygen gas (Ox R Industrial Oxygen, Airgas, Woodland, CA) was delivered into the wine tanks under pressure using a fluorinated ethylene propylene tubing FEP-188x250 (Ozone Solutions, Inc. 451 Black Forest Rd. Hull, IA 51239 USA). The oxygen was delivered into the 23 L stainless tanks through a sterile ingress on the lid of the tank. Oxygen delivery was measured using a hydroalcoholic solution, assessing the increased oxygen concentration over 2 weeks.

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Wine sampling system. The sampling system was designed to provide an aseptic sample avoiding both the loss of wine volatile compounds and introducing air or contaminants (e.g. micro-organisms). Samples were collected through a septum (Agilent Technologies, Inc LSCA New Castle DE, USA) by means of sterilized syringes. During MOx treatment the wines were sampled at 2, 5, 10, 15, 20, 25 and 30 days after MOx commenced for acetaldehyde, SO_2 and phenolics analyses. The eight control wines were sampled only after 30 days of storage in tanks under the same temperature used for MOx treated wines.

Dissolved oxygen analyses. To measure dissolved oxygen in the wine while keeping the tank closed at all times, a system including a NMR tube (Vineland, NJ USA) fixed on the internal face of the lid, with the bottom end immersed in the wine. A PreSens Pst3 oxygen sensor for measurement of trace oxygen was placed on the external part of the flat bottom of the NMR tube in contact with wine. DO at bottling was measured by means of oxo-luminenscence, using a Nomasense oxygen analyzer (Nomacorc SA, Thimister Clermont, Belgium). The Nomasense fiberoptic trace oxygen meter (FIBOX 3LCD trace V7) was inserted into the NMR tube, thus avoiding opening the tank. Measurements were taken one hour after filling the tanks and every day during MOx treatments. Recently it has been shown that DO measurements were affected by several wine components (del Alamo-Sanza et al. 2014). However, in this study we performed a comparative trial the use of compensation values is not necessary.

MOx trial. Each tank was purged with nitrogen immediately prior to filling. A layer of nitrogen was maintained over the wine as it was transferred into the tanks. Before sealing, the headspace volume was displaced with nitrogen. To ensure consistency, all the tanks were kept in a thermostated room at 19.5±0.1 °C. Since MOx treatment can induce significant DO gradients - (Nevares et al. 2010), each tank was continuously stirred using magnetic stirrers. An insulating

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plate was placed between the stirrers and the tanks to eliminate any heat transfer to the fluid. Oxygen was introduced at a rate of 15 mL/L/month by setting the oxygen pressure at 430 kPa. This value was chosen based on the results of previous replicate trials in which the oxygen was supplied to a hydroalcoholic solution in a sealed 23 L tank. .

Sulfur Dioxide measurements. The concentration of free and total sulfur dioxide was determined using the aspiration method (Iland et al. 2004).

Spectrophotometric Analyses. Chromatic characteristics and spectrophotometric measures were determined using an Agilent 8453 UV−vis spectrophotometer (Agilent Technologies, Palo Alto, CA). Colorant intensity, Abs420, Abs520, Abs620 and hue were evaluated according to the Glories methods (Glories, 1984) Tannins, total anthocyanins and large polymeric pigments (LPP) were determined by the Harbertson–Adams assay (Harbertson et al. 2003). Briefly, pH changes allowed the evaluation of total anthocyanins while the large polymeric pigments (LPP) were obtained combining analysis of supernatant obtained after protein precipitation using bovine serum albumin BSA (Spectrum Chemical, Gardena, CA, USA) with the bisufite bleaching of pigments in wine. For Vanillin Reactive Flavans (VRF) the method described by Di Stefano and Guidoni (Di Stefano and Guidoni 1989) was scaled down and volumes were adjusted to decrease the consumption of organic solvents. The modified method required two 1.5 mL microfuge tubes for each sample. One hundred μL of wine were previously diluted 1 to 10 with methanol. The first tube was made up by dispensing 125 μL of diluted wine and then adding 750 μL of a solution of vanillin (4% in methanol). After 5 minutes the tube was placed in cold water (4°C) and 375 μL of concentrated hydrochloric acid was added. After a 15 minute incubation of the mixture at room temperature (20°C) the absorbance was determined at 500 nm. For the second tube the procedure was the same apart the fact that 750 μL of pure methanol was used

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instead of the solution of vanillin. The 500 nm absorbance of this tube is considered the blank. Concentrations were calculated as (+)-catechin (mg/L) by means of a calibration curve. Vanillin was purchased from Alfa Aesar (Ward Hill, MA, USA).

High-Performance Liquid Chromatography (HPLC) analyses of acetaldehyde. Acetaldehyde was analyzed using a newly modified method (Han et al. 2015) Briefly, wine sample aliquots (100µL) were dispensed to a vial, followed by 20 µL of freshly prepared 1120 ppm sulfur dioxide solution, then 20 µL of 25% sulfuric acid was added followed by 140 µL of 8g /L DNPH reagent. After mixing, the solution was allowed to react for 15 min at 65 ℃ and then promptly cooled to room temperature. Analysis of carbonyl hydrazones was conducted by HPLC (HP 1100 series, Agilent Technologies, Wilmington, DE) coupled to a tandem mass detector (HP 1100MSD series, Agilent Technologies) equipped with an ESI interface for identification and UV detection for quantification, monitoring at 365 nm. A ZORBAX Rapid Resolution HT, SB-C18 column (1.8µm, 4.6×100mm, Agilent Technologies) was used for separation. The chromatographic conditions were: sample injection volume, 15 µL; flow rate, 0.75 mL/min; column temperature, 35°C; mobile-phase solvents, (A) 0.5% formic acid in water and (B) acetonitrile; gradient elution protocol, 35% B to 60% B (t=8 min), 60% B to 90% B (t=13 min), 90% B to 95% B (t=15 min, 2-min hold), 95% B to 35% B (t=17 min, 4-min hold), total run time, 21 min. Eluted peaks were compared with derivatized carbonyl standards. Data analysis and peak integration was carried out using the Agilent Chemstation (A 09.03) software package.

Cyclic acetals analysis. Heterocyclic acetal isomers from glycerol and acetaldehyde were analyzed using a newly optimized method (Peterson et al. 2015). Wine sample aliquots (2 mL) were dispensed into vials (19 mm x 65 mm) and spiked with synthesized deuterium-labelled acetal isomers (25 µL of 800 mg/L in water) as internal standards. Acetals were extracted twice with 1

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mL of ethyl acetate by placing capped vials on a 14-19 mm foam tube holder attached to a vortex mixer. Samples were shaken for 10 minutes and the organic layers were combined and dried over sodium sulfate (0.5-1 g). Dried extracts were analyzed by GC-MS using an Agilent model 6890N gas chromatograph coupled to a 5973 mass-spectrometric detection system. Isomers were separated on a DB-WAX capillary column (30 m x 0.25 mm i.d., film thickness 0.5 µm). One microliter of the sample extract was injected at 240ºC in splitless mode with a purge flow of 40 mL/min to the split vent at 2.0 min. The oven temperature program was as a follows: 1 min at 40 ºC, 10 ºC/min up to 80 ºC, 2 ºC/min up to 150 ºC, 10 ºC/min to 250 ºC, and finally held at this temperature for 5 min. The carrier gas was helium with a constant flow at 0.8 mL/min. Mass spectrometric conditions included electron impact with 70 eV, an ion source temperature of 150 ºC, and an emission current of 200 µA. The MSD was operated in full scan (*m/z* 50-150) for ion selection. The molecular (*m/z* 117), quantitative (*m/z* 103), and qualitative (*m/z* 87/88) ions for the four glycerol acetal isomers were measured in selective ion monitoring mode (SIM). Quantitation of the deuterium-labelled acetal isomers was performed by measuring the *m/z* 91 and 92 ions in SIM.

High-Performance Liquid Chromatography (HPLC) Analyses of monomeric and polymeric phenolics. HPLC separation and quantification of monomeric and polymeric phenolics was carried out according to the Waterhouse et al. method (Waterhouse et al. 1999) as successively modified (Peng et al. 2002). Analyses were performed using a Hewlett-Packard (Agilent Technologies, Palo Alto, CA, USA) 1100 series high-performance liquid chromatograph equipped with a diode array detector coupled to Chemstation software 10.02 (Hewlett Packard, Waldbronn, Germany). An Agilent PLRP-S 100- Å reversed phase polystyrenedivinyl benzene column (4.6 x 150 mm, 3 µm particle size) protected with a guard cartridge with the same packing material

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(PLRP-S, 5 x 3 mm) kept at 35 °C was used as the stationary phase. The HPLC solvents were: solvent A: 1.5% v/v ortho-phosphoric acid (EMP Chemicals, Gibbstown, NJ, USA) and solvent B consisting of 80% acetonitrile (HPLC grade, Honeywell, Muskegon, MI, USA) with 20% of solvent A. The following gradient was established: 0 time conditions, B 6%; 73 min, B 31%; 78 min, B 62%, staying constant until 86 min; 90 min, B 6%. This zero-time solvent mixture was followed by 15-min equilibrium period prior to injecting the next sample. The flow rate of the mobile phase was 1 mL/min. Twenty µL of wine or calibration standards were injected onto the column. All the samples were filtered through 0.20 µm MicroLiter PTFE membrane filters (Wheaton, NJ 08332 USA) into dark glass vials and immediately injected into the HPLC system. Detection was carried out by monitoring the absorbance signals at 520 nm for free native anthocyanins, pyranoanthocyanins and polymeric anthocyanins. 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. The anthocyanins and pigments concentrations were expressed as mg/L of malvidin-3-monoglucoside. Polymeric tannins were quantified at 280 nm as mg/L of (+)-catechin (Sigma, St. Louis, MO, USA). Two calibration curves were obtained by injecting 7 solutions (in triplicate) containing increasing concentrations of malvidin-3-monoglucoside and catechin. Calibration curves were characterized by a determination coefficient (R^2) > 0.999. The analyses of wine samples were carried out in duplicate. The identification of monomeric and polymeric phenolic was made by comparison with retention time and chromatographic profile reported by Peng et al. (Peng et al. 2002) and by comparison of pyranoanthocyanins UV-vis spectra reported by Blanco-Vega et al. (Blanco-Vega et al. 2011). The content of monomeric and polymeric phenolics of red wine before MOx treatment is reported in Table 1.

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Statistical Analyses. Quantitative data relative to the phenolic compounds of the treated wines were compared using Fisher's least significant differences (LSDs) procedure. When the variances were not homogeneous, data were analyzed using Kruskall–Wallis test. When results of the "Kruskal–Wallis" test were significant ($P<0.05$), the significance of between-group differences was determined by the "Bonferroni–Dunn" test (5% significance level). 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).

Results and Discussion

MOx trial. A comparison among the dissolved oxygen (DO) concentration of wines treated with MOx under the different experimental treatments is shown in Figure 2.

Although the concentration of DO were strongly influenced by the content and kind of antioxidant compounds used, a general trend for all wines can be observed. In the first 3 days of treatment, wines with a high content of GSH and/or SO_2 showed no increase or a slight decrease in DO, suggesting that, at the beginning of MOx treatment, these preservatives result in an oxygen consumption rate comparable to the oxygen dissolution. Starting at day 4 a slight increase of DO was detected for all tanks. Between 15 and 21 days DO reached the maximum value for MLL, MLH and MHL followed by a decrease, but one that was very rapid for MLL.

A decrease of free SO_2 by aeration oxidation was detected during the treatment and, after about 20 days, concentrations fell below the limit of detection for wines MLL and MLH, which both had an initial free SO_2 content of 25 mg/L (Figure 3). A similar trend for free SO_2 has been

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277 reported during the introduction of similar quantities of O_2 in three different MOx experiments
278 carried out on Merlot (Tao et al. 2007) and Cabernet Sauvignon (Fell et al. 2007) wines. As
279 expected, both free SO_2 and bound SO_2 reacted during MOx due to the partial release of the bound
280 form (Table 2). Considering that the oxygen supplied during MOx is equivalent to 20 mg/L, and
281 that it would remove about 80 mg/L of total SO_2 if the SO_2 reacted both with quinones and H_2O_2,
282 the amounts of consumed total SO_2 detected in this study confirms previous evidence that at least
283 2 mole equivalents of SO_2 were consumed during wine oxidation (Danilewicz 2011, Danilewicz
284 and Wallbridge 2010).

285 Here, the loss of free SO_2 led to a very rapid decrease of DO. Thus, initially, for the first
286 15 days, higher quantities of GSH are associated with lower levels of DO, but when the free SO_2
287 is exhausted, oxygen consumption is greatly stimulated. The chemistry of non-enzymatic wine
288 oxidation may explain these results (Figure 4). In a simplified reaction mechanism the oxidation
289 pathway can be summarized in two main phases. In a first step wine hydroquinones undergo iron
290 or copper catalyzed oxidation reactions, reducing oxygen to hydrogen peroxide and producing
291 quinones. In the second step iron (II) species react with hydrogen peroxide to form hydroxyl
292 radicals in the Fenton reaction giving a great number of oxidation reactions with wine components.
293 Therefore the first step of wine oxidation can be considered governed by the chemistry of quinones
294 while the second one by the presence of hydrogen peroxide. SO_2 and GSH may affect both quinone
295 and hydrogen peroxide reactivity but in an opposite manner. For quinones, the rate of reaction of
296 oxygen is markedly accelerated by SO_2 due to fact that quinone is reduced back to flavanol
297 (Danilewicz et al. 2008, Danilewicz and Wallbridge 2010). In addition, both preservatives used,
298 SO_2 and GSH, react with quinones (Nikolantonaki and Waterhouse 2012) shifting the oxygen
299 consumption equilibrium towards the products, as resulting in an increase in oxygen consumption

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300 (Danilewicz and Wallbridge 2010), explaining the lower levels of oxygen in the high SO_2
301 solutions.

302 Looking at the Fenton reaction, sulfite is known to quickly react with hydrogen peroxide,
303 thus competing with Fe(II), and reducing the formation of the hydroxyl radical. Previous studies
304 conducted by using spin trapping techniques showed that sulfur dioxide inhibits the formation of
305 free radical species due to Fenton reaction in a concentration dependent manner (Elias et al. 2009,
306 Elias and Waterhouse 2010), supporting this theory. It appears that GSH may be able to slow wine
307 oxidation reactions with hydrogen peroxide in a similar manner. This hypothesis is supported by
308 numerous past studies (Armstrong and Brechanan 1978, Winterbourn and Metodiewa 1999) in
309 which looked at the loss in the nutritional quality of foods during oxidations. Reaction of GSH
310 with hydrogen peroxide was used as a model to study the oxidation of cysteine in proteins
311 (Armstrong and Buchanan 1978). It is known that the rate of reaction for both preservatives with
312 hydrogen peroxide is characterized by a strong pH dependence. In the pH range 2-6, lower pH
313 values increase the rate of reaction for SO_2 (Drexler et al. 1991) while the oxidation rate of
314 glutathione by hydrogen peroxide increases with increasing pH (Finley et al. 1981). XXHowever,
315 taking into account the low amount of GSH used in this experiment and the high O_2: GSH molar
316 reaction ratio it seems that the more relevant effect produced by GSH is due to other reaction than
317 to its reaction with H_2O_2 likely due to its capability of reacting rapidly with quinones
318 (Nikolantonaki and Waterhouse 2012) and other organic compounds such as catechin,
319 hydroxycinnamic acidsand carbonyls involved in wine oxidation (Bouzanquet et al. 2012, Sonni
320 et al. 2011b).

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321 According to proposed chemical mechanisms involved, the behaviors detected during the
322 MOx may be the results of several phenomena. During the first 3 days of treatment the lower
323 values of DO detected for MLH and MHH suggest that reactions involving quinones are dominant
324 in determining oxygen consumption and so GSH accelerates consumption by rapidly reacting with
325 quinones, probably acting as "oxidation promoting nucleophiles" -(Danilewicz 2011). When both
326 preservatives are no longer available, rapid consumption of oxygen occurs due to the production
327 of radicals by the Fenton reaction, and it is these radicals that react most rapidly with oxygen.

328 In the MLL wines, after few days during which the oxygen was almost all consumed, the
329 DO started to increase. A similar trend for DO has been reported by Laurie et al. (Laurie et al.
330 2008) in an experiment conducted on red wine after malolactic fermentation (post-MLF)
331 completion. These results seem to indicate that each wine has a maximum capacity of oxygen
332 uptake, and before reaching this limit, the quantity of oxygen consumed is limited only by the
333 amount introduced. When oxygen-consuming reactants begin to be depleted, the oxidation
334 reactions slow and oxygen starts to accumulate.

335 As observed in prior Mox studies an increase in acetaldehyde has been detected in the later
336 stages of micro-oxygenation, as observed here for wines MLL, MLH and MHL (Carlton et al.
337 2007, Fell et al. 2007). The acetaldehyde levels started to increase after 20 days of treatment and
338 followed the order MLL>>MLH>MHL. The fact that this dramatic evolution started
339 contemporaneously with the rapid decrease in DO and when the content of SO_2 is close to zero, is
340 an important clue to understanding the operative chemical reactions. This also reveals key
341 indications of the role played by SO_2 in regulating the production of acetaldehyde in Mox
342 treatments, by preventing the Fenton reaction (Figures 2 and 3). Here our results agree with data

reported in a previous experiment showing that the production of 1-hydroxyethyl radicals in aerated wine model solution by Fenton reaction could be completely stopped when SO_2 was present at 1000 μM while lower concentration (500 μM SO_2) inhibited the production of radicals by 87.2% compared to the control (Elias and Waterhouse 2010). Experiments carried out on beer (Andersen et al. 2000) and wine (Elias et al. 2009) also showed a suppression of 1-hydroxyethyl radicals by sulfur dioxide. The evidence that in the MHL treatment acetaldehyde co-exists with free SO_2 despite strong binding, is in agreement with previous findings by Shmidtke et al. 2011. However this is the first observation that GSH can inhibit acetaldehyde production in wine oxidation during MOx. It is known that GSH binds acetaldehyde (Sonni et al. 2001) and that oxidation of GSH yields several oxidation products when it reacts with hydrogen peroxide in aqueous solution at pH values close to that of wine (Finley et al. 1981). So, it seems possible that this molecule exerts a protective function in wine by sevral possible mechanisms: i) scavenging hydrogen peroxide, ii) by direct reaction with wine phenolics (Bouzanquet et al. 2012; Sonni et al. 2011a, Sonni et al. 2011b) via quinones (Nikolantonaki and Waterhouse 2012) and iii), binding acetaldehyde to a less extent with respect to SO_2. (Sonni et al. 2011b). A synergic effect of SO_2 and GSH is also not ruled out. More studies are necessary to better understand the role played by GSH in controlling the Fenton reaction. The effect of SO_2 and GSH on the evolution of total anthocyanins and monomeric anthocyanins is reported in Figure 5A. As expected a decline in total and monomeric anthocyanins was detected owing to the fact that they can: combine or condense with acetaldehyde and other oxidation products, forming stable cycloaddition products (pyranoanthocyanidins), participate in condensation reactions with other phenolic compounds or self-condense to give other more complex pigments, 2) produce polymeric anthocyanins from condensation reaction between anthocyanins and/or flavan-3-ols directly or mediated by aldehydes

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366 and 3) disappear due to an oxidative mechanism involving direct reaction with peroxide and free
367 radicals and/or through reactions with the oxidized components of the media to yield colorless or
368 brown products (Fulcrand et al. 2004, He et al. 2012, Jackman et al. 1987).

369 Sulfur dioxide exhibited a protective effect against the decline in free native anthocyanins,
370 and in fact the data suggests a small enhancement in anthocyanin levels as a result of SO_2
371 treatment, perhaps by trapping acetaldehyde that had bound to anthocyanins (Drinkine et al. 2007).
372 After 20 days a smaller effect was detected for total anthocyanins, and in both cases the decline
373 was more pronounced after 20 days of treatment when SO_2 levels reached zero in MLL and MLH.
374 A similar effect on anthocyanins in low SO_2 wines during micro-oxygenation has been reported
375 by Tao et al. (Tao et al. 2007) where they detected a faster loss of total pigments after 40 days of
376 treatment with an oxygen flow of 10 mL/L/month. In an industry scale post-malolactic
377 fermentation MOx trial performed on South African wines, the level of free anthocyanins
378 decreased after 10 weeks of treatment with 3 mg/L/month of oxygen (du Toit et al. 2006). For
379 MLL and MLH wines the more rapid loss of free anthocyanins was coincident with the increase
380 of pyranoanthocyanins and polymeric pigments, observed after 20 days of treatment (Figure 5B).
381 For MHH and MHL wines, no formation of new pigments during the entire treatment was observed
382 indicating that in these wines the anthocyanin stabilization reactions are inhibited by the higher
383 levels of SO_2. Comparing these data with the evolution of acetaldehyde (Figure 6), it seems that
384 the formation of acetaldehyde, as well as other likely carbonyls from Fenton reaction is the limiting
385 factor in color stabilization, and SO_2 is a major inhibitor. The evidence that a strong influence of
386 SO_2 and, to a less extent, of GSH, was observed is further confirmation of the fact that these
387 compounds are very reactive in inhibiting the Fenton formation of aldehydes and they also prevent
388 reactions with anthocyanins by diverting the aldehydes to bound forms.

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389 Apart from color stabilization, MOx is widely used in the wine industry to diminish tannins
390 astringency more quickly than can be obtained by several months barrel aging. Such changes are
391 closely related to variations of complex mixture of macromolecules constituting wine tannins with
392 the formation of modified structures that can exhibit different organoleptic properties. In MLL and
393 MLH wines an increase of polymeric tannins by HPLC was measured (Figure 7). Recently it has
394 been shown that under oxidative conditions different reaction pathways such as intra- vs
395 intermolecular reactions of native proanthocyanidins giving high polymerized and branched
396 tannins occurs (Mouls and Fulcrand 2012, Vernhet et al. 2014). In addition the formation of
397 ethylidene-bridged flavan-3-ol products (Jesus Cejudo-Bastante et al. 2011) and of
398 anthocyanins/tannins adducts (Fulcrand et al. 2006) can take place. The increase of polymeric
399 tannins detected only for MLL and MLH wines suggest that reactions involving acetaldehyde such
400 as the formation of ethylidene bridge flavan-3-ols probably occurred. These data further support
401 the occurrence of an accelerate oxidation of wine phenolics when acetaldehyde increases after SO_2
402 levels reached zero. The evidence that at the end of the MOx the content of polymeric tannins is
403 lower for MLH than MLL, confirm the role played by GSH in the modulation of phenolics
404 oxidation and it is in agreement with previous results of Sonni et al. (2011b) suggesting a
405 substantial impact of GSH on carbonyl-derived polymerization reactions in wine

406 **Effects on color parameters, tannin, and phenolic composition.** Anthocyanins,
407 anthocyanin-related red pigments and polymeric tannnins were significantly affected by
408 preservative treatments whether or not MOx was being applied (Table 3). A decrease of all native
409 free anthocyanins with MOx was detected in agreement with previous results (Cejudo-Bastante et
410 al. 2011a, Gambuti et al. 2013). For each native anthocyanin the relative loss followed the order:
411 MLL>MLH>MHL>MHH. The percentage of decrease was four time higher in MLL compared to

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MHH wines (e.g. for Mv3glc the percentage of decrease was 25% in MLL, 16% in MLH, 13% in MHL and 8% in MHH). Therefore it is possible to say that anthocyanins are protected by both SO_2 and GSH, and, for the first time a specific protective role has been demonstrated for GSH. The sensitivity to MOx is different for each anthocyanin and the acylated ones were more affected, suggesting a possible role of copigmentation in these reactions (Boulton 2001). The fact that malvidin-3-*O*-glucoside and peonidin-3-*O*-glucoside reacted as much as the others confirms the hypothesis that the key reactions with aldehydes and ketones are not on the B-ring but on the C and A rings.

Wines in the treatments that initially contained the lower level of SO_2, 25 mg/L, (MLL and MLH) ended up with higher content of pyranoanthocyanins. However, Asenstorfer et al. (Asenstorfer et al. 2003) showed that higher sulfur dioxide amounts added before alcoholic fermentation resulted in wines with higher content of vitisin A. This apparent contradiction can be explained by considering alternate sources of pyruvic acid. In a post FML trial, as in this experiment, it is likely that pyruvate is formed by chemical oxidation of malic acid, and SO_2 inhibits this process by limiting the Fenton reaction. However, during fermentation pyruvic acid is produced by micro-organisms and its concentration in wine is enhanced through the formation of a weak bisulfite-addition complex. In this latter case, more SO_2 results in a greater accumulation of pyruvic acid through the shift of free and bound SO_2 when the wine is subsequently oxidized. Also, compared to MHH, VitB was increased by about 20% in MLL and MLH wines, likely due to the increased formation of acetaldehyde in these wines. In concurrence with these results Cejudo-Bastante et al. (Cejudo-Bastante et al. 2011a) showed that an oxygen addition increased the concentrations of anthocyanin-ethylflavan-3-ols, anthocyanin-ethyl-B-type-procyanidins and B-type vitisins, in particular just after the micro-oxygenation treatment. In addition the MOx

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435 treatment resulted in an increase in polymeric pigments and tannins, and the presence of
436 preservatives limit their formation in agreement with observations of Bakker et al. (Bakker et al.
437 1998), showing a clear effect of SO_2 on the polymerization processes. So, the observations noted
438 here concur with others in that the MOx treatment increases the yield of stabilized pigment from
439 carbonyl reactions, while sulfite decreases their yield.

440 As already observed by other authors (Cejudo-Bastante et al. 2011b) the addition of oxygen
441 results in a decrease of color intensity with an increase of Abs at 420nm and hue (Table 4). In
442 addition some authors observed an increase of CI several months after the treatment (Cano-Lopez
443 et al., 2008; Gambuti et al., 2013) suggesting that time dependent reactions create a general
444 hyperchromic effect in micro-oxygenated wines. As expected, a significant bleaching effect of SO_2
445 was observed as demonstrated by the lower values of 520 nm absorbance and CI for wines with
446 higher SO_2. Further supporting these results, total anthocyanins as measured by the Harbertson
447 Adams assay is reduced by MOx treatment, and preserved by SO_2 addition (Table 5). Oxygen
448 addition also resulted in a significant decrease of SPP, perhaps because they were converted to
449 higher high molecular weight structures by cross-linking reactions.

450 Oxidation reduces vanillin reactive flavonoids (VRF), while both SO_2 and GSH reduce
451 these losses. These results can be interpreted by chemical reactions between oxidation products
452 such as acetaldehyde with the C8 and C6 positions on A ring of flavanols (Fulcrand et al. 2004).
453 The vanillin assay utilizes the aldehyde vanillin, and it reacts with the same positions on the
454 flavanol A-ring to produce a colored product (Hagerman et al. 1997). Therefore the measurement
455 by VRF may be considered an indirect, inverse measure of the oxidative polymerization of
456 flavanols. Thus, the VRF assay may well be a good marker for tannin oxidation. By the same

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457 analysis the reduction in tannin is due at least in part to acetaldehyde bridging reactions that inhibit
458 protein binding.

459 In this study, all of the variables tested had the ability to significantly affect the main
460 phenolic parameters linked to wine evolution: the sum of free anthocyanins and
461 pyranoanthocyanidins, as well as the content of polymeric phenolics (Table 6). Among the factors,
462 MOx has the most significant effect. It generally resulted in wines with a higher content of
463 pyranoanthocyanidins, more polymers, less free anthocyanins (significant at $p < 0.001$). On the
464 other hand the added SO_2 and, to a less extent, GSH both have an opposite effect on these
465 parameters, resulting in a general protection of wine from oxidation and polymerization reactions.
466 Apart the sum of free anthocyanins, interactions among all the variables considered were
467 significant, indicating that both SO_2 and GSH are able to modulate MOx. For the first time a
468 significant effect of GSH on red wine pigments and on the formation of polymeric tannins has
469 been demonstrated.

470 **Effects on acetals.** The concentrations of the acetaldehyde-glycerol acetals have been
471 correlated to the aging of a wine (da Silva Ferreira et al. 2002), indicating their connection with
472 wine oxidation. Due to the formation of acetaldehyde through non-enzymatic oxidation of ethanol,
473 these compounds are expected to increase in response to continuous oxygen exposure. The
474 concentrations of the four isomers *cis*- and *trans*-1,3-dioxane and *cis*- and *trans*-1,3-dioxolane in
475 the experimental wines are reported in Table 7. A subset of this data was previously reported with
476 the method on the acetal analysis (Peterson et al., 2015). Higher values for all the acetal isomers
477 were detected in wines that underwent the MO treatment and particularly those not protected by
478 SO_2. Micro-oxygenated wines treated with high levels of SO_2 (MHH and MHL) contained

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479 comparable acetal concentrations to control wines, demonstrating the antioxidant properties of
480 SO_2. Da Silva Ferreira et al. (da Silva Ferreira et al. 2002) observed a similar effect of SO_2 on the
481 evolution of these acetals during the storage of a Port wine and model solutions. The authors added
482 SO_2 prior to barrel-aging the samples. They ascribed the results to the binding of SO_2 to
483 acetaldehyde, thus inhibiting the acetalization reaction. In our experiment, the effects of SO_2 and,
484 to a lesser extent GSH, were also due to their activity in inhibiting the production of acetaldehyde
485 during MOx or binding after production. A difference in the relative abundance of the dioxane
486 isomers versus dioxolane isomers was detected in both oxygenated and control wine samples. The
487 total content of the 6-membered acetal rings (dioxanes) were found to be the most abundant
488 isomers in the non-oxygenated wines, while the introduction of oxygen to the wines resulted in
489 acetal contents dominated by the 5-membered acetal rings (dioxolanes).. This inversion of isomer
490 abundances was most evident in micro-oxygenated samples containing low levels of SO_2 and GSH.
491 The data confirms that the 5-membered rings are more kinetically stable and thus formed at a faster
492 rate under conditions of rapid oxidation. In control wine samples, the isomers are able to
493 equilibrate to a mixture dominated by *cis*-1,3-dioxane (and dioxanes in general) due its higher
494 thermodynamic stability (Aksens and Albriktsen 1966). Thus, a higher concentration of the
495 dioxolane isomers in a given wine sample indicates continuous, high oxygen exposure (micro-
496 oxygenation) while a higher *cis*-dioxane content indicates that the acetal isomers have reached
497 equilibrium due to low oxidative conditions. The results from the acetal analysis of MOx wine
498 samples confirm that these compounds are good markers of oxidation, most notably in techniques
499 involving consistent additions of oxygen to wine samples. Although the sum of four acetals do not
500 exceed the sensory threshold (100 mg/L), a potential future impact of these compounds on wine
501 aroma could occur if adequate oxidation occurs during aging (da Silva Ferreira et al. 2002, Martins

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et al. 2013). These substance have a sensory character of old-port like odor, and their concentration of is correlated with barrel aging of port (da Silva Ferreira et al. 2002). Since acetaldehyde is the major product of the Fenton reaction, a determination of the accumulated formation of acetaldehyde and its products in a wine may be considered a marker of the oxidative evolution of a wine (Carlton et al. 2007).

Conclusion

In conclusion this study provides clear evidence for a strong link between levels of SO_2 and GSH in wine and reduced development of oxidation by-products during MOx treatment. The loss of free SO_2 during MOx led to a dramatic acceleration of wine oxidation, signaled by a rapid loss of DO. This is likely due to the efficient consumption of oxygen by the Fenton reaction as suggested by a concomitant increase in acetaldehyde. The simultaneous increase in a series of other oxidation products linked to the presence of acetaldehyde, such as pyranoanthocyanidins, polymeric pigments and acetals further support the observation of accelerating oxidation.

A strong relationship between the appearance of various oxidation products and the loss of DO during MOx suggests that monitoring DO, and/or free SO_2 may be useful parameters to monitor the rate of oxidation reactions induced by the MOx process. Glutathione also played a very influential role in most of the parameters measured and a synergistic effect was observed in combination with SO_2. Wines with reduced SO_2 but added GSH showed an intermediate degree of oxidation, with enhanced production of stable phenolics but reduced production of acetals. In addition GSH appeared to mitigate the loss of DO when SO_2 was depleted, suggesting that the consumption of peroxide in the Fenton reaction is reduced. This could arise from GSH reduction of peroxide, providing another tool to manage MOx. Therefore the use of GSH to modulate red

wine evolution during oxidative treatments or perhaps also during aging may be an interesting complement to SO_2 use; further studies could help develop usage protocols to provide winemakers with some direction on how to use various combinations of these two preservatives.

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American Journal of Enology and Viticulture (AJEV). doi: 10.5344/ajev.2015.15005
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Table 1 Content of monomeric and polymeric phenolics (mg/L) of red wine before the MOx treatment measured by RP- HPLC.

Compound	mg/L
Dp3glc	25.17 ± 0.09
Pt3glc	26.41 ± 0.23
Pn3glc	12.86 ± 0.04
Mv3glc	246.97 ± 1.54
Dp3acglc	13.46 ± 0.34
VitA	1.49 ± 0.16
VitB	1.57 ± 0.02
Pn3acglc	4.65 ± 0.11
Mv3acglc	93.22 ± 0.85
Pn3cmglc + Mv3cmglc	23.92 ± 0.56
Polym Pigm	68.14 ± 3.54
Tot. anthocyanins	525.27 ± 4.45
Polym tannins	695.82 ± 20.82

Compounds: Dp3glc = delphinidin 3-glucoside. Pt3glc = petunidin 3-monoglucoside. Pn3glc = peonidin 3-monoglucoside. Mv3glc = malvidin 3-glucoside. Dp3acglc = delphinidin 3-(6^{II}-acetyl)-glucoside. VitA= vitisin A. VitB= vitisin B. Pn3acglc = peonidin 3-(6^{II}-acetyl)-glucoside. Mv3acglc = malvidin 3-(6^{II}-acetyl)-glucoside. Pn3cmglc = peonidin 3-(6^{II}-coumaroyl)-glucoside. Mv3cmglc = malvidin 3-(6^{II}-coumaroyl)-glucoside. Polym Pigm = polymeric pigments.

Table 2 Content of free, bound and total SO_2 (mg/L) of MOx wines.

	Free SO_2	Bound SO_2	Total SO_2
0 time			
MLL (Microox - LowSO_2 -Low GSH)	20.1 ± 4.2	31.7 ± 2.1	51.8 ± 6.3
MLH (Microx-LowSO_2-LowGSH)	22.8 ± 2.5	31.7 ± 3.0	54.5 ± 5.5
MHL (Microx-High SO_2-Low GSH)	64.8 ± 2.0	53.3 ± 2.3	118.2 ± 4.3
MHH (Microx-High SO_2-High GSH)	74.9 ± 0.0	53.3 ± 2.7	128.3 ± 2.7
End of treatment (30days)			
MLL (Microox - LowSO_2 -Low GSH)	0.0 ± 0.0	9.7 ± 2.0	9.7 ± 2.0
MLH (Microx-LowSO_2-LowGSH)	0.0 ± 0.0	7.3 ± 1.5	7.3 ± 1.5
MHL (Microx-High SO_2-Low GSH)	6.9 ± 5.9	21.5 ± 0.4	28.4 ± 5.9
MHH (Microx-High SO_2-High GSH)	11.8 ± 1.0	16.3 ± 0.5	28.1 ± 1.5

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Table 3 Effect of micro-oxygenation. SO_2. and glutathione on monomeric and polymeric phenolics (mg/L) of red wine after 30 days of treatment measured by RP- HPLC.

Control Wines				
	HH	HL	LH	LL
Dp3glc	25.65 ± 0.55a	25.31 ± 0.17a	21.89 ± 0.10b	22.04 ± 0.14b
Pt3glc	27.46 ± 0.52a	27.28 ± 0.17a	23.33 ± 0.15b	23.42 ± 0.11b
Pn3glc	13.19 ± 0.29a	13.04 ± 0.04a	11.37 ± 0.03b	11.28 ± 0.05b
Mv3glc	262.96 ± 5.19a	260.94 ± 1.73a	222.96 ± 2.43b	223.96 ± 1.02b
Dp3acglc	7.21 ± 0.23a	7.13 ± 0.02a	6.04 ± 0.06b	5.91 ± 0.06b
Pyr1				
VitA	2.88 ± 0.06c	2.95 ± 0.01b	3.27 ± 0.00a	3.28 ± 0.02a
VitB	1.26 ± 0.09b	1.66 ± 0.19a	0.92 ± 0.06c	0.89 ± 0.04c
Pyr4				
Pn3acglc	4.80 ± 0.15a	4.69 ± 0.03a	4.44 ± 0.05b	4.30 ± 0.05c
Mv3acglc	100.75 ± 2.14a	100.15 ± 0.54a	84.09 ± 0.56b	84.45 ± 0.36b
Pn3cmglc + Mv3cmglc	30.90 ± 0.68a	30.94 ± 0.30a	24.43 ± 0.33c	25.80 ± 1.08b
Polym Pigm	63.93 ± 1.28a	63.46 ± 0.88a	61.35 ± 3.32ab	59.98 ± 1.79b
Tot. anthocyanins	540.98 ± 10.86a	537.54 ± 2.12a	464.08 ± 4.08b	465.31 ± 1.33b
Polym tannins	675.35 ± 10.64b	674.18 ± 6.78b	723.85 ± 6.47a	706.96 ± 16.74a
MO Wines				
	MHH	MHL	MLH	MLL
Dp3glc	23.79 ± 0.23a	22.72 ± 0.89a	18.56 ± 1.09b	16.66 ± 0.28c
Pt3glc	25.39 ± 0.28a	23.91 ± 1.16b	19.37 ± 1.12c	17.26 ± 0.27d
Pn3glc	12.30 ± 0.18a	11.67 ± 0.54b	9.94 ± 0.36c	9.04 ± 0.14d
Mv3glc	241.89 ± 2.47a	227.27 ± 10.64b	186.86 ± 9.63c	167.44 ± 2.92d
Dp3acglc	6.76 ± 0.03a	6.33 ± 0.34b	5.14 ± 0.36c	4.47 ± 0.11d
Pyr1[a]				2.23 ± 0.25
VitA	2.88 ± 0.09b	2.70 ± 0.19b	3.80 ± 0.14a	3.64 ± 0.04a
VitB	1.85 ± 0.12d	1.43 ± 0.10c	2.03 ± 0.11b	2.22 ± 0.07a
Pyr4[b]				0.81 ± 0.13
Pn3acglc	4.51 ± 0.02a	4.26 ± 0.19a	3.47 ± 0.26b	2.93 ± 0.08c
Mv3acglc	92.48 ± 0.95a	85.77 ± 5.30b	68.82 ± 3.61c	61.25 ± 1.15d
Pn3cmglc + Mv3cmglc	27.00 ± 0.45a	24.38 ± 2.13b	17.42 ± 1.67c	14.55 ± 0.33d
Polym pigm	66.54 ± 0.52c	69.06 ± 1.32c	77.84 ± 3.23b	86.31 ± 1.62a
Tot. anthocyanins	505.39 ± 5.14a	479.49 ± 20.14ab	413.81 ± 14.19b	383.13 ± 2.95c
Polym tannins	704.76 ± 4.58d	728.16 ± 8.12c	783.25 ± 15.28b	832.59 ± 8.41a

Wines treatments: LL (LowSO_2-Low GSH); LH (LowSO_2-High GSH); HL (High SO_2-Low GSH); HH (High SO_2-HighGSH); MLL (Microox - LowSO_2 -Low GSH); MLH (Microx-LowSO_2-High GSH); MHL (Microox-High SO_2-Low GSH); MHH (Microx-High SO_2-High GSH).
Compounds: Dp3glc = delphinidin 3-glucoside. Pt3glc = petunidin 3-monoglucoside. Pn3glc = peonidin 3-monoglucoside. Mv3glc = malvidin 3-glucoside. Dp3acglc = delphinidin 3-(6^{II}-acetyl)-glucoside. Pyr1= unknown pyranoanthocyanins (retention time=39.7min). VitA= vitisin A. VitB= vitisin B. Pyr4= unknown pyranoanthocyanins (retention time=47.47min). Pn3acglc = peonidin 3-(6^{II}-acetyl)-glucoside. Mv3acglc =

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malvidin 3-(6^{II}-acetyl)-glucoside. Pn3cmglc = peonidin 3-(6^{II}-coumaroyl)-glucoside. Mv3cmglc = malvidin 3-(6^{II}-coumaroyl)-glucoside. Polym Pigm = polymeric pigments. [a]=partially coeluted with Pyr2. [b]=partially coeluted with Pyr3. *Different letters indicate statistical differences (p<0.05). Latin small letters (a. b. c) are used to compare control and MO wines treated with low and high levels of GSH and SO_2.*

Table 4 Effect of micro-oxygenation. SO_2. and glutathione on chromatic characteristics of red wine after 30 days of treatment.

Control Wines	Abs420nm	Abs520nm	Abs620nm	CI	Hue
LL	5.83 ± 0.02a	9.22 ± 0.02a	2.86 ± 0.01a	17.91 ± 0.05a	0.63 ± 0.00c
LH	5.83 ± 0.06a	9.16 ± 0.05a	2.84 ± 0.01a	17.83 ± 0.12a	0.64 ± 0.00b
HL	5.41 ± 0.06b	8.40 ± 0.10b	2.67 ± 0.03b	16.48 ± 0.18b	0.64 ± 0.00a
HH	5.44 ± 0.05b	8.40 ± 0.06b	2.67 ± 0.01b	16.51 ± 0.11b	0.65 ± 0.00a
MO Wines					
MLL	5.75 ± 0.04a	7.77 ± 0.03a	2.48 ± 0.01a	15.99 ± 0.06a	0.74 ± 0.00a
MLH	5.72 ± 0.07a	7.71 ± 0.05a	2.44 ± 0.03a	15.87 ± 0.14ab	0.74 ± 0.00a
MHL	5.62 ± 0.15a	7.71 ± 0.14a	2.35 ± 0.05b	15.68 ± 0.35b	0.73 ± 0.01b
MHH	5.42 ± 0.01b	7.47 ± 0.03b	2.27 ± 0.00c	15.15 ± 0.03c	0.72 ± 0.00b

Wines treatments: see table 1.*Different letters indicate statistical differences (p<0.05). Latin small letters (a. b. c) are used to compare control and MO wines treated with low and high levels of GSH and SO_2.*

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Table 5 Effect of 1 month micro-oxygenation (30 mg O_2). SO_2. and glutathione on anthocyanins. SPP pigments (evaluated using the Harbertson-Adams assay) and vanilline reactive flavans (VRF) of red wine.

Control Wines	Anth.(mg/L)	SPP	VRF (mg/L CE)
LL	452.9 ± 3.0c	1.26 ± 0.01b	816 ± 15*a
LH	446.4 ± 4.3d	1.28 ± 0.02a	782 ± 37*ab
HL	511.9 ± 4.9b	1.23 ± 0.01c	726 ± 33*b
HH	518.7 ± 2.6a	1.25 ± 0.01b	792 ± 25*ab
MO Wines			
MLL	259.4 ± 5.4c	1.22 ± 0.01a	621 ± 18*b
MLH	283.1 ± 2.0b	1.08 ± 0.01a	625 ± 9*b
MHL	353.4 ± 3.9a	1.04 ± 0.00b	798 ± 18*ab
MHH	358.1 ± 3.3a	1.04 ± 0.00b	892 ± 22*a

Wines treatments: See Table 1. *Different letters indicate statistical differences ($p<0.05$). Latin small letters (a. b. c) are used to compare control and MO wines treated with low and high levels of GSH and SO_2.*

Table 6 F values and significance[a] of variables SO_2. GSH and MO for main phenolic compounds.

	Σ free anth.		Σ pyranoanth.		Polym. pigments		Polym. tannins	
SO_2	268.98	***	547.09	***	216.62	***	342.26	***
GSH	20.11	***	15.16	**	32.06	***	54.13	***
MO	341.33	***	535.42	***	489.39	***	278.89	***
SO_2 x GSH	0.35	ns	20.71	***	9.39	**	6.88	*
SO_2 x MO	16.34	**	727.32	***	206.48	***	44.36	***
MO x GSH	16.52	**	15.61	**	14.17	**	28.02	***

[a]: *. $p<0.05$; **. $p<0.01$; ***. $p<0.001$; ns. not significant.

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Table 7 Effect of micro-oxygenation. SO_2. and glutathione on acetaldehyde-glycerol acetals (mg/L) after 30 days of treatment measured by GC-MS.

Control Wines	HH	HL	LH	LL
cis-5-hydroxy-2-methyl-1.3-dioxane	0.332 ± 0.016	0.328 ± 0.0024	0.333 ± 0.0054	0.330 ± 0.0028
trans-5-hydroxy-2-methyl-1.3-dioxane	0.133 ± 0.0029	0.133 ± 0.0019	0.136 ± 0.0024	0.133 ± 0.0022
cis-4-hydroxymethyl-2-methyl-1.3-dioxolane	0.163 ± 0.016	0.159 ± 0.0010	0.169 ± 0.0017	0.167 ± 0.0029
trans-4-hydroxymethyl-2-methyl-1.3-dioxolane	0.161 ± 0.0023	0.157 ± 0.0014	0.164 ± 0.0031	0.160 ± 0.0021
MO Wines	MHH	MHL	MLH	MLL
cis-5-hydroxy-2-methyl-1.3-dioxane	0.440 ± 0.0051	0.619 ± 0.0080	0.843 ± 0.015	1.29 ± 0.019
trans-5-hydroxy-2-methyl-1.3-dioxane	0.176 ± 0.0090	0.255 ± 0.0060	0.323 ± 0.0070	0.493 ± 0.011
cis-4-hydroxymethyl-2-methyl-1.3-dioxolane	0.354 ± 0.0029	0.531 ± 0.0064	0.708 ± 0.011	1.30 ± 0.023
trans-4-hydroxymethyl-2-methyl-1.3-dioxolane	0.267 ± 0.0030	0.405 ± 0.049	0.543 ± 0.0059	0.923 ± 0.017

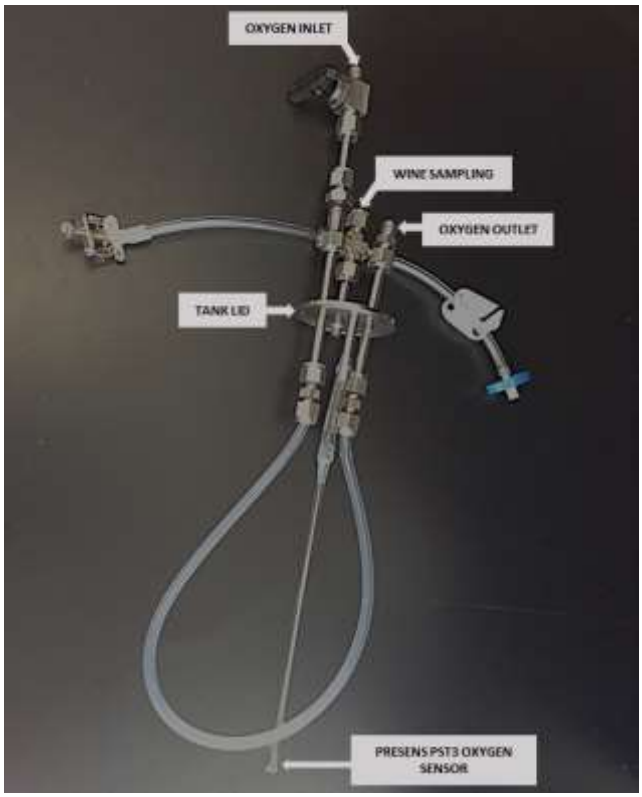

Figure 1 Stainless steel lid fittings.

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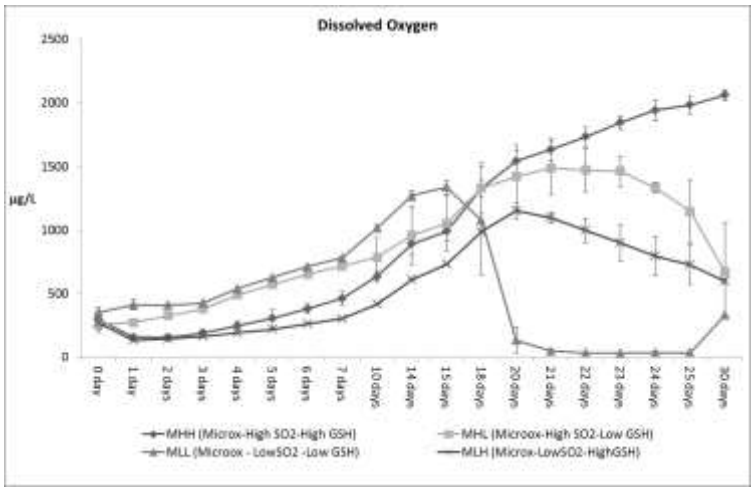

Figure 2 Dissolved Oxygen changes for wines with different initial addition of SO_2 and GSH during MOx.

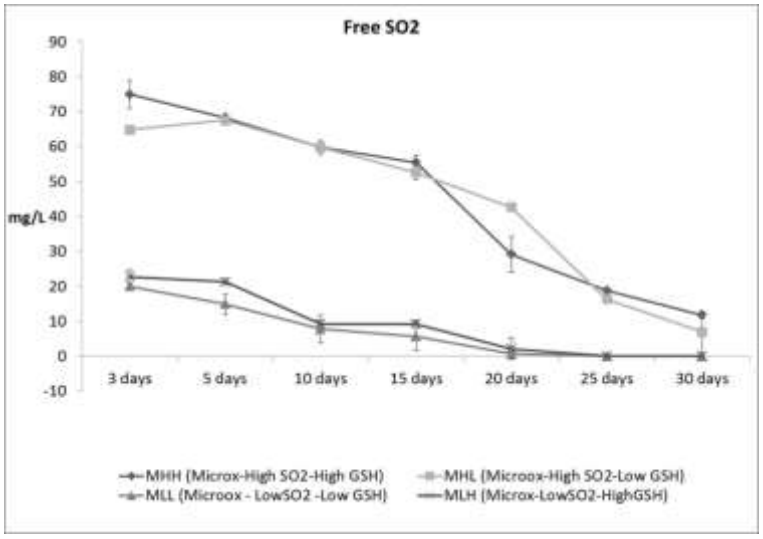

Figure 3 Levels of free SO_2 for wines with different initial addition of SO_2 and GSH during MOx.

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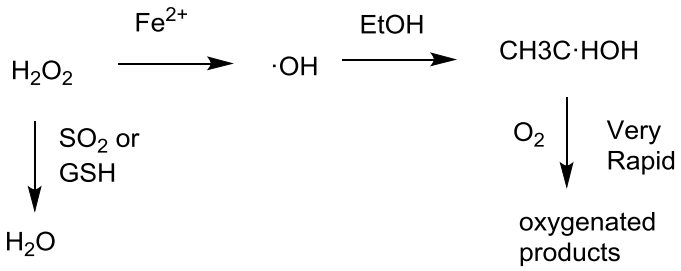

Figure 4 Mechanism for rapid oxygen loss when SO_2. GSH are depleted.

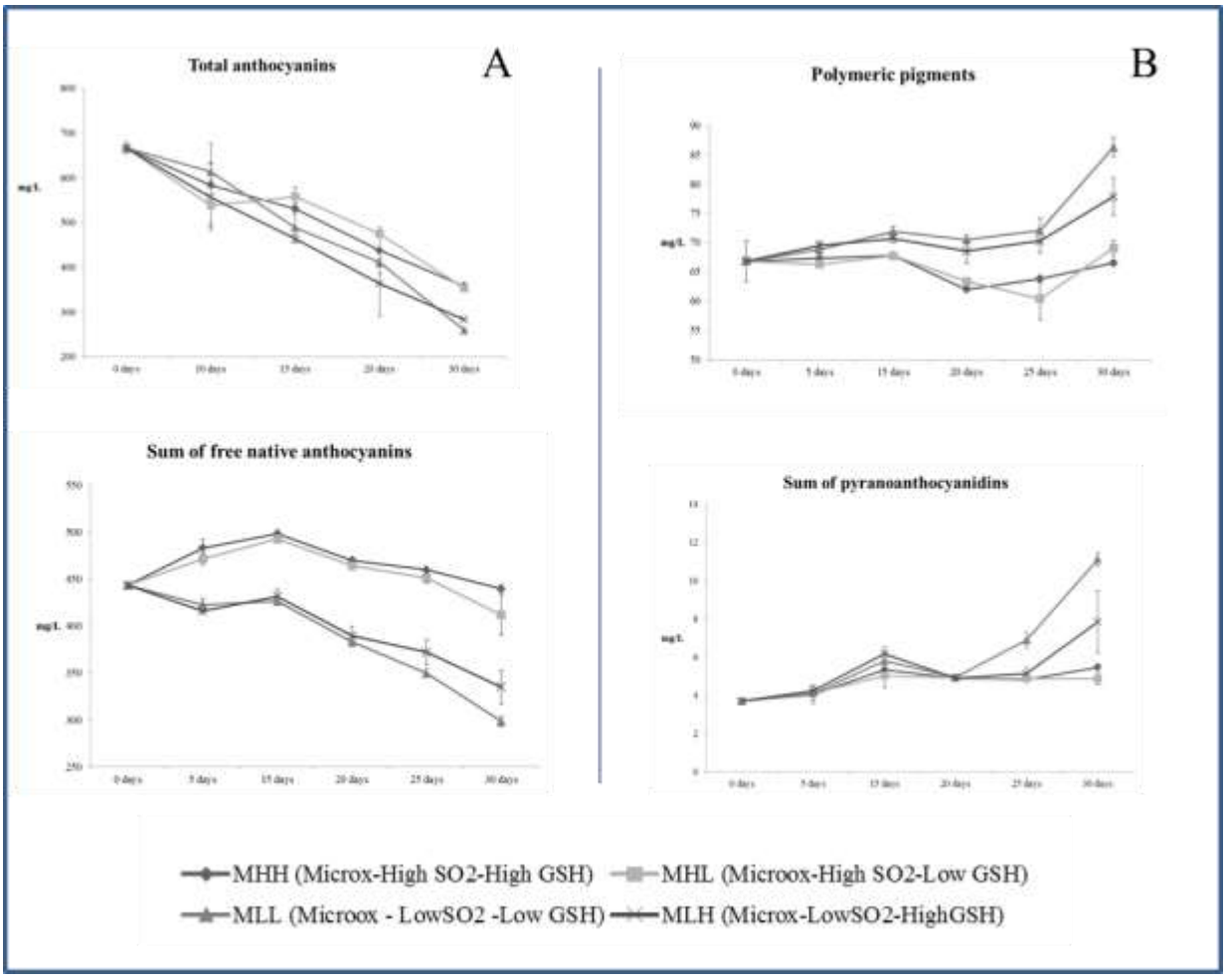

Figure 5 Effect of SO_2 and GSH on the loss of total anthocyanins (Harbertson Adams) and native anthocyanins (HPLC) (**A**) and the formation of polymeric pigments (Harbertson Adams) and pyranoanthocyanis (HPLC) (**B**) during MOx.

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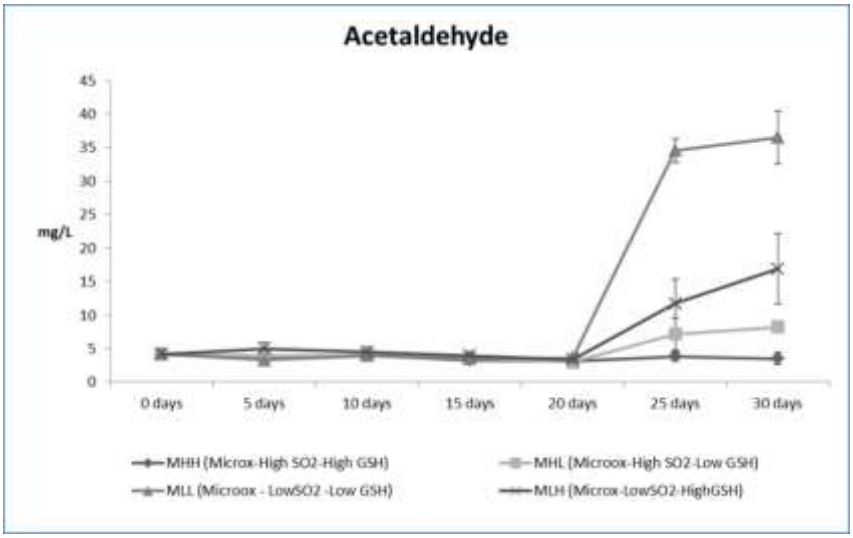

Figure 6 Levels of acetaldehyde for wines with different initial addition of SO_2 and GSH during MOx.

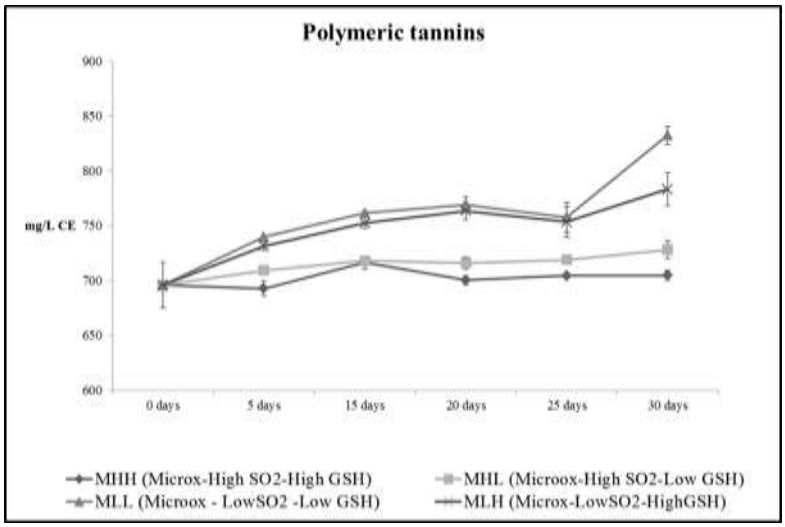

Figure 7 Effect of SO_2 and GSH on the formation of polymeric tannins during MOx.