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Using LC-MSMS To Assess Glutathione Levels in South African White Grape Juices and Wines Made with Different Levels of Oxygen

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The effect of oxygen on the levels of glutathione, an important antioxidant, in must and wine was studied using a novel LC-MSMS method for the analysis of reduced and oxidized glutathione. This study found that the storage of grape juice at high SO_2 and ascorbic acid levels at −20 °C did not lead to a decrease in reduced glutathione levels. The effect of varying the oxygen levels in South African white grape juices, which included a reductive treatment (less than 0.3 mg/L dissolved O_2 added), a control treatment (between 1.0 and 1.5 mg/L dissolved O_2 added), and an oxidative treatment (3.5−4 mg/ L dissolved O_2 added, without SO_2) on reduced glutathione levels in the juice and resulting wine was also investigated. A custom build press was used to press whole bunches of two different Sauvignon Blanc and Colombard grapes. Alcoholic fermentation and oxygen additions to the must led to lower reduced glutathione levels in the wine. Reduced glutathione levels were only significantly higher in the wine made from reductive juice that had the highest initial reduced glutathione levels in the grapes.

KEYWORDS: Glutathione; LC-MSMS; oxygen; wine

INTRODUCTION

Glutathione has received much attention in the past due to its important role in many detoxification processes in living cells. It has a strong antioxidative effect and prevents cellular damage by maintaining certain thiols in their reduced state, scavenging hydrogen peroxide and hydroxyl radicals, and maintaining the redox potential in cells (*1*, *2*). Glutathione binds toxins such as heavy metals, organic solvents, and pesticides and is thus more easily taken up in bile and urine (*3*).

In addition, glutathione plays an important role during the oxidation of white must to form the grape reaction product (GRP), which prevents browning to a certain extent. This is due to the reduction of oxidized phenol molecules to a reoxdizable phenol (the GRP). One molecule of caffeic acid consumes 3.4 atoms of oxygen, which increases to 8.5 when glutathione is added (*4*). Glutathione acts in generating a reoxdizable product by reducing the quinone to caffeic acid and by substituting the quinone to regenerate the hydroquinone form of 2-*S*-glutathionyl-caftaric acid (*5*). Depletion of glutathione leads to browning of white musts. The compounds responsible for certain tropical fruit flavors of certain Sauvignon Blanc wines are sulfur containing compounds, of which the production may be linked to glutathione. The catabolism of *S*-3-(hexan-1-ol)-glutathione leads to the formation of *S*-3-(hexan-1-ol)-L-cysteine, which is the precursor of 3-mercaptohexan-1-ol (*6*). The latter compounds form the characteristic passion fruit aroma in Sauvignon Blanc wine. The yeast can also change the glutathione levels of the wine during fermentation (*7*).

Recent advances in glutathione analysis in wine and must include an automated HPLC method using a precolumn derivatization with fluorescence detection, which proved to be a fast and accurate method (*7*). However, according to our knowledge, a rapid LCMSMS method to analyze reduced and oxidized glutathione in must and wine has not been developed. The effect of treating South African white grape musts very reductively and oxidatively, on reduced and oxidized glutathione levels before and after fermentation, is also not well-known. The main aims of this study were thus to develop a rapid and repeatable LCMSMS method for glutathione analyses in must and wines and to assess the effect of different oxygen additions to must, as well as its effect on reduced and oxidized glutathione levels in the juice and wine.

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MATERIALS AND METHODS

Reagents and Chemicals. Reduced L-glutathione (Fluka 49750) and oxidized l-glutathione (Fluka 49740) were purchased from Sigma-Aldrich.

Juice and Winemaking Treatments. Two different Sauvignon Blanc and one Colombard grapes were used in the experiments. These were designated wine A (Sauvignon Blanc of origin Elgin), wine B (Colombard, of origin Robertson), and wine C (Sauvignon Blanc of origin Stellenbosch). The grapes were all grown according to standard viticultural practices and picked by hand when ripe. The grapes were stored at 4 °C overnight. The grape bunches were mixed homogenously, and rotten berries were removed by hand. The bunches were then divided into three groups, designated for reductive, control, and oxidative juice treatments. A custom-built small scale press was used to extract the juice with minimum oxygen pick up (**Figure 1**). This was achieved by placing 9 kg of whole grape bunches around a central plastic membrane in the press. The press was filled with grapes, sealed, and filled with water. The water was displaced with CO_2 (Afrox SA) to achieve an inert atmosphere around the grapes before pressing. The plastic membrane was subsequently filled with nitrogen gas (Afrox SA). The pressure applied in the plastic membrane was slowly increased to 2 bar. The nitrogen gas was released through a pressure release valve, and the grapes were pressed again to 2 bar. An extra in- and outflow valve on top of the press allowed continuous flow of CO_2 through the press during the procedure. Around 3.5 L of the juice generated by this pressing action was accumulated in a 4.5 L glass bottle that previously had been filled with water and displaced with CO_2. Sulfur dioxide (60 mg/L) and ascorbic acid (50 mg/L) were added in the 4.5 L glass bottles just before filling with juice from the press. In spite of two pressings of up to 2 bar each, quite low percentages (40%) of juice yields were obtained in all treatments. Oxygen (O_2) concentrations were measured in the headspace of the press with a Micro III G202 of Gesellschaft für Gerätebau according to the supplier's recommendations. Oxygen concentrations in the juice in the 4.5 L glass containers were measured with an Oxi 330i oxygen meter with a cell ox 325 probe (Wissenschaftlich-Technische Werkstätten). Oxygen levels during pressing were kept below 1% inside the headspace of the press and less than 0.3 mg/L in the juice during the treatment. The O_2 concentrations in the headspace of the glass bottles were also monitored just before and after the juice was collected in the glass bottles and were lower than 1%. These treatments were considered the reductive juice treatments. The control juice treatment was conducted in the same manner, but the press and 4.5 L bottles were not filled with water and displaced by CO_2 prior to the pressing of the juice. Sulfur dioxide (60 mg/L) and ascorbic acid (50 mg/L) were also added into the juice just after pressing. The oxygen pick-up in the control juices was between 1.0 and 1.5 mg/L. The oxidative juice treatments were also pressed without water and CO_2 in the press and 4.5 L glass bottles. No SO_2 or ascorbic acid was added to these juices. After pressing, the juices were racked 4 times into a plastic 20 L bucket to encourage O_2 pick up. This resulted in 3.5−4 mg/L O_2 dissolved in the juice in total, measured just after four air rackings. Juice yields were the same between the reductive, control, and oxidative treatments. All treatments in all three grape types were performed in triplicate.

After settling overnight, the juice was racked from the grape lees with CO_2. It was then inoculated with the yeast strain Vin13 (Anchor Yeast Biotechnologies) at 0.3 g/L according to the supplier's recommendations and fermented at 15 °C until the accumulated mass loss was seized. Two days after the start of fermentation, diammonium phosphate was added at 0.5 g/L.

At the end of fermentation, an additional 50 mg/L SO_2 was added to all treatments, and the wine was racked with CO_2 from the yeast lees and bottled in green 750 mL wine bottles under CO_2 gas and sealed with screw tops.

Sampling Procedure from Different Juice Treatments. Samples destined for LC-MSMS analyses were taken from the juice in the reductive treatments just after the juice was collected in the 4.5 L glass bottles. This was done by transferring 80 mL of juice, with CO_2 pressure, from the 4.5 L glass bottles into 100 mL glass vials containing 1000 mg/L SO_2 and 500 mg/L ascorbic acid. The 5% SO_2 solution

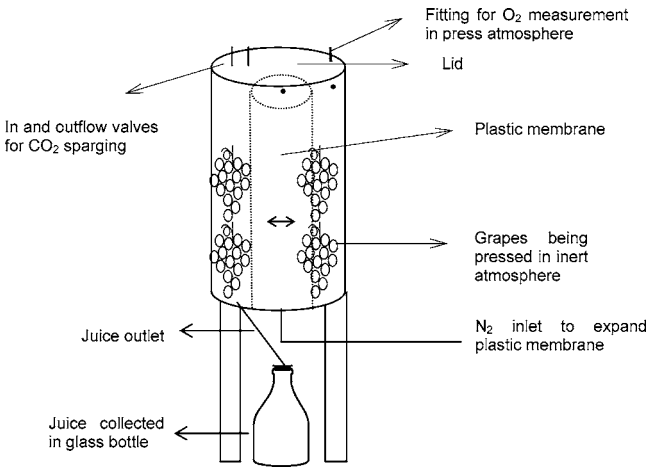

Figure 1. Press used to obtain juice under reductive conditions.

was made from $K_2S_2O_5$ (Riedel-de Haën) and 1.6 mL was added to 80 mL of juice to obtained the desired concentration. A 5% ascorbic acid solution (Sigma-Aldrich) was also used and prepared daily before starting the experiments. Such high SO_2 and ascorbic acid concentrations were used to completely inhibit any residual phenolic oxidase or laccase activity in the samples. Carbon dioxide was also blown inside the 100 mL glass vials before and after the juice was transferred into the glass vials. This was done to completely inhibit any residual oxidation enzyme activity. Additional CO_2 was blown on the headspace, and the vials were sealed hermitically. The vials were then frozen at −20 °C until they were analyzed. Samples from the oxidative treatments were stored in the same manner but were collected only after 8 h to allow the enzymatic oxidative reactions to take place.

Samples were thawed on the day of the LC-MSMS analyses and filtered through a 0.45 um syringe filter. Ethanol was evaporated from the wine samples under reduced pressure at 40 °C, and wine samples were redissolved to the initial volume with deionized water.

Recovery of Reduced Glutathione in Juice and Wine using the LC-MSMS Method. The recovery of the LC-MSMS method was assessed by adding reduced glutathione to grape juice and white wine at 0, 20, and 40 mg/L. The juice consisted of Shiraz grapes that were crushed under CO_2, and 1000 mg/L SO_2 and 500 mg/L ascorbic acid were added and filtered through a 0.45 μm syringe filter, spiked, and injected onto the LC-MSMS system. The wine was spiked, ethanol evaporated at 40 °C under vacuum for 10 min, water was used to adjust the sample to the initial volume, and the sample was subsequently injected on the LC-MSMS system.

Stability of Reduced Glutathione Juice over Time. The stability of reduced glutathione over time was also assessed in the following manner. Nine kilograms of Hanepoot table grapes was pressed in the reductive manner, and 3.5 L of juice was collected into a 4.5 L glass bottle as described previously. Eighty milliliters of this juice was then immediately transferred into 100 mL glass vials as described previously. Three of these vials contained juice with no SO_2 and ascorbic acid, while three more vials contained the same juice with the addition of ascorbic acid and SO_2 (at 500 and 1000 mg/L, respectively). The reduced glutathione concentrations of these six vials were analyzed by the LC-MSMS system on the same day. Additional vials containing SO_2 and ascorbic acid were also stored at 4 or −20 °C and analyzed after 4 weeks of storage. In addition, three vials, stored at −20 °C with SO_2 and ascorbic acid, were thawed after 2 weeks, refrozen, and thawed again for analyses after 4 weeks of storage. This was done to asses the effect of repeated thawing on reduced glutathione levels. We decided not to analyze the glutathione concentration in the same vial over time, but rather in separate vials, due to possible degradation reactions that might take place with repeated thawing.

LC-MSMS Method. A Waters API Quattro microtriple quadropole mass spectrometer with a 2690 Alliance HPLC was used for LC-MSMS analysis. Separation was achieved on a Waters Atlantis C18, 3 μm, 2.1 × 150 mm column using 0.1% formic acid (solvent A) to an acetonitrile (solvent B) gradient at a flow rate of 0.2 mL/min. The

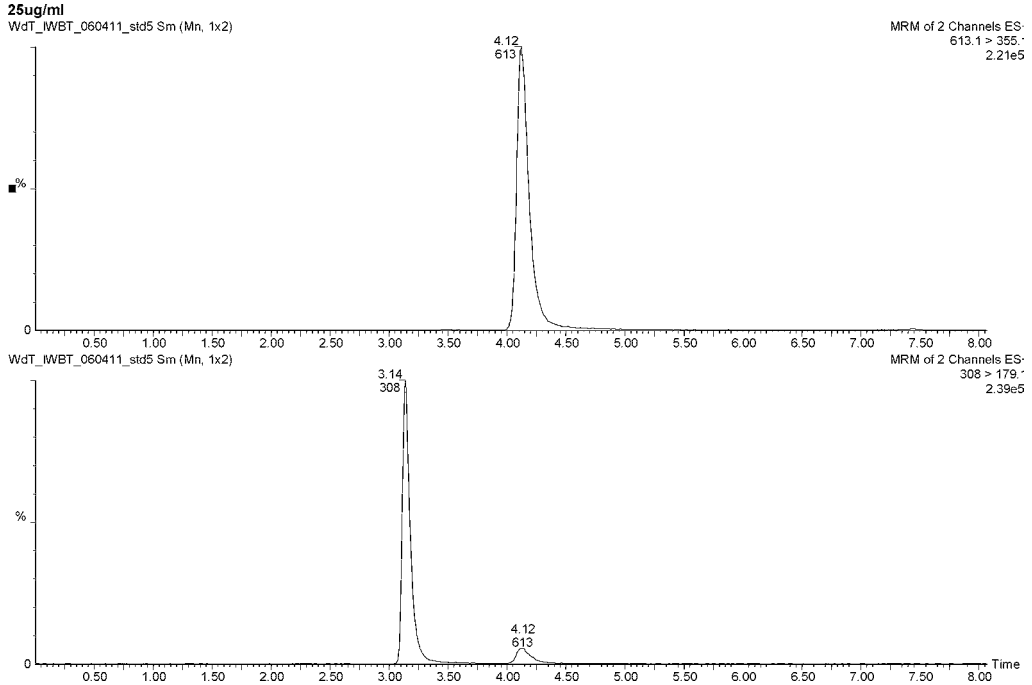

Figure 2. LC-MSMS chromatograms of oxidized (4.12 min) and reduced (3.14 min) glutathione standards.

solvent composition was kept at 100% solvent A for the first 0.5 min, followed by a linear gradient over 6.5 min to 80% solvent B and re-equilibration to 100% A for 7 min. Reduced glutathione eluted at 3.14 min and oxidized at 4.12 min.

The MS method consisted of two multiple reaction monitoring (MRM) functions with electrospray ionization in the positive mode, a capillary voltage of 3.5 kV, and argon as the collision gas. The first MRM monitored the reduced glutathione with a *m*/*z* 308 > 179.1 transition at a collision energy of 17 eV and cone voltage of 18 V. The second monitored the oxidized glutathione with a *m*/*z* 613.1 > 355.1 transition at a collision energy of 20 eV and cone voltage of 30 V.

These settings were selected to be able to quantify both compounds in one injection even though the concentration of the oxidized glutathione was much less. The transition settings for the reduced glutathione were selected at a collision energy that was not optimum (the optimum collision energy was 20 eV and cone voltage was 30 V) but that allowed a linear calibration at higher concentrations.

Statistical Analyses. The glutathione concentrations in the stability test were statistically analyzed with one way ANOVA analyses (with Tukey HSD post hoc tests) using STATISTICA software (*20*). In the cases where juice and wine values were compared across treatments, repeated measures of ANOVA were used.

RESULTS AND DISCUSSION

LC-MSMS chromatograms of oxidized and reduced glutathione standards are shown in **Figure 2**. Recovery of reduced glutathione from the juice and wine was significantly lower for the wine: 77% for the 20 mg/L reduced glutathione addition and 82% at 40 mg/L but not for the juice with a 91 and 95% recovery at 20 and 40 mg/L reduced glutathione addition, respectively ($p \leq 0.05$). The lower recovery for the wine can be attributed to the ethanol removing step, which was not performed for the juice. The ethanol was removed to reduce the solvent affect that resulted in distorted peaks. The dilution necessary to overcome this solvent effect was too much for the detection of expected levels of oxidized glutathione in wine, hence the decision to remove the ethanol.

The limit of detection for the method (signal-to-noise ratio of 1:3) was 0.2 mg/L for oxidized and 0.4 mg/L for reduced glutathione at an injection volume of 5 μL with the upper limit

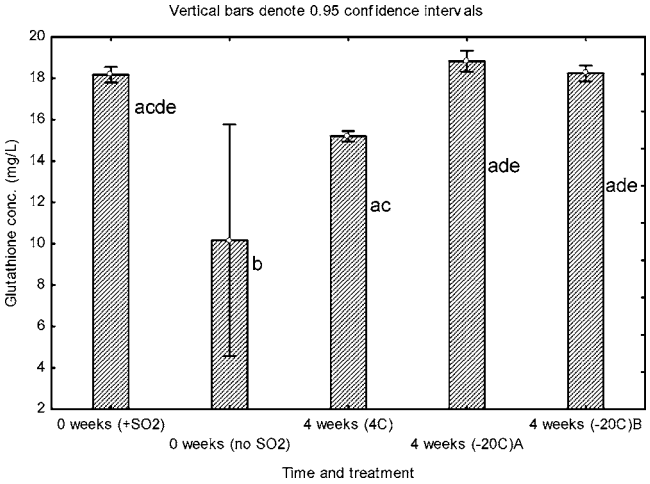

Figure 3. Reduced glutathione levels in grape juice stored under different conditions. 0 Weeks (+SO_2): samples analyzed immediately after pressing, with 1000 mg/L SO_2 and 500 mg/L ascorbic acid added; 0 weeks (no SO_2): samples analyzed immediately after pressing, with no SO_2 or ascorbic acid added; 4 weeks (4 °C): stored at 4 °C for 4 weeks; 4 weeks (–20 °C) A: stored at –20 °C for 4 weeks, sample thawed only once and 4 weeks (–20 °C) B: stored at –20 °C for 4 weeks, sampled thawed twice. All the samples analyzed at week 4 had 1000 mg/L SO_2 and 500 mg/L ascorbic acid added initially. Different letters indicate significant differences ($p \leq 0.05$).

of the linear range of 200 mg/L. The relative standard deviation for six injections was 0.4% in the 40 mg/L range, and the limit of quantitation for both compounds was 0.8 mg/L. The limit of quantitation was taken as the lowest concentration where the % RSD of six injections was better than 20%.

In **Figure 3**, the reduced glutathione concentrations during the stability test can be seen. It is clear that the samples in the absence of SO_2 and ascorbic acid oxidized rapidly, with reduced glutathione levels dropping significantly as compared to the same samples that received SO_2 and ascorbic acid. After 4 weeks, reduced glutathione concentrations in the samples stored at 4 °C decreased but remained constant in the samples frozen

Table 1. Oxidized Glutathione Concentrations (mg/L) of Three Grape Juices Made with Different Oxygen Additions[a]

treatment	juice A	juice B	juice C
reductive	0.46 ± 0.06ab	1.73 ± 0.06ab	1.43 ± 0.7a
control	0.47 ± 0.03ab	1.73 ± 0.05ab	1.35 ± 0.9b
oxidized	0.80 ± 0.05c	2.93 ± 0.0.49c	2.33 ± 0.12c

[a] Different letters indicate significant differences ($p \leq 0.05$).

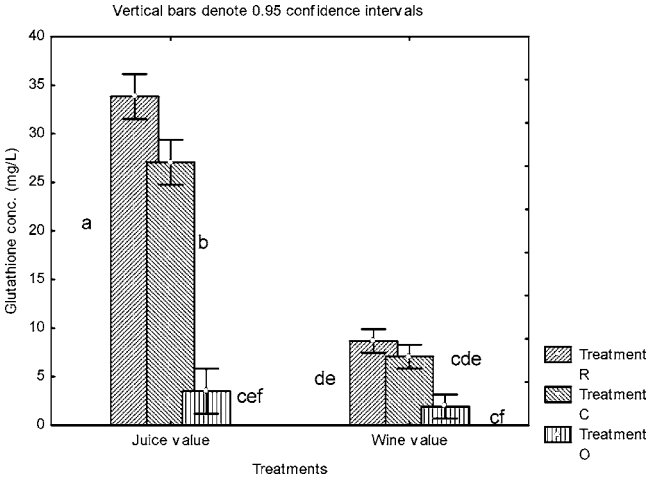

Figure 4. Reduced glutathione concentrations in juice and wine A, which underwent different treatments. Treatment R: reductive treatment of juice; treatment C: control treatment of juice; and treatment O: oxidized treatment of juice. Different letters indicate significant differences ($p \leq 0.05$).

at −20 °C to which high concentrations of SO_2 and ascorbic acid were added. Even when samples were thawed twice before analyses, the reduced glutathione levels stayed relatively constant over the 4 week period. Enzymatic oxidation of caffeic acid in grape juice is a rapid process, with the oxidized caffeic acid being reduced by the addition of glutathione to form GRP (*8*−*10*). However, 75 mg/L SO_2 additions to must led to 90% inhibition of phenol oxidase activity. Laccase, an oxidation enzyme normally associated with mold-infected grapes, is more resistant toward the inhibitory effect of SO_2 (*11*). The grapes used in this study had very little mold infection, and the high levels of SO_2 and ascorbic acid added to the juice were sufficient to prevent oxidation of the reduced glutathione. Ascorbic acid also competes with oxidation enzymes for oxygen and removes oxygen rapidly from wine or must (*12*, *13*). All these factors will prevent enzymatic oxidation of phenolic molecules in the juice and the incorporation of glutathione in GRP. Storage at 4 °C does, however, not seem optimal, even in the presence of high concentrations of SO_2 and ascorbic acid (**Figure 3**).

In **Table 1**, it is clear that the oxidative different treatments led to significantly higher oxidized glutathione levels, although these were still relatively low as compared to the reduced glutathione levels found in the different wines.

In **Figures 4**−**6**, the reduced glutathione levels can be seen of the reductive, control, and oxidative treatments in the juice and corresponding wines after alcoholic fermentation. The glutathione profile during the fermentation was not analyzed. However, a decrease in glutathione concentration after fermentation shows that yeast plays a significant role in glutathione metabolism. Levels in the three different juices ranged from 33 to 71 mg/L, which is similar to previously published data (*14*). Park et al. (*7*, *15*), however, found glutathione levels in Sauvignon Blanc juice to be between 0.001 and 1.28 mg/L,

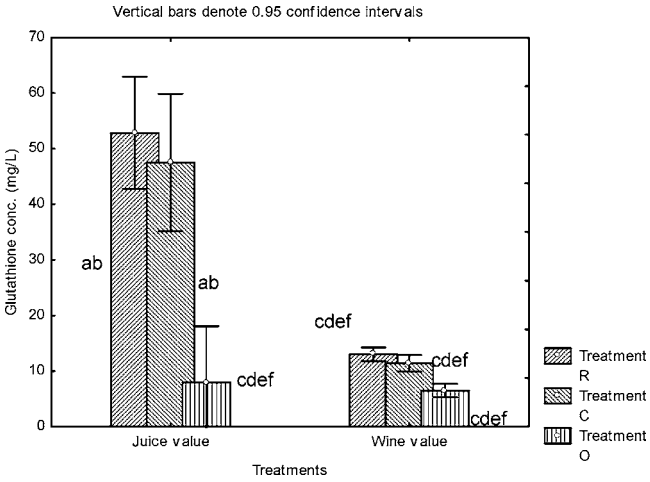

Figure 5. Reduced glutathione concentrations in juice and wine B, which underwent different treatments. Treatment R: reductive treatment of juice; treatment C: control treatment of juice; and treatment O: oxidized treatment of juice. Different letters indicate significant differences ($p \leq 0.05$).

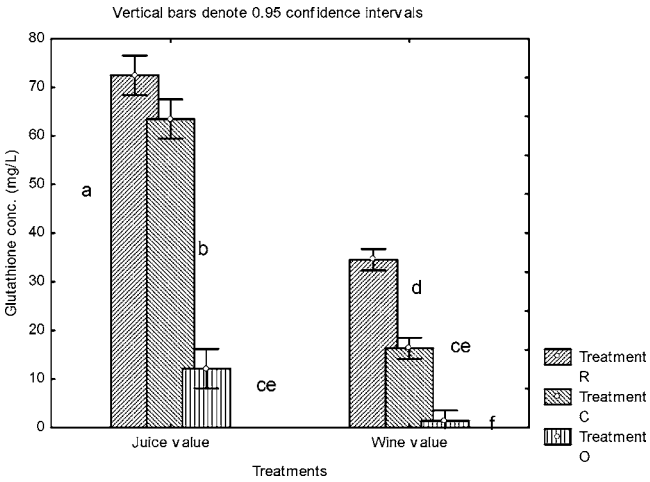

Figure 6. Reduced glutathione concentrations in juice and wine C, which underwent different treatments. Treatment R: reductive treatment of juice; treatment C: control treatment of juice; and treatment O: oxidized treatment of juice. Different letters indicate significant differences ($p \leq 0.05$).

which is much lower than what we found. The authors were not clear on how the grapes were crushed and whether it was done under very reductive or more oxidative conditions, with the latter possibly leading to degradation of the reduced glutathione before the start of fermentation. Our reductive treatments led to the highest levels of reduced glutathione in the juice, with control treatments having lower values in wines A and B and significantly lower values in wine C. Low concentrations of O_2 contact as in the control (about 1.0−1.5 mg/L) led to a reduction in reduced glutathione levels. This is low in terms of commercial winemaking, where O_2 levels of a few milligrams per liter often come in contact with the must, which is observed with standard commercial crushers and presses (*16*). Sulfuring was also performed very quickly after crushing, which can inhibit enzymatic oxidation and thus consumption of reduced glutathione. At high O_2 concentrations, such as in the oxidative treatment, reduced glutathione levels, however, dropped drastically in the juice, which is due to the absence of SO_2 and higher concentration of dissolved O_2. Reduced glutathione levels in the wines were constantly lower

than in the corresponding juice in all the treatments. Significant differences in reduced glutathione concentrations, especially in juice C, between different juices treated with different O_2 concentrations were also reflected in the corresponding wines. Park et al. (*7*, *15*) found glutathione levels to increase during alcoholic fermentation, which is contradictory to our findings. They reported that glutathione production is directly correlated with both nitrogen and assimilable amino acid concentration. The differences in initial amino acid composition as well as the large difference in initial glutathione levels could explain the difference between our results and that of other workers. Yeast strains could also differ in their glutathione metabolism. Only in juice C, which had the highest initial value of reduced glutathione, did the reductive treatment lead to relatively high reduced glutathione levels in the wine. It thus seems that the reductive treatment of Sauvignon Blanc as is currently being conducted in several wine producing countries should be reassessed. Marais (*17*) investigated the reductive and oxidative treatments of Sauvignon Blanc juice and its effect on the aroma and composition of the resulting wine. They found that wines made reductively gave the best quality wine but that the oxygen levels in the headspace of the press and in the juice were not, according to our knowledge, monitored in this work. However, 2-methoxy-3-isobutylpyrazine levels, which give the typical green pepper aroma to Sauvignon Blanc, were not influenced by the addition of hydrogen peroxide to wine, a strong oxidizing agent. It seems that 3-(hexan-1-ol)-glutathione is catabolized to *S*-3-(hexan-1-ol)-L-cysteine in vines. The latter compound is the precursor for 3-mercaptohexan-1-ol, which has a passion fruit aroma, typical of certain Sauvignon Blanc wines (*8*).

The addition of glutathione to dry white wine prevented the decline of linalool and α-terpineol when this wine was exposed to air. However, the oxidation led to a statistically lower concentration of linalool and α-terpineol in the control wines without glutathione addition (*18*). Currently, commercial yeast extract additions, containing glutathione, can also be purchased, which is reputed to extend the shelf life, delay yellow or brown formation, and preserve the flavor of white wines. It is also thought that Sauvignon Blanc wines with higher glutathione levels have a longer bottle maturation potential (*19*).

Glutathione thus seems to play an important role in the winemaking process and an accurate method to analyze for glutathione is thus of great importance. The novel method described in this work should address this need, as well as extend the knowledge on the interaction between O_2 and glutathione in must and wine, especially under South African conditions.

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